Original Paper

Isolation and molecular identification of native As-resistant bacteria: As(III) and As(V) removal capacity and possible mechanism of detoxification

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Abstract

The study of arsenic (As)-resistant microorganisms with high As removal capacity is fundamental for the development of economically sustainable technologies used for the treatment of water contaminated with metalloid. In the current study, four bacterial strains were isolated from As-contaminated water samples of the Xichu region, Mexico. Based on 16S rRNA gene sequencing and phylogenetic analysis of the isolated strains, *Rhodococcus gordoniae*, *Microbacterium hydrocarbonoxydans*, *Exiguobacterium indicum*, and *Pseudomonas kribbensis* were identified as potential As removal strains. *R. gordoniae* shows the highest growth capacity in both As(III) and As(V). *R. gordoniae*, *M. hydrocarbonoxydans*, and *E. indicum* removed approximately 81.6, 79.9, and 61.7% of As(III), as well as 77.2, 68.9, and 74.8% of As(V), respectively. *P. kribbensis* removed only about 80.2% of As(V). This study contributes to the possible detoxification mechanisms employed by these bacteria. Such insight could be crucial in the successful implementation of in situ bioremediation programs using these little-known bacteria.

Keywords *Rhodococcus gordoniae* · *Microbacterium hydrocarbonoxydans* · *Exiguobacterium indicum* · *Pseudomonas kribbensis* · Xichu · Ars C · Aio · Arr

Introduction

Arsenic (As) is a toxic metalloid present in high concentrations in natural waters due to various hydrogeological, geochemical conditions and anthropogenic processes, causing serious environmental health problems worldwide (Kumari et al. 2019). One of the main problems is the use of As-contaminated water (from groundwater or river water) for drinking or domestic consumption (Arroyo-Herrera et al. 2021; Muñoz et al. 2016; Osuna-Martínez et al. 2020). The main critical effects of humans’ long-term ingestion of inorganic arsenic are cancer, skin lesions, cardiovascular diseases, neurotoxicity, renal diabetes, and hematological and respiratory disorders (Cavalca et al. 2013). The World Health Organization (WHO) recommends a maximum concentration of 10 μgL⁻¹ of As in drinking water (World Health 2018); however, some countries, including Mexico, India, and Bangladesh, continue to allow from 25 μgL⁻¹ to 50 μgL⁻¹ of As (Kumari et al. 2019; Loredo-Portales et al. 2017). Around the world, it is estimated that more than 40 million people are at risk of drinking As-contaminated water (Nordstrom 2002). For instance, in Europe, more specifically in the Pannonian Basin (Hungary, Serbia, and Romania), more than 600,000 residents are at risk of drinking water containing high As concentrations. Also, in Asia, the countries most contaminated by arsenic are Bangladesh, China, India, Nepal, and Vietnam (Alka et al. 2021; Osuna-Martínez et al. 2020). In Africa, elevated As concentrations have been reported in both the surface and groundwater (Mudzielwana et al. 2020). In Latin America, the estimated population at risk of As exposure exceeds ~14 million people, with hundreds of recorded cases of exposure in countries, such as Chile, Argentina, and Mexico (Castrejón et al. 2020; Muñoz et al. 2016; Osuna-Martínez et al. 2020). Within this context, in Mexico, it is estimated that

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approximately 450,000 people are regularly exposed to As-contaminated water (Arroyo et al. 2013). For instance, in the Northeast of the State of Guanajuato, high As levels have been detected in the municipality of the Xichu mining district (up to 62,302 μg −1) (Osuna-Martínez et al. 2020). The inorganic forms of As predominantly exist as trivalent arsenite (As(OH)3, As(OH)4−, AsO2OH2−, and AsO33−) and pentavalent arsenate (AsO43−, HAsO42−, and H2AsO4−) in natural waters. Pentavalent species of As (As(V)) are stable in oxygen-rich aerobic environments, whereas the more soluble and toxic trivalent arsenite (As(III)) is prevalent in moderately reducing anaerobic environments such as groundwater (Kumari et al. 2019; Prasad et al. 2011). As(III) is 70 times more toxic than methylated species and 10 times more toxic than As(V) due to higher solubility. As(III), when interacting with thiol groups of proteins, inhibits enzyme functions (Cavalcà et al. 2013).

Existing technologies for As removal are primarily based on physicochemical methods, such as precipitation and/or adsorption of As in the form of As(V), lime softening, ion exchange, and membrane separation (Alka et al. 2021). Most of these methods involve the chemical pre-oxidation of As(III) to As(V), since the negatively charged arsenate adsorbs easily to solid surfaces, thereby, facilitating precipitation and removal from contaminated matrix (Lee et al. 2003). However, this may result in the production of undesirable by-products in such a way as to generate environmentally unfriendly technologies; additionally, they may be costly to apply (Weerasundara et al. 2021).

Biosorption of metal ions is an example of the wide variety of potential and actual applications of the bioremediation technique in polluted water treatments (Pandey and Bhatt 2016b). The use of microorganisms for the biosorption of As ions from water is an extremely efficient process. Bioaccumulation can also be considered a part of the process of metal and/or metalloid sequestration by living biomass (Kumari et al. 2019).

In a geographical context, native bacteria inhabit contaminated areas. These possess the innate ability to tolerate high concentrations of As by the process of extrusion after microbial oxidation, reduction, and methylation, collectively termed as biotransformation processes. Microbial transformation of As(III) into As(V) is an environmentally friendly and economically viable alternative to the aforementioned treatment methods (Anna Corsini et al. 2014; Kumari et al. 2019). On the other hand, in bacteria, the genes involved in As metabolism include the ars (arsenic resistance system), aox (arsenite oxidase), and arr (arsenate respiratory reduction) operons, which can be found in both plasmids and chromosomes. Tolerance or transformation mechanisms in some bacteria include a chromosomal- or plasmid-coded arsenite operon with three or five genes. The operon includes a regulatory gene (arsR), a coding gene for a transmembrane ejection pump specific for arsenite (arsB), and a coding gene for an arsenate reductase (arsC) where the reduction from As(V) to As(III) is carried out by the same gene (Kruger et al. 2013). The arr operon includes arrA and arrB genes; in dissimilatory As(V)-reducing bacteria, these genes are sometimes flanked by ars genes, forming clusters of arsenic-metabolizing genes in their genomes (arsenic-metabolizing gene island) (Tsuchiya et al. 2019).

The objective of the present work was to analyze the resistance, growth, and removal capacity of As(III) and As(V) by four native species. These bacterial species were isolated and molecularly identified, and their genes (arsenic oxidizers and reducers) were amplified to propose a possible mechanism of detoxification against As. It is worth mentioning that this work is the first to investigate the As removal capacity of little studied bacteria (Rhodococcus gordoniae, Microbacterium hydrocarbonoxydans, Exiguobacterium indicum, and Pseudomonas kribbensis) as well as their possible detoxification mechanism properties.

Materials and methods

Study area and sampling

Water samples were collected along the Xichu River, within the mining area “La Aurora”, corresponding to the municipality of Xichu, Guanajuato, Mexico. The location points were AJ (21°19′40.04″N, 100°01′57.18″O), J (21°19′48.13″N, 100°01′57.89″O), DJ (21°19′57.81″N, 100°01′48.87″O), and EQ (21°19′52.88″N, 100°01′54.09″O). Figure S1, in the supplementary material, shows the geographical map of the sampling sites. Water samples for microbiological and physicochemical analyses were collected using sterile Falcon tubes. For this, the tube was inserted into the river (below the surface, − 20 cm to − 30 cm) upside down and then filled by rotating it sideways and upwards. Afterward, it was sealed before being removed from the water to avoid contamination. Samples were placed in a cooler containing dry ice and kept at low temperatures (~ 4 °C) during transport to the laboratory (PROY-NMX-AA-121/1-SCFI-2008, 2008).

Analysis of the water samples

Physicochemical characterization

Samples were analyzed in situ and according to the parameters (anions, cations, pH, temperature, electrical conductivity, dissolved oxygen) established in the Mexican Norm NOM-127-SSA-2017 (NOM-127-SSA 2019). The quantifications of Fe2+, S2−, SO42−, NO3−, K+, Mg2+, Mn2+, and Zn2+ were done by handheld colorimeter (HACH DR 900®,
USA). Temperature (°C), electrical conductivity (µScm⁻¹), and dissolved oxygen (mgL⁻¹) were quantified by a water analysis potentiometer (Thermo scientific®, USA). The determination of total As was carried out by atomic absorption spectrometry.

**Determination of total As concentration**

The acid digestion process is oriented toward the dissolution of the analytes (in this case arsenic) to be analyzed by susceptible analytical equipment such as the Atomic Absorption Spectrometer. This equipment works with samples in solution, analyzing metals and/or metalloids (Edgell 1989). For the analytes to be in the optimum state for analysis, they must be in complete solution (Edgell 1989; Thompson et al. 2020).

Water samples collected from rivers or other bodies of water present quantities of organic matter that interfere with the analysis of metals and metalloids (Element 2007). The digestion equipment was used to obtain analytes from the complete solution of water samples. Therefore, water samples were subjected to digestion processes using digestion equipment (microwave digestion system Titan MPS 8 Position Microwave Sample Preparation System MPS™ N3130110, Perkin Elmer, USA), aiming to destroy all organic matters using acidic media and high temperatures, based on USEPA method 3015A for the determination of metals and metalloids (US EPA 2007). All samples were digested with concentrated HNO₃ (Nitric acid 70%, purified by re-distillation, ≥ 99.999% trace metals basis, Sigma Aldrich, USA). Deionized water (18.2 MΩcm⁻¹, resistivity) was used for all dilutions, obtained in a purification system (Milli-Q® direct 8/16 system, France). The flasks and all the materials used in the preparation of the solutions were carefully cleaned in 15% HNO₃ for 48 h, rinsed in deionized water, and dried in laminar flow. Quality control for the determination of metals was carried out by analyzing the standard reference materials (SRM) LGC6027 (Trace Elements in water) (Cartwright 2016).

The calibration curve was established using the standard solutions prepared in 1 mol L⁻¹ HNO₃ by dilution from stock solutions (As Standard for AAS, TraceCERT®, 1000 mg/L As in nitric acid, USA). A PerkinElmer PinAAcle 900 T Atomic Absorption Spectrometer equipped with MHS-15 Mercury/Hydride System (HG-AAS) was used in this study. High-purity argon (99.999%) was used as carrier gas (at a flow rate of 300 mL/min) and as shielding gas (at flow rate of 700 mL/min). A hollow cathode lamp operating at 18 mA was used, and a spectral bandwidth of 0.7 nm was selected to isolate the 193.7 nm As line. Peak height was used for quantitation. The detection limit (LOD) was calculated from the standard deviations of the blank solution (mostly nitric acid 0.2% of HNO₃) using the criterion of three times the standard deviations and ten times the standard deviations, respectively. All analyses were performed in the Environmental Engineering Laboratory of Sustainable Innovation of the UG-Guanajuato Campus (clean room, class 1000).

**Isolation of bacteria on selective As-enriched media**

Nutrient Agar (NA) (BIOXON®, Mexico) was prepared with sodium arsenate (Na₃AsO₄) (Sigma Aldrich-Merck, USA) and sodium arsenite (NaAsO₂) (Sigma Aldrich-Merck, USA). The media were adjusted to pH 8.5 by adding 0.1 M NaOH (Fisher Scientific, USA), the value was quantified among the water samples at the sampling points. A quantity of 100 µl of water sample was inoculated to two NA plates, one containing 3.5 mM of As(III) and one containing 3.5 mM of As(V); the spread plate method was used to obtain single colonies. The plates were incubated under aerobic conditions at 30 °C (Aguilar et al. 2020; Kumari et al. 2019). Bacterial colonies with different forms, colors, and margins were purified on solid medium containing As at the same concentration, at least three times, and stored at 80 °C in a solution of 30% glycerol. The strains were characterized according to morphological characteristics like shape, margin, chromogenesis, optical detail, and surface with the help of an optical microscope (Primo Star Zeiss GmbH, Germany). The 3dp.rocks/lithophane software was used for image enlargement and analysis. According to standard procedures, Gram staining was performed with a Gram staining set (Millipore Sigma; cat REF 77,730, USA) (Gerhardt et al. 1994). Subsequently, one bacterial species from each site was selected for further analysis from the isolated bacteria.

**Molecular identification of as resistant isolates**

Ribonucleic acid (RNA) extraction from fresh cells was performed using TRIzol and following the manufacturer’s instructions (Rio et al. 2010). For RNA recovery, the upper phase of the TRIzol was transferred to new micro-tubes and precipitated with 3 M sodium acetate (18 µL) and 600 µL of isopropanol. The micro-tubes were placed at − 20 °C overnight and centrifuged at 12,000 rpm for 15 min at 4 °C (Invitrogen®). Electrophoresis was performed in 1% agarose gel, stained with ethidium bromide to check the quality of the RNA. After purifying it, the RNA was transcribed into DNA using a reverse transcriptase (RT SuperScript® IV/Invitrogen®), where for each mRNA molecule, a single-stranded cDNA molecule was synthesized and later converted to double-stranded using a DNA polymerase (Invitrogen®).
Amplification procedure

DNA dilutions were added in a PCR mix containing: 2.5 µl of 10X PCR buffer; 2 µl of direct and reverse initiators; Midex D and Midex R, respectively (final concentration, 1 µM each); 0.5 µl of de-oxynucleoside triphosphates (dNTPs) (final concentration, 200 µM); 2.0 µl of MgCl₂ (final concentration 2.0 mM); and 3.0 U of Taq polymerase (Invitrogen®) (Saiki 1990). Water Milli-Q® was added, giving a final volume of 25 µl. The mixture was placed in a thermal cycler (Multigene LabNet®), with the following PCR conditions: initial denaturation at 95 °C for 3 min, 30 cycles of 95 °C for 30 s, 59 °C for 30 s and 72 °C for 3 min, followed by a final extension at 72 °C for 5 min (45). Based on the supplier’s instructions, the purification of the PCR products was carried out with GE Healthcare® kit: GFX PCR DNA and Gel Band Purification. Then, all purified PCR products were sequenced at the National Laboratory of Agricultural, Medical, and Environmental Biotechnology (IPICYT-Mexico) using the applied biosystems brand sequencer, capillary model 3500, which applies the Sanger method (Men et al. 2008). Finally, the sequences were compared to those in the National Biotechnology Information Center (NCBI) database (Sayers et al. 2021a, b). The evaluation of the available 16S rRNA gene sequences via a BLASTN search. This program compares nucleotide sequences of DNA and/or RNA (Schwartz et al. 2000). This application is available on the NCBI website. The evaluation of the electropherograms and the alignment of the direct chain with the reverse was carried out. Multiple alignments of the sequences of the strain with other bacterial species were carried out using the CLUSTAL X program (Larkin et al. 2007). Finally, the 16S rRNA phylogenetic tree was constructed using the distance method Neighbor-Joining (Saitou 1987), which relates bacterial sequences from references selected in GenBank® (Sayers et al. 2021a, b).

Minimum inhibitory concentration of the isolates

The selected isolates’ As(III) and As(V) resistance was evaluated using minimum inhibitory concentration tests under aerobic conditions. The minimum inhibitory concentration (MIC) is the lowest concentration of both inorganic As species that completely inhibits growth. MIC for four bacterial cultures was determined in nutrient broth medium (NB) (BIOXON®, Mexico) containing different concentrations of As(III) and As(V), which ranged from 1 to 20 mM (Kumari et al. 2019). The medium was inoculated with cell suspensions taken from fresh cultures in the log phase and incubated at 120 rpm in an incubation shaker (Thermo Scientific MaxQ 4000, USA) at 30 °C for 96 h. Two controls were prepared for each experiment (As(III) and As(V)): (i) Nutrient broth supplemented with only bacteria (without arsenite or arsenate) and (ii) Nutrient broth with arsenite or arsenate, and without bacteria. Optical density of cell cultures was taken at 24 h intervals for 96 h to determine the bacterial growth responses at different concentrations of As(III) and As(V). Bacterial growth was measured by determining the optical density at 600 nm (OD₆₀₀) in a UV–Vis Spectrophotometer (DR 3900, HACH, USA). Each experimental set-up was prepared in triplicate.

Effect of pH on the growth of bacteria culture

The isolated bacteria were inoculated into culture media with different pH (3.5–12) at 30 °C (shaking condition 120 rpm). Bacterial growth was measured by determining OD₆₀₀ after a 24 h growth period (Kumari et al. 2019).

Gene expression analysis

The presence of the arr (respiratory arsenate reduction system) and aox (respiratory arsenite oxidation system) genes was detected through amplification using the DNA of the isolated bacterial strains (Chang et al. 2010). PCR amplification was performed with primers: arrA-CVF1 and arrA-CVR1 (direct and reverse respectively) for arsenate reductase A. As an initial step, these primers were tested and evaluated in silico for virtual amplification of arrA genes in clones and, subsequently, for amplification and sequencing of arrA genes from groundwater samples, since the monitoring of the arrA gene can provide useful information about the variations of the arsenate-reducing microbial species. The samples were then amplified with the primers aoxA and aoxB (direct and reverse, respectively) for arsenite oxidase AB. The primer “a” (5’-AATGACACCTTCAGGGCG-3’), annealing 48 bp upstream of the aoxA, stops codon; and the primer “b” (5’-AGCCTACGATCTTTTGCAG-3’), annealing 872 bp downstream of the aoxB, starts codon. The amplification program was set to the following conditions. First, the initial denaturation temperature was 94 °C for 3 min (1 cycle). The temperature was then cycled to 94 °C for 30 s, to 60 °C for 40 s, and then to 72 °C for 1 min. This process was repeated for other 30 cycles, followed by a final extension at 72 °C for 10 min. Finally, the amplification of the genes was verified in a 1% agarose gel stained with ethidium bromide (Mirza et al., 2017).
Comparison of the growth profile among the four bacteria under As(III) and As(V) stress

The assays for growth kinetics were designed using a factorial design “A*B” of two-factor 8 × 4 (time and strain), where the “A” factor has eight levels (0–168 h), and the “B” factor has four levels (A1, S3, A4, A11) (Kenny 2003). The above was intended to identify the main effects of strains and time on microbial growth in the presence of As(III) and As(V). Growth kinetics were planned in 30 ml of YEM broth, with an aliquot of each culture taken every 24 h. Three experiments were carried out as follows: (1) growth kinetics without As, (2) growth kinetics in YEM with As(III), and (3) growth kinetics in YEM with As(V). The concentration of arsenic was defined according to the result obtained by the MIC. Incubation conditions in Falcon tubes were inoculated with bacteria culture (cell density of about 10^7 CFU/ml) at 30 °C and 120 rpm for 168 h. Absorbance was measured at 600 nm in a UV–Vis Spectrophotometer (DR 3900, Hach, USA).

Statistical analysis

Initially, the determination of normality (Shapiro–Wilk test), homogeneity (Bartlett test), and independence (Durbin–Watson test) of the data obtained was carried out (Kirchman et al. 1982). The experiments in this study were performed in triplicate (n = 3). The values of the growth kinetics are given as means ± standard deviation (SD) using Microsoft excel software, version 2010. Data obtained from the factorial design were analyzed by a two-way analysis of the variance test (ANOVA) to test significance of bacterial growth in the presence of As(III) and As(V). Tukey’s multiple range test was performed to identify the strains with the highest potential for interaction with As (III) and As (V) and their optimal growth times. Significance of differences was defined as p < 0.05, with a 95% confidence interval, using Minitab software version 19.

Removal of As(III) and As(V) by isolated strains

Isolated strains that showed the best growth in the presence of As was selected and cultured under aerobic conditions. The concentration of As used was in relation to the result obtained by the MIC. Synthetic water was prepared from the analyzed constituents of the water samples from the studied site based on the average concentrations of the results obtained “Physicochemical characterization”. Biosorption assays for As(III) and As(V) were performed using the lyophilized cell pellet of the selected strains (Kao et al. 2013). From each strain, the lyophilized cell pellet (5 g/l) was added to 125 ml Erlenmeyer flasks with 30 ml of culture medium containing As (III), pH was adjusted to 8.5 by adding dilute solutions of NaOH, while the contact time was 168 min. The flasks were shaken (120 rpm/min) at 30 °C. Samples were withdrawn every 24 h and centrifuged at 4000 rpm at 4 °C for 15 min. In parallel, the same procedure was performed for the case of As(V). The supernatants were analyzed to determine the concentrations of As(III) and As(V) by the methodology described in “Analytical method”. All experiments were conducted in triplicate. The removal of As(III) and As(V) in percentages was calculated using Eq. (1) (Kumari et al. 2019).

Bioremoval As(III)or As(V) % = \frac{Ci - Cf}{Ci} × 100

Ci—Initial concentration of As(III) or As (V), Cf—Final concentration of As (III) or As (V).

Analytical method

The determination of As levels was performed by HG-AAS (described in “Analysis of the water samples”). The methodology for the determination of As(III) and As(V) was carried out by the combination of co-precipitation and atomic absorption spectrometry by hydride generation, as described in a previous work (Tuzen et al. 2009). As(V) was quantitatively recovered on aluminum hydroxide precipitate. After the oxidation of As(III) using dilute KMnO4 (Sigma Aldrich-Merck, USA), the developed co-precipitation was applied to determine total As. As(III) was calculated as the difference between the total As and As(V) content. The limits of detection (LOD) were based on three times the blank sigma (n = 20). The same certified reference materials were used for validation as described in “Determination of total As concentration”.

Analysis of possible mechanism of biological removal of arsenic

A bioinformatic search of the genes of the isolated species was carried out from the National Center for Biotechnology Information (NCBI) databases. This platform includes the database (BD) Public library called GenBank®, which contains an extensive collection of nucleotide sequences obtained from more than 300,000 species (Sayers et al. 2021a, b).

Results and discussion

Physicochemical characterization in water samples

The results of the water characterization are shown below in Table 1. The average pH value obtained in the water samples was 8.73 ± 0.52, which shows that, within the sampling
region in “La Aurora” mine, a highly alkaline medium predominates. The average values for the parameters of C.E., O.D., and temperature were 725.25 ± 155.06 μScm−1, 4.75 ± 0.66 mgL−1, and 28.73 ± 0.96 °C, respectively. For K+, Mg2+, Mn 2+, Fe2+, Zn, SO4 2−, and NO3 −, the values were 1.97 ± 0.67, 33.55 ± 3.17, 0.20 ± 0.16, 0.05 ± 0.02, 0.03 ± 0.01, 136.88 ± 21.15, and 4.64 ± 4.27 mgL−1, respectively.

Determination of total As in water samples

The arsenic found in the water samples from the sampling sites (Table 2), quantified by hydride generation atomic absorption spectrometry (HG-AAS), indicates that the values are above the maximum permissible values (World Health 2018). The mean As concentration was 58.48 ± 33.68 μgL−1, with a maximum value of 108 ± 2.1 μgL−1 (DJ) and a minimum value of 35 ± 0.2 μgL−1 (AJ). This heterogeneity in element concentration in different samples is common in mining areas due to constant movement and excavation of materials being mined (Ono et al. 2016). The arsenic concentrations found in all water samples are above the maximum permissible limit of both the WHO (10 μgL−1) (Nordstrom 2002; World Health 2018) and the Mexican Norm NOM-127-SSA (25 μgL−1) (World Health 2018). In this study area, the range of As concentration reported in water samples ranges from 98 to 163 μgL−1 (Loredo-Portales et al. 2017; Muñoz et al. 2016; Rodríguez et al. 2019).

Few studies have reported on the quantification of As in surface water from the Xichu area; most studies have analyzed in soil, sediment, and rock samples (Osuna-Martínez et al. 2020). It is important to mention that the main sources of water in this region are the rivers, streams, and groundwater. At approximately 100 m from the mining area, there are some active corn fields where this water is used for irrigation (Loredo-Portales et al. 2017) and domestic consumption.

Morphology and molecular identification of isolated bacteria

A total of four morphologically distinct bacteria capable of growing on As(III) and As(V) were selected (one from each sampling point) and isolated. The bacteria were labeled as A1-AJ, A1-J, A4-DJ, and S3-EJ.

Figure S2, in the supplementary material, shows the morphological characteristics of the isolated strains. A1-AJ, A1-J, and A4-DJ were Gram-positive, while S3-EJ was Gram-negative. For A1-AJ, the colonies were yellow and rod-shaped. In addition, A1-J presented a light pinkish red color with a coccoidal ovoid shape. A4-DJ had an orange color and a stick shape. In the case of S3-EJ, the colonies were circular, convex, mucoid, and translucent beige. All strains were aerobic.

DNA and RNA extractions and amplification of 16S ribosomal genes from arsenic-resistant bacterial isolates are shown in Figures S3 and S4 (in the supplementary material). After PCR, the amplicons were purified for sequencing. Subsequently, the genetic tree was elaborated based on the NCBI
Platform. Finally, the respective comparisons were made with the nucleotide sequences deposited in the GenBank® employing the Nucleotide BLAST tool.

Figure 1 shows the phylogenetic tree of the 16S rRNA gene, where the relationship of the isolates is observed with support from the sequence database GenBank® (Sayers et al. 2021a, b).

The analysis of 16S rRNA sequence showed that A1-J belongs to the Rhodococcus gordoniae W4937 T (99.34% identity) (Jones et al. 2004). The strain S3-EJ showed 99.23% similarities with the Pseudomonas kribbensis 46-2 T (D. H. Chang et al. 2016). The strain A4-DJ showed 100% similarities with several Exiguobacterium, but according to the phenotypic characteristics, it can presumably be identified as the Exiguobacterium indicum HHS 31 T (Chaturvedi and Shivaji 2006). The strain A1-AJ showed 99.78% similarities with the Microbacterium hydrocarbonoxydans NBRC 103,074 T (Schippers et al. 2005). The summary of the molecular identification of the isolates is represented in Table 3. In this same context, based on the morphology observed in this study for each strain, there is an agreement with the results cited in other works (D.-H. Chang et al. 2016; Jones et al. 2004). It should be noted that, for the first time, the molecular

Fig. 1 16S rRNA sequence-based phylogenetic tree for the strains: 1DA*, 3DS**, 4DA*** and 13DB**** compared to other bacteria in GenBank® using the neighbor-joining method. Asterisks indicate our isolates

Table 3 Summary of the Molecular Identification

| Keys of isolated strains | Percent identity (%) | Scientific name | Strain designation | Access numbers | Genes | Reference |
|-------------------------|----------------------|-----------------|--------------------|----------------|-------|-----------|
| 1DA                     | 99.34                | Rhodococcus gordoniae | W4937 T | NR_025730.1 | n.d | (Jones et al. 2004) |
| 3DS                     | 99.23                | Pseudomonas kribbensis | 46–2 T | KT321658.1 | ars C, aioA** | (Chang et al.2016) |
| 4DA                     | 100                  | Exiguobacterium indicum | HHS 31 T | MT256271.1 | ars C, aioA** | (Chaturvedi and Shivaji 2006) |
| 13DB                    | 99.78                | Microbacterium hydrocarbonoxydans | NBRC 103,074 T | MK424292.1 | ars C, aioA** | (Schippers et al. 2005) |

**Genes identified in this work
n.d not detected
identification of native bacterial strains in this geographic region has been reported.

**Minimum inhibitory concentration of the isolated**

The arsenic tolerance of the four bacterial cultures was tested by determining their minimum inhibitory concentration (MIC). The percentage analysis was in relation to the growth of the control group. 

*R. gordoniae*, *E. indicum*, and *M. hydrocarbonoxydans* showed favorable growth on media enriched with 10 mM As for 96 h. On As(III) media, they showed an average growth of 93.1 ± 7.2%, and for As(V) the average growth was 95.2 ± 4.4%. Bacterial growth decreased in media enriched with 12 mM As. For *R. gordoniae*, a slight growth was observed in 12 mM enriched media, both in As(III) (32.5%) and As(V) (39.8%). For *E. indicum* and *M. hydrocarbonoxydans*, mean growth rates of 4.3 ± 0.8% and 9.9 ± 3.1% for As(III) and As(V), respectively, were observed. *P. kribbensis* presented a MIC of 5 mM (69.7%) for As(III) and 10 mM (88.4%) for As(V). The genus of these species has previously been reported to be highly resistant to As (Kumari et al. 2019; Pandey and Bhatt 2015). Notably, this is the first work to report the resistance of bacterial species of *R. gordoniae*, *E. indicum*, *P. kribbensis*, and *M. hydrocarbonoxydans* on As(III)- and As(V)-enriched media.

**Effect of pH on the growth bacteria culture**

The pH of the surrounding medium plays a critical role in determining As mobilization and toxicity, and it influences the occurrence and distribution of the microorganisms themselves. In addition, pH controls the energy yields of redox reactions commonly found in anoxic environments in microbial systems, including syntrophic oxidation, iron reduction, sulfate reduction, and methanogenesis (Cavalcá et al. 2013). Figure 2 shows the results of the effect of pH on the growth of the four bacterial cultures. All bacterial cultures grew at pH values ranging from 5.5 to 10.0. Feeble change was observed within the pH range of 4.0 to 5.5. At acidic pH < 4 no growth was observed. The maximum OD$_{600}$ value occurred for *R. gordoniae*, followed by *M. hydrocarbonoxydans*, *P. kribbensis*, and *E. indicum* at pH 8.5, 8.0, 7.5, and 7.5, respectively. The results of the present study are consistent with previous reports. Kumari et al. (Kumari et al. 2019) observed that As-resistant bacteria grew in a wide pH range between 5 and 10. However, these bacteria were unable to tolerate conditions with extreme pH values (pH = 2 or pH = 11).

**Gene expression analysis**

In this work, we searched for the presence of genes expressing As-resistance in the bacterial species studied by PCR. Aio genes encoding the enzyme Aio (arsenite oxidase) were detected in *M. hydrocarbonoxydans*, *P. kribbensis*, and *E. indicum*, but not in *R. gordoniae* (Table 3). The *Rhodococcus* strain failed to give positive amplification, probably due to mismatches between the tested primers and the gene sequence (Anna Corsini et al. 2014). However, it presented a significantly higher growth in terms of biomass generation and growth rate. Therefore, *R. gordoniae* could metabolize arsenic in a chemoautotrophic manner. It is also noteworthy that the genus *Rhodococcus* is reported not only with the presence of mostly reducing genes but also
with the presence of As(III)-oxidizing genes (Kumari et al. 2019; Prasad et al. 2011; Wang et al. 2020).

Studies have reported that the presence of genes conferring arsenic resistance in microorganisms is influenced by the environmental conditions to which they are exposed (Wang et al. 2020). Consequently, the presence of arsenic in water induces the expression or presence of a group of genes (operon) that confer arsenic resistance in the microorganisms (Wang et al. 2020). One example is that, in conditions with high sulfate reduction activity, genes—such as aioA and aoxB—enriched by oxic environments—are inhibited by this activity (Andres and Bertin 2016).

In accordance with previous results (A. Corsini et al. 2014), these data corroborate the hypothesis that the ars operon could be slightly different, even though the bacteria are categorized in the same genera, supporting the hypothesis of a horizontal transfer of the arsenic genes within bacterial population.

**Comparison of the growth profile among the four bacteria under As(III) and As(V) stress**

Based on the results obtained in the MIC, the bacteria were grown on control, 10 mM As (III), and 10 mM As(V) media. All experiments were carried out at 168 h. The growth patterns of *R. gordoniae*, *M. hydrocarbonoxydans*, *E. indicum*, and *P. kribbensis* are shown in Fig. 3.

It was observed that *R. gordoniae* was the bacterium with the highest growth capacity in the presence of As(III) and As(V), showing a statistically higher growth ($p < 0.05$) compared to the other bacteria under study (Figs. 3b and 3c).

In the presence of As(III), an interesting phenomenon was observed between 120 and 168 h for *M. hydrocarbonoxydans* (Fig. 3b). This bacterium showed a biphasic behavior similar to diauxic growth. This is possibly because As(III) as a stress stimulus activates an adaptive response that increases the resistance of the bacterium to a higher level of stress. Such response may involve the expression of genes encoding cytoprotective proteins, namely chaperones, heat shock proteins, antioxidant enzymes, and growth factors (Andres and Bertin 2016).

Relevantly, *E. indicum* showed a different behavior. In the presence of As(III), it showed a significant ($p < 0.05$) and continuous growth up to 96 h; but in the presence of As(V), it showed an exponential growth in 24 h and entered to stationary phase until 168 h without significant differences ($p \geq 0.05$) (Fig. 3b and 3c).

In the case of *P. kribbensis*, good growth is observed in the presence of As(V), reaching maximum growth at 168 h (Fig. 3c). In contrast, in the presence of As(III), growth is completely inhibited (Fig. 3b).

Based on Tukey’s test, the results obtained are as follows (in order from the highest to the lowest growth capacity):
an adaptation phase to overcome the toxicity caused by arsenic in their metabolism.

**Removal of As(III) and As(V) by isolated bacteria**

The concentration of arsenic in the medium was based on the result of the MIC (10 mM was used for As(III) and As(V)). The pH used was 8.5 since this is the average pH present in the contaminated area of study. The results of this study revealed different behaviors between the percentages of As(III) and As(V) removal by isolated bacteria (Fig. 4).

**Exiguobacterium indicum**

This bacterium was more efficient in removing As(V) than As(III). At the end of the experiment (168 h), *E. indicum* successfully removed approximately 74.83 ± 1.2% of As(V) from the medium, while only 61.77 ± 0.8% removal was achieved in the case of As(III) (Fig. 4a). For this genus, as in previous studies, removal percentages of 99% for As(V) and 90–99% for As(III) (Pandey and Bhatt 2016a; 2016b) have been reported, depending on the method developed (Table 4). Bacteria of the genus *Exiguobacterium* are adapted to use multiple strategies to maintain themselves under the stress conditions they face in their environments, namely the high toxicity generated by As (Andreasen et al. 2018). For this, bacteria use a large arsenal of proteins related to protein synthesis, detoxification, energy generation, transport, and global stress (Pandey and Bhatt 2015). The differences between the level of resistance exhibited by the isolated strains and the reported strains may have resulted from the variations in the levels of contamination, source of contamination, period of exposure, characteristics of the environment, and variations of metal or metalloid bioavailability (Aguilar et al. 2020).

**Rhodococcus gordoniae and Microbacterium hydrocarbonoxydans**

For the cases of the bacteria belonging to the Actinobacteria phylum, *R. gordoniae* and *M. hydrocarbonoxydans* were slightly more efficient in removing As(III), with values of 81.6 ± 1.1 and 79.98 ± 2.2%, whereas for As(V) the values were 77.21 ± 0.9 and 68.93 ± 2.8%, respectively (Fig. 4b, d).
| Bacteria                     | Source of isolation                                                                 | Media used for isolation         | Method of remediation employed by bacteria                                                                 | Remediation efficiency | Reference                                      |
|------------------------------|--------------------------------------------------------------------------------------|---------------------------------|----------------------------------------------------------------------------------------------------------|------------------------|------------------------------------------------|
| Exiguobacterium sp.          | Soil from Rajnandgaon, Chhattisgarh, India                                           | Basal Salt Medium (BSM)         | Biosorption                                                                                              | 99% of As(V), 90% of As(III) | (Pandey and Bhatt 2016b)                        |
| Exiguobacterium sp.          | Subsurface soil from Chhattisgarh, India                                             | Basal salt medium (BSM)         | Biosorption, bio-oxidation                                                                             | 26.2 and 29.4 mg g⁻¹ biomasses, respectively | (Pandey and Bhatt 2015)                        |
|                            | Subsurface soil from Chhattisgarh, India                                             | Planktonic cultures and biofilms| Remediaion efficiency of various bacterial species                                                      |                        |                                                |
| Exiguobacterium sp.          | Subsurface soil from Chhattisgarh, India                                             | Basal salt medium (BSM)         | Biosorption, bio-oxidation                                                                             | > 90% of total As was removed by 0.3 g/L BMnOx | (Liang et al. 2013)                           |
| Exiguobacterium profundum    | Wastewater samples were collected from the outer drain of an industrial paint plant, Kasur, Pakistan | Planktonic cultures and biofilms| Biosorption, bio-transformation                                                                       | 25.2 and 29.4 mg g⁻¹ biomasses, respectively | (Pandey and Bhatt 2019)                       |
| Microbacterium paraoxydans   | Soil samples from districts of Chhattisgarh, India                                   | Basal salt medium (BSM)         | Biosorption                                                                                              | 87 ± 0.9% of As(III)     | (Pandey and Keshavkant 2019)                   |
| Pseudomonas genus, As7325    | Sediment and groundwater from Southern Zhuoshui River                                | Artificial groundwater          | Bio-oxidation                                                                                            | 77.3 mg/g of As(III)     | (Kao et al. 2013)                             |
| Pseudomonas aeruginosa, AT-01| Groundwater from Pakistan                                                            | Artificial groundwater          | Bio-oxidation                                                                                            | 80.23% of As(V)          | (Tariq et al. 2019)                           |
| Rhodococcus sp.              | Subsurface water sample from contaminated site of West Bengal, India                | Batch scale by adding a different amount of sorbent (Biomass) | Biosorption                                                                                              | 75 mg l⁻¹ As(III) in 48 h | (Anna Corsini et al. 2014)                    |
| Rhodococcus gordoniae        | Water samples from the Province of Cremona, Lombardy, Italy                         | BBWM culture medium             | Biosorption, bio-oxidation accompanied with bio-oxidation                                              | 61.77% of As(III), 74.83% of As(V) | (This study)                                  |
|                            | Water samples from the Xichu River                                                  | Artificial water                | Bio-oxidation                                                                                            | 70.98% of As(III), 68.93% of As(V) | (This study)                                  |
|                            | Artificial water (Based on the analysis of the Xichua River)                        | Artificial water                | Bio-oxidation                                                                                            | 81.6% of As(III), 77.21% of As(V) | (This study)                                  |
|                            | Artificial water (Based on the analysis of the Xichua River)                        | Artificial water                | Bio-oxidation                                                                                            | 80.2% of As(III)         | (This study)                                  |
c). For both genders, in comparison with previous works (A. Corsini et al. 2014; Pandey and Bhatt 2016b; Prasad et al. 2011), the results obtained fell within the reported range, going from 48.43% to greater than 90% (Table 4).

The possible explanation for the removal capacity of As(III) and As(V) by R. gordoniae and M. hydrocarbonoxydans, observed in this study, is that they can present systems capable of reducing and oxidizing As. The transformation reactions are affected by the bacterial functional enzymes but also by the reduction/oxidation potential in the environment, which is greatly influenced by the presence of microbial communities. There is information on As redox cycling by microorganisms which play a significant role in controlling As speciation and mobility in high As environments (Andres and Bertin 2016; Cai et al. 2020).

**Pseudomonas kribbensis**

This microorganism was able to remove As(V) up to 80.23 ± 0.3% (Fig. 4d); however, it was not able to remove As(III). One of the possible explanations is that the toxicity of As depends on its chemical form, with the inorganic forms being the most toxic. In most reported cases, the percentage of As adsorbed by bacteria is higher with As(V) because it has lower toxicity than As(III) (Aguilar et al. 2020). Arsenate is taken up into the cell membrane by phosphate transporters; once As(V) binds to phosphate, the cell cannot detect its presence, even when it is highly accumulated in the cell. Moreover, this behavior is related to the fact that the bacteria can use the energy generated by As(V) reduction.

It is worth mentioning that this bacterium has been little studied, since it was recently discovered (D. H. Chang et al. 2016). Therefore, this is the first time that As removal studies by P. kribbensis have been presented.

**Possible mechanisms that play a role in removal of As in isolated bacteria**

Continuous exposure of microorganisms to toxic metalloids in the environment provides a selective pressure to evolve resistance genes. Microorganisms have developed various genetic systems to cope with arsenic toxicity. These systems include ars operons, which are groups of genes widely distributed in bacterial species (Escudero et al. 2013; Gu et al. 2018). The distribution of arsenic resistance genes in microorganisms is a manifestation of the presence of arsenic in nature.

Based on a bioinformatic analysis carried out on the bacteria identified in this work, it appears that the detoxification of arsenic for R. gordoniae possibly uses the system operon ars, which transports arsenite out of the cell (A. Corsini et al. 2014). These bacteria have two ars operons on its chromosome: arsRBC and arsRDABC; in contrast, E. indicum HHS 31 and P. kribbensis CHA-19 possibly have only one ars operon: arsRBC. For the case of M. hydrocarbonoxydans SA35, it has been reported that the mechanism of As(III) oxidation by this bacteria is related to As detoxification rather than to energy generation (Macur et al. 2004). Arsenate is acquired by an organism through endogenous inorganic phosphate (Pi) transport systems. Inside the cell, arsenate is reduced to the thiol-reactive form arsenite. Glutathione (GSH)-conjugates of arsenite may be extruded from the cell or sequestered in vacuoles by members of the ATP-binding cassette (ABC) family of transporters (LeBelanc et al. 2013). Figure 5 shows the likely mechanism of microbial transformations of arsenic: (a) In detoxification and energy generation, arsenite oxidase (Aio) is responsible for the oxidation of As(III), arsenite functions as an electron donor in the initiation of a membrane respiratory chain; (b) in arsenate reduction and energy generation, arsenate reductase (Arr) participates in the reduction of As(V), arsenate functions as a terminal electron acceptor for an anaerobic respiratory chain; and (c) As(V) is taken up by organisms through phosphate transporters. As(V) is then reduced to As (III) by bacterial ArsC. Glutathione and glutaredoxin serve as a source of potential reduction. Possibly, arsenite is extruded from cells only by ArsB, where energy is supplied by the cell membrane potential or by an arsenite translocating ATPase (ArsAB ATPase).

The ArsA protein is an ATPase that interacts with ArsB to form the arsenite expulsion pump energized by ATP hydrolysis. The main function of Ars D is related to its ability to bind arsenite and transfer it to the ArsA ATPase prior to oxiidxion by the ArsB pump. In general terms, it is worth mentioning that, in the analysis of arsenic removal by microorganisms, the stress mechanisms employed by the microorganisms for their survival and acclimatization in arsenic-rich environments must also be considered.

**Conclusion**

The application of microorganisms in the field of treatment of water contaminated by metalloids or metals is immense, since remediation using microbial ecological methods is emerging as a promising technology. Bacteria from As-contaminated sites can tolerate high levels of metalloid and are able to remove it. The results of the present study indicate that native microorganisms could be good candidates for the field of in situ bioremediation. R. gordoniae, M. hydrocarbonoxydans, E. indicum, and P. kribbensis were found to be able to tolerate arsenic as well as to remove arsenite and arsenate. Of the four bacterial species investigated, R. gordoniae and M. hydrocarbonoxydans...
were adapted to a wider pH range, were able to tolerate higher As(III) and As(V) stress, and could be chosen as potential candidates in bioremediation. However, the study of *E. indicum* and *P. kribbensis* also offers a potential field of research for As removal under optimal conditions.

On the other hand, the distribution of arsenic resistance genes in native micro-organisms is an indicator of the presence of arsenic in nature.

Finally, knowledge of the mechanisms of detoxification by bacteria offers future research for the sustainable application of molecular tools and technologies. Recent advances in genetic engineering, including techniques, such as gene editing, systems biology, metabolic engineering, and nanzyme-based bioremediation, provide further insight into microbial metabolism and could be employed to improve bioremediation capabilities.

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**Declarations**

**Conflict of interest** The authors declare that they have no conflicts of interest.

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