Environmental Science and Pollution Research (2024) 31:39063–39076
https://doi.org/10.1007/s11356-023-30361-7

CLEANER PRODUCTION AND SUSTAINABLE PROCESSES FOR ENVIRONMENTAL REMEDIATION

Insight into the genome of an arsenic loving and plant growth-promoting strain of *Micrococcus luteus* isolated from arsenic contaminated groundwater

Ashutosh Kabiraj[1] · Urmi Halder[1] · Annapurna Chitikineni[2,3] · Rajeev K. Varshney[2,3] · Rajib Bandopadhyay[1]

Received: 17 January 2023 / Accepted: 5 October 2023 / Published online: 21 October 2023

Abstract
Contamination of arsenic in drinking water and foods is a threat for human beings. To achieve the goal for the reduction of arsenic availability, besides conventional technologies, arsenic bioremediation by using some potent bacteria is one of the hot topics for researchers. In this context, bacterium, AKS4c was isolated from arsenic contaminated water of Purbasthali, West Bengal, India, and through draft genome sequence; it was identified as a strain of *Micrococcus luteus* that comprised of 2.4 Mb genome with 73.1% GC content and 2256 protein coding genes. As the accessory genome, about 22 genomic islands (GIs) associated with many metal-resistant genes were identified. This strain was capable to tolerate more than 46,800 mg/L arsenate and 390 mg/L arsenite salts as well as found to be tolerable to multi-metals such as Fe, Pb, Mo, Mn, and Zn up to a certain limit of concentrations. Strain AKS4c was able to oxidize arsenite to less toxic arsenate, and its arsenic adsorption property was qualitatively confirmed through X-ray fluorescence (XRF) and Fourier transform infrared spectroscopy (FTIR) analysis. Quantitative estimation of plant growth-promoting attributes like Indole acetic acid (IAA), Gibberellic acid (GA), and proline production and enhancement of rice seedling growth in laboratory condition leads to its future applicability in arsenic bioremediation as a plant growth-promoting rhizobacteria (PGPR).

Keywords Arsenic · Bioremediation · *Micrococcus luteus* · Purbasthali · Oxidation · PGPR

Introduction

Arsenic, a notoriously toxic, highly soluble, silent killer in environment that commonly hampers the agricultural (Ali et al. 2022; Majumdar et al. 2023) and public health (Ali et al. 2022; Ro et al. 2022). Living beings in Asian countries like India, Bangladesh is menacing of that absolute poison. Distribution of arsenic in various oxidative forms (like +V, +III, 0, -III) and concentrations mostly varies in accordance of the soil and water quality, abundance of microflora, and the presence of other heavy metals as well. Anthropogenic activities and natural processes are main causes of arsenic contamination in groundwater and agricultural soils. Arsenic accumulation within the grains or other parts of plants is one of the primary sources of arsenic toxicity among animals, and rice grains among other cereals potentially accumulate ten times more arsenic from the environment (Roychowdhury et al. 2018). People of many Asian countries use rice as their staple food. Arsenic present in rhizospheric soil is easily absorbed by rice roots and eventually transported to aerial parts. A portion of absorbed arsenic gets accumulated into the rice grains (Majumdar et al. 2021).

There is no affective method to remove arsenic from foods and drinking waters except few conventional methods based on adsorption, membrane filtration, and ion exchange.

Responsible Editor: Diane Purchase

✉ Rajib Bandopadhyay
rajibindia@gmail.com

[1] Microbiology Section, Department of Botany, The University of Burdwan, Bardhaman, West Bengal 713104, India

[2] Center of Excellence in Genomics and Systems Biology, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Hyderabad, India

[3] State Agricultural Biotechnology Centre, Centre for Crop and Food Innovation, Murdoch University, Murdoch 6500, Australia

Conventional methods for detoxification of arsenic is very expensive that usually depends on a time-consuming oxidation step governed by ozone, chlorine, hydrogen per oxide, etc. and also produces hazardous by-products. So, there is always a need to develop sustainable and cost-effective approach, and potent arsenic oxidizing microorganisms could play a significant role to cope up with this problem (Bahar et al. 2012; Khanikar and Ahmaruzzaman 2023). In recent past, it has been reported that bacterial species like *Pseudomonas*, *Thiobacillus*, and *Anaeromyxobacter* contribute in arsenite oxidation and provide plant growth-promoting attributes as well (Li et al. 2023; Lin et al. 2023). Other than that, various bacterial metabolisms like transformation (oxidation, reduction, methylation), adsorption, and accumulation in the environment could partially immobilize arsenic (Kabiraj et al. 2023a). In rhizosphere, these activities of bacteria indirectly hinder uptake of arsenic through the plant root system. In arsenic rich environment, there is a possibility to exempt arsenic biosorption through plant root (one of the major entry points of this poison to humans) by introducing selective arsenic resistant bacterial species. Beside application of arsenic resistant plant growth-promoting bacteria, application of biochar on rice fields is also a cost-effective and sustainable approach that can reduce availability and toxicity of arsenic to the rice plants as well as enhance their nutrient uptake through roots (Majumdar et al. 2023). Exogenous addition of thiourea on rice field has positive impact on rice grain yield. It also helps to reduce arsenic accumulation in the grains. The antioxidant properties of this chemo-priming agent reduce the negative impact of reacting oxygen species (ROS) (Upadhyay et al. 2022).

There are some potent plant growth-promoting rhizobacteria (PGPR) that can alleviate the arsenic toxicity from rice plants (Singh et al. 2016; Ghosh et al. 2018; Banerjee et al. 2020; Bist et al. 2022). Bacterial species may reduce the availability of arsenic metalloid in root periphery by accumulating it within the cells and accelerating plant growth-promoting attributes (Singh et al. 2016; Ghosh et al. 2018).

Micrococcus luteus has wide range of distribution and can tolerate high concentrations of different heavy metals (or metalloids). Members of *M. luteus* have high GC content (more than 70%) which is an interesting feature of this species. Whole genome of many strains of *M. luteus* has been studied extensively in the last decade on different aspects (Young et al. 2010; Sher et al. 2020a; Martínez et al. 2021). However, comprehensive genomic characterization of *M. luteus* in respect to arsenic and heavy metal tolerance along with plant growth-promoting attributes has not been elucidated so far. In this perspective, we have isolated an arsenic-resistant species of *M. luteus* from arsenic contaminated groundwater and performed its physico-biochemical as well as genomic characterizations. The capability of this species to adsorb arsenic and probable applicability as plant growth stimulator on rice seedling growth have been screened. This study has demonstrated the potentiality of arsenic tolerance and multi-metal resistance of *M. luteus*. Furthermore, insight into its genomic features provides a clear image of its resistance against arsenic and other metal tolerance and useful to develop bioengineered cells for the possible remediation of arsenic.

Materials and methods

Sampling

Water sample was collected aseptically from a tube well after 5 minutes pumping of Purbasthali village (23° 23′ 26.43″ E and 88° 19′ 43.50″ N); situated in Purba Bardhaman district of West Bengal, India. At the time of sampling, pH, TDS, and temperature of the sample were measured and finally the sample was stored at 4 °C for further analyses. Electro conductivity (EC), quantitative analyses of Ca, K, and Na ions were done using flame photometer (Systronics, 128, India).

Isolation

To isolate arsenic tolerant bacteria, water sample (after 10^{-3} times dilution) was spreaded on Luria Bertani (LB) (HiMedia, India) Agar media (pH 7.0) associated with 20 mg/L, 40 mg/L, 60 mg/L, 80 mg/L, and 100 mg/L concentrations of sodium arsenate dibasic heptahydrate (Na_2AsHO_4, $7H_2O$) (HiMedia, India). After overnight incubation aerobically for 48 h at 37 °C, the best isolate (on the basis of arsenic tolerance) was selected among various colonies for further studies.

Physico-biochemical parameters and antibiotics susceptibility screening

Extracellular enzyme production (catalase, amylase, protease, citrase (citrate utilization test)), carbon source utilization (glucose, fructose, galactose, mannitol), colony morphology, motility, endospore staining, and Gram character of the selected isolate (i.e., AKS4c) were studied according to the standard protocols (Tittsler and Sandholzer 1936; Skerman 1959; Benson 1990). A total of 23 antibiotics of different groups were analysed to decipher antibiotics susceptibilities (Halder et al. 2022) of the isolate.

Study of multi-metal tolerance ability

Isolate AKS4c was grown in LB media (pH 7.0) against 100 to 1000 mg/L concentration of various metals. Heavy metal salts of Cd (cadmium chloride, $CdCl_2$); Cr (chromium trioxide, CrO_3), Cu (copper sulphate, $CuSO_4$, $5H_2O$), Hg (mercuric chloride, $HgCl_2$), Mo (sodium

molybdate dihydrate, Na_2MoO_4, $2H_2O$), Mn (manganese sulphate, monohydrate, $MnSO_4$, H_2O), Pb (lead (II) acetate 3-hydrate $(CH.COO)_2$ $Pb.3H_2O$), Mg (magnesium sulphate heptahydrate, $MgSO_4$, $7H_2O$), Fe (ferrus sulphate, $FeSO_4$, $7H_2O$), Co (cobalt chloride hexahydrate, $CoCl_2$, $6H_2O$), and Zn (zinc chloride, $ZnCl_2$) was selected for this study. Further methods were followed as described by Halder et al. (2022).

Maximum tolerance concentration (MTC) screening of arsenate and arsenite salts

Isolate AKS4c was grown in LB media (pH 7.0) for 72 h in continuous shaking at 130 rpm at 37 °C against different concentrations (0 to 78,000 mg/L) of sodium arsenate heptahydrate (Na_2HAsO_4, $7H_2O$) and sodium (meta) arsenite ($NaAsO_2$) (HiMedia, India) (concentration varied from 0 to 650 mg/L). Absorbances were taken at 8 h of intervals at 600 nm of wave length by using a visible spectrophotometer (Lasany, Model LI-721, India).

Study of arsenic biotransformation ability

Arsenic biotransformations (oxidation and reduction) by the isolate were screened by silver nitrate test after growing it on Petri plates (Dey et al. 2016; Banerjee et al. 2021) and in liquid culture (Das et al. 2014).

Microscopic analyses

Control, low (100 mg/L), and high (300 mg/L) concentrations of $NaAsO_2$-treated bacterial cells were studied under field emission scanning electron microscopy (FE-SEM, Sigma 300; Zeiss, Germany) and transmission electron microscopy (TEM, JEM 1400 plus, Japan) after proper sample preparation (Majhi et al. 2023).

FTIR and XRF analyses

Arsenic interacts with bacteria and sometimes gets adsorbed by the cells, as a result arsenic immobilization takes place by bacterial biomass. To screen arsenic adsorption capability, isolate AKS4c was grown in without arsenic (control) and supplemented with 100 mg/L arsenite in LB media (pH 7.0) and after 48 h of incubation at 37 °C at 120 rpm; the pellets were harvested after centrifugation at 8000 × g. Then, the pellets were dried properly for further analyses. Dried samples were analysed by Fourier transform infrared spectroscopy (FTIR, IR-Prestige 21, Shimadzu, Japan) and X-ray fluorescence (XRF, Bruker nano; ARTAX, Germany).

Study of growth under various pH, NaCl concentration, and temperature

AKS4c was grown (both control and 100 mg/L arsenite-treated conditions) in different pH (4.0–11.0) and NaCl concentrations (5.0 to 60 g/L) in LB, and absorbance was taken at 600 nm after overnight shaking at 120 rpm at 37 °C for 24 h. For temperature optimization, isolate was grown in 10–50 °C temperature. For each case, results were taken against 600 nm of wavelength.

Genome sequencing and assembly

Strain AKS4c was incubated in NB medium and from exponential growth phase, genomic DNA was extracted using a Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, USA). The purity and concentration of the obtained DNA was measured with a NanoDrop 1000 (Thermo Scientific, USA). Agarose gel electrophoresis (1.5%) was used to check the DNA integrity (Halder et al. 2022). Genomic DNA was fragmented using Covaris followed by end repair and adapter ligation. TruSeq™ DNA PCR-Free library preparation kit (Illumina, Inc., USA) was used to construct sequence libraries. After denaturation of libraries, the genome was sequenced on Illumina MiSeq (Illumina, USA). Then, the paired end raw reads were trimmed with Trimmomatic v.0.39 (Bolger et al. 2014), and sequence quality was checked with FastQC v.0.11.5 (Andrews 2010). For the de novo assembly, SPAdes v.3.13.0 (Bankevich et al. 2012) tool was used, and the draft assembly quality was checked with QUAST v.5.0.2 (Gurevich et al. 2013); furthermore, the quality of the genome was estimated by using CheckM v1.2.0 (Parks et al. 2015).

Genome annotation

The genus and species level identification were carried out using the Type (Strain) Genome Server (TYGS) (Meier-Kolthoff and Göker 2019). Further, a pairwise average nucleotide identity (ANI) values between close relatives of AKS4c were calculated using JSpeciesWS (Richter et al. 2016), and an ANI heatmap was generated in R (4.1.1) using 'pheatmap' package. OrthoVenn2 (Xu et al. 2019) was used to compare orthologous gene clusters between the species clustered with genome of AKS4c. A circular genome view of the assembled AKS4c genome was generated using CGView server (Grant and Stothard 2008). Functional gene annotation and functions of the predicted proteins were analysed by NCBI

Prokaryotic Genome Annotation Pipeline (PGAPP) (Tatusova et al. 2016), Rapid Annotation using Subsystem Technology (RAST) (Aziz et al. 2008), and Rapid Prokaryotic Genome Annotation (PROKKA) v.1.14.5. The 'PHASTER' online tool (Arndt et al. 2016) was used to identify prophage regions, and IslandViewer 4 (Bertelli et al. 2017) was used to analyse genomic islands.

Data availability

The whole-genome shotgun project was deposited at DDBJ/ENA/GenBank under the accession number JAIQWC000000000.1 (BioProject number PRJNA762542 and BioSample number SAMN21397803).

Study of plant growth-promoting traits of the isolate

Quantifications of indole acetic acid (IAA), Gibberellic acid (GA), were performed as described by methods of Bric et al. (1991) and Das et al. (2014) for IAA; and Abou-Aly et al. (2019) for GA. For proline estimation, 48 h grown bacterial culture was centrifuged, and supernatant was used. Then, its quantification was done by the described method of Theriappan et al. (2011). There were two sets of experiments, control and arsenite (300 mg/L) treated.

Ammonia production and phosphate solubilizing activities were qualitatively screened following described methods of Kumar et al. (2012). Nitrogen fixation test was screened by growing AKS4c in nitrogen free medium. Siderophore production was screened (Podschun et al. 1992) where the media was associated with chrome azurol S (CAS).

Study of the efficacy of AKS4c on rice seedlings growth promotion

Khitish (IET 4094) variety of rice (*Oryza sativa* L.)seeds were collected from Crop Research and Seed Multiplication Farm (Burdwan University). Seeds were sterilized by using 0.1% mercuric chloride solution ($HgCl_2$). In the Petri plates, total 25–30 uniformed sized seeds were placed. Total four sets of experiments were considered here, i.e. control, only bacteria-treated (only AKS4c), arsenic-treated (As), and finally, both arsenic and bacteria-treated (As + AKS4c). For bacterial treatment purpose, strain AKS4c was grown in NB, and at the middle of log phase (OD 0.5, ~ 10^6 CFU/mL), the culture was centrifuged at 6000 × g. Pellets were taken after rigorous washing using phosphate buffer saline (PBS). After that, pellets were resuspended in double distilled water and treated on the Petri plates. The rice seedlings were grown in controlled condition as described by Ghosh et al. (2021). Only double distilled water was supplied throughout the experiment. After 10 days, root and shoot lengths were measured.

Results and discussion

Sampling and isolation

The water sample was associated with TDS 211 ppm, pH 6.5, temperature 22 °C, EC 349, Ca^{2+} 138.70 mg/L, K^+ 6.64 mg/L, and Na^+ 22.09 mg/L. From the sample, seven bacteria were isolated; among them, isolate AKS4c was selected on the basis of its maximum arsenic salt tolerance capability. Colony morphology of this Gram-positive isolate showed circular colony, margin-entire, colour-yellow, elevation-convex, opaque, and texture were smooth.

Arsenic contamination in Purbasthali groundwater and its negative impact on agriculture and human beings were studied by different researchers (Nag et al. 1996; Biswas 2010; Dey et al. 2016; Ghosh et al. 2017; Chatterjee et al. 2022). The availability and concentration of arsenic vary seasonally and geographically. Biswas (2010) reported presence of 80–120 µg/L arsenic in groundwater of Purbasthali collected from tube wells of different depths; whereas, about 50.6 µg/L concentration of arsenic was reported after the study of Ghosh et al. (2017). Two bacterial strains, viz. *Bacillus* sp. SW2 and *Aneurinibacillus aneurinilyticus* SW4 were isolated from arsenic contaminated water of the same region (Dey et al. 2016). But, comprehensive studies by whole genome analyses with respective genes related to arsenic resistance are not elucidated so far.

Physico-biochemical parameters and antibiotics susceptibilities screening

The isolate was nonmotile, catalase, and lipase positive and can utilize glucose and fructose as carbon sources. It was protease and amylase negative and cannot utilize citrate, mannitol, and galactose as primary carbon source. As per previous report, no endospore was found to be produced by the isolate.

This bacterium was resistant to nalidixic acid and aztreonam, but it was susceptible to 21 other antibiotics (Supplementary file Table S1). Less than 10-mm inhibition zone was considered as resistant (R). Bacterial susceptibility to antibiotics is considered as an important trait of bacteria for application in field. Another strain of *M. luteus* was susceptible to gentamycin, erythromycin, and ciprofloxacin, whereas resistant to co-trimoxazole, clindamycin,

and augmentinantibiotic (Munawar 2021). Isolate AKS4c was also susceptible to gentamycin and erythromycin. A strain of the same species was isolated from bovine frozen semen, and it was also sensitive to amoxicillin, erythromycin, gentamicin, neomycin, etc. (Abro et al. 2021).

Multi-metal tolerance

The bacterium was highly resistant (> 1000 mg/L) to heavy metals (HMs) salts like Fe, Mo, and Pb. Bacterial growth was inhibited about 1000 mg/L concentration of another three HMs (Cr, Co, and Mn). The isolate was able to tolerate > 400 mg/L cadmium salt (Fig. 1c), but was highly sensitive to Hg, where the bacterial growth was totally inhibited at 100 mg/L. HMs like Pb and Hg are highly toxic elements for any kind of organisms. On the other hand, optimum concentrations of HMs like Fe and Cu are essential factors for various cellular metabolisms. But, more than optimum concentration, these elements exert negative impacts. In general, bacteria use to deal with different challenging situations throughout their life cycle; HM stress is one of them. Subsequently, bacteria adopted multiple mechanisms to overcome the HMs stress. It may be genetic or physio-morphological; for example, bacterial cell walls sometimes adsorb metallic ions passively and restrict their entry (Nanda et al. 2019). Otherwise, they might accumulate metals after active uptake or transform in different oxidative forms (Kabiraj et al. 2022). MIC values for *M. luteus* strain AS2 against multiple metals such as Pb, Cd, Cr, Hg, and Co were (in mM) 5.0, 3.0, 4.0, 1.50, and 5.0, respectively (Sher et al. 2019).

MTC screening of arsenate and arsenite salts

The bacterium was able to tolerate more than 46,800 mg/L arsenate [As (V)] and 390 mg/L arsenite [As (III)] salts (Fig. 1 a, b). More than these concentrations (i.e. under 62,400 mg/L for arsenate and 650 mg/L arsenite concentrations), a little growth (O.D. ~ 2.1, 4.2×10^5 CFU/mL for arsenate and O.D. ~ 2.3, 4.6×10^5 CFU/mL for arsenite) was found after 40–48 h of continuous shaking. As (III) is about hundred times more toxic than As (V). So, bacterial tolerating concentration against As (III) is significantly lower than As (V). Significant growth retardation had been observed in arsenic stress. Cell division under arsenic stress is highly affected; consequently, log phase of its growth is retarded. *M. luteus* strain AS2 could tolerate quite higher concentration of As (V) 275 mM (~ 85,800 mg/L) and 55 mM (~ 7,150 mg/L) As (III) salts (Sher et al. 2019) than our current strain. Strain BPB1 could tolerate about 650 mM (~ 202,800 mg/L) and 7.5 mM (~ 975 mg/L) As (V) and As (III) concentrations, respectively (Vijay et al. 2017). *Micrococcus* sp. EIKU8 was resistant up to 400 mM (124,800 mg/L) As(V) and 8 mM (1,040 mg/L) As(III) salts (Bhakat et al. 2019). So, this species has a well-established background on arsenic resistant

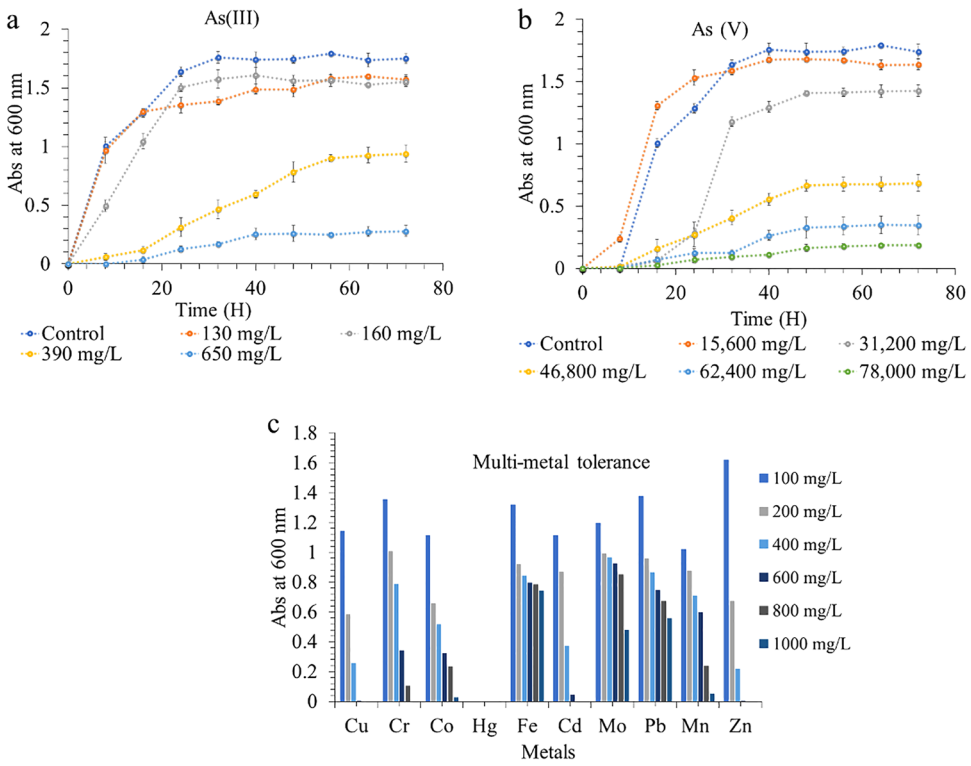

Fig. 1 Growth of *M. luteus* strain AKS4c under arsenic and HM stresses against **a** arsenite; **b** arsenate; and **c** multiple metals

capability. Unlike strain AS2, isolate AKS4c showed slow growth at the lag phase, and then, the graph uplifted gradually in As (III)-treated condition (Fig. 1 a, b).

Study of growth under various pH, NaCl concentration and temperature

Optimum pH for the strain was 7.0, and it can tolerate more than 40 g/L sodium chloride concentration. Bacterial growth was found higher in 30 °C and 40 °C (Fig. 2). So, this strain is mesophilic in nature. Salt stress hampers osmotic balance of cell. Bacteria adapted themselves by activating some genes associated with alleviation of salt stress.

Biotransformation

Silver nitrate screening on biotransformation of arsenic compounds showed that the bacterium was able to oxidize arsenite [As(III)] to arsenate [As(V)]. The confirmation experiment also suggested the same result. It was unable to reduce arsenate. Oxidation of As(III) could be considered an important trait, because this metabolism releases less toxic form of arsenic, i.e. As(V). Two genes, i.e. *aioA* and *aioB* (encodes arsenite oxidase), are mainly responsible for arsenic oxidation. After transformation to As(V), the product releases in the media, and after reacting with silver, it produces brownish coloured precipitation (Banerjee et al. 2021). Brownish colour was also found in confirmation experiment as reported by Das et al. (2014). Two species, viz. *Bacillus* sp. SW2 and *Aneurinibacillus aneurinilyticus* SW4, were isolated from water of Purbasthali, West Bengal, India, which also showed As(III) oxidizing capabilities (Dey et al. 2016). *Brevundimonas aurantiaca* PFAB1, isolated from a hotspring of West Bengal, was able to reduce and oxidize arsenic compounds (Banerjee et al. 2021). *M. luteus* strain NE2E1, isolated from plant roots of heavy metal contaminated region of Mexico, reduced 94% As(V) in experimental condition (Román-Ponce et al. 2018). In this study, the bacterium oxidized As(III), but was unable to reduce As(V).

Microscopic analyses

Cell shape of the bacterium was round and usually present in tetrad condition. However, diplococci were also abundant. There was no such morphological difference found in SEM and TEM analyses (Fig. 3). Studies indicate that under 100 mg/L of arsenic stress, sizes of bacterial cells increased; but more than this concentration (300 mg/L), cell size was decreased. Cell size of control set was ~ 1103 nm, whereas under 300 mg/L arsenite salt stress, it was ~ 757 nm (Supplementary file Fig. S1). Reduction of cell size in arsenic stress is also reported by Dey et al. (2016).

FTIR and XRF

IR analyses revealed that presence of plenty number of functional groups like C = C, C-O, C-F, N–O, C = N, N–H, O–H, etc. in the surface of cellular mass, indicating its complexity (Vijay et al. 2017). Among them, due to interactions with arsenic molecules, some sharp shipment of peaks was noticed which have reported by different authors for *Bacillus aryabhattai* MCC3374, *M. luteus* strain BPB1, *Staphylococcus* sp. strain AS6, and *M. luteus* strain AS2 (Vijay et al. 2017; Ghosh et al. 2018; Sher et al. 2020a, 2020b). In this study, absorbance at 2349 cm^{-1} (indicates carbon di oxide) was present in control, whereas absent in treated (Fig. 4a) cell biomass. Additively, the peak of 1637 cm^{-1} was shifted to 1700 cm^{-1}. The hydroxyl bond at 3749 cm^{-1} shifted to 3747 cm^{-1} that might be due to interaction with arsenic; same type of result was also found in strain BPB1 (Vijay et al. 2017). Amino, hydroxyl, and carboxyl groups were also found in cell wall of arsenic-resistant *M. luteus* AS2 (Sher et al. 2020a). Cellular proteins and fatty acids are the main sources of these functional groups. Positively charged arsenic ions possibly get attracted with the negatively charged functional groups containing macromolecules within the cell walls of the strain AKS4c. Beside electrostatic interactions, ion exchange (e.g. replacement of positively charged ions with other positively charged

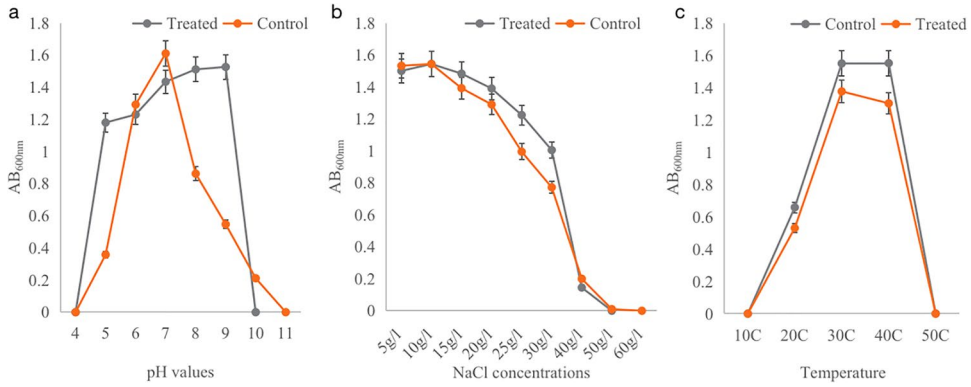

Fig. 2 Growth of stain AKS4c under various **a** pH, **b** salt concentrations, and **c** temperatures

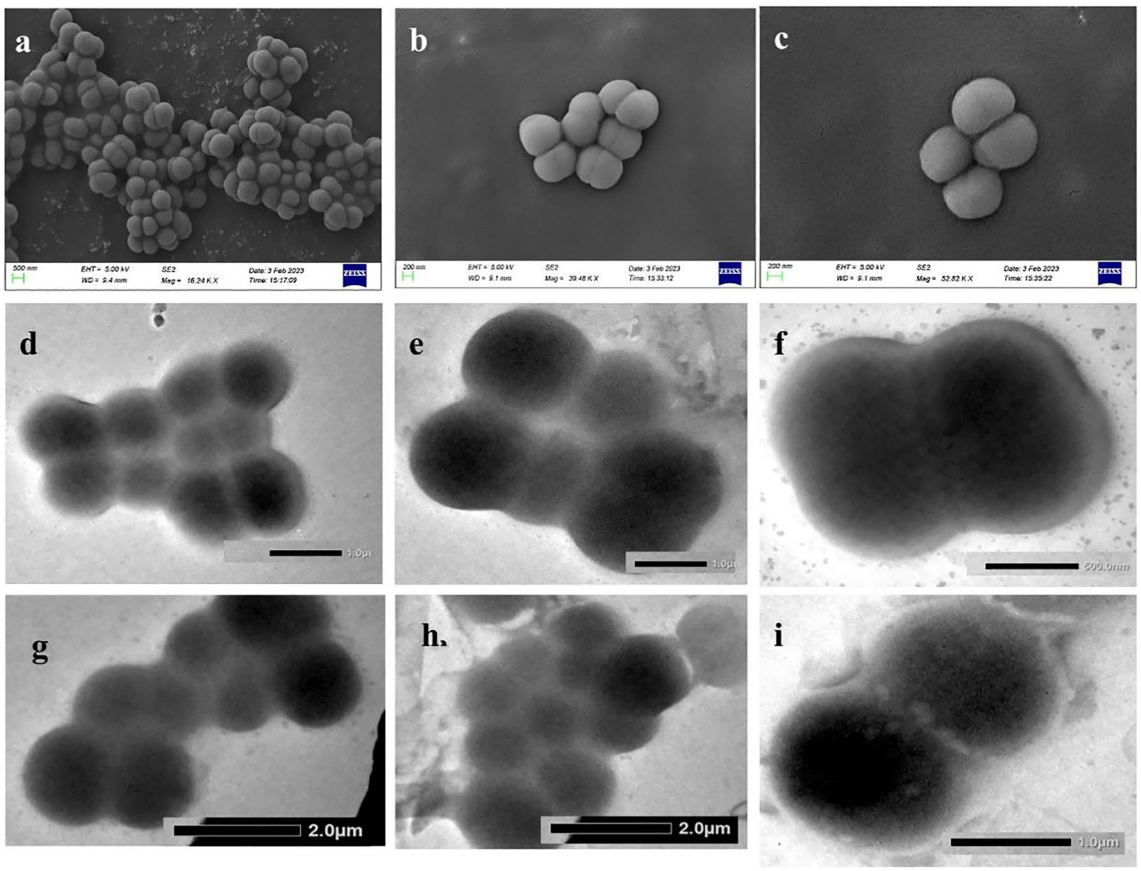

Fig. 3 SEM and TEM showing morphology; **a** SEM micrograph of control samples, **b–c** SEM micrograph of 300 mg/L arsenic-treated cells; **d–f** TEM micrograph of control; and **g–i** TEM micrograph of 300 mg/L arsenic-treated cells

ion) may play pivotal role in arsenic adsorption (Durve and Chandra 2014). By this process, immobilization of arsenic takes place. At the time of treatment, salts of arsenic were given as an arsenic source. So, salt stress might have a preliminary role in peak shipment. But, on the basis of previous studies conducted on various bacterial species (Vijay et al. 2017; Ghosh et al. 2018; Kabiraj et al. 2023b), it could be assumed that arsenic would be the key player for FTIR peak shipment. XRF showed sharp peak of arsenic for treated cell mass; however, no such peak was found in control sample (Fig. 4b). The XRF analyses were conducted in 50 kiloelectron volts (keV) voltage and 698 microampere (µA) current with 1579 count per second (cps) count time. Peak of arsenic was found within the graph (in treated condition) at around 10 keV of energy (Fig. 4b). Arsenic tolerable strains of *B. aryabhattai*, *Pantoea dispersa*, and *Bacillus pacificus* also showed such type of results in XRF analyses (Ghosh et al. 2018, 2021; Kabiraj et al. 2023b). These results support arsenic adsorption capability of this strain which could be applicable in arsenic bioremediation.

Genomics analyses

Quality analysis by using CheckM of the genome of *Micrococcus luteus* AKS4c revealed that the completeness of the genome is around 87.63% and contamination is about 0.46%. The completeness percentile of the genome could be considered as 'substantial' (as the genome completeness was in between ≥ 70 and 90%) and contamination is 'low' (≤ 5%) (Parks et al. 2015). The genome of strain AKS4c consists of 2.42 Mb genome with 73.2% GC content. There are 2256 genes present within the genome among them, 2152 are protein coding genes, 38 are pseudogenes, 13 rRNAs, 48 tRNAs, and 3 other RNAs. Overview of the genome is given in Fig. 5. TYGS and ANI heatmap values indicate this strain is highly similar with the species *Micrococcus luteus* (Fig. 5 a, c). There are 1623 orthologous genes shared by different species under the genus *Micrococcus* and strain AKS4c bears 3 unique genes (Fig. 5 d). According to the RAST analyses, on the basis of 37% of coverage, within 236 subsystems, amino acids and derivatives contain highest number genes (237) which is followed by protein metabolism (154),

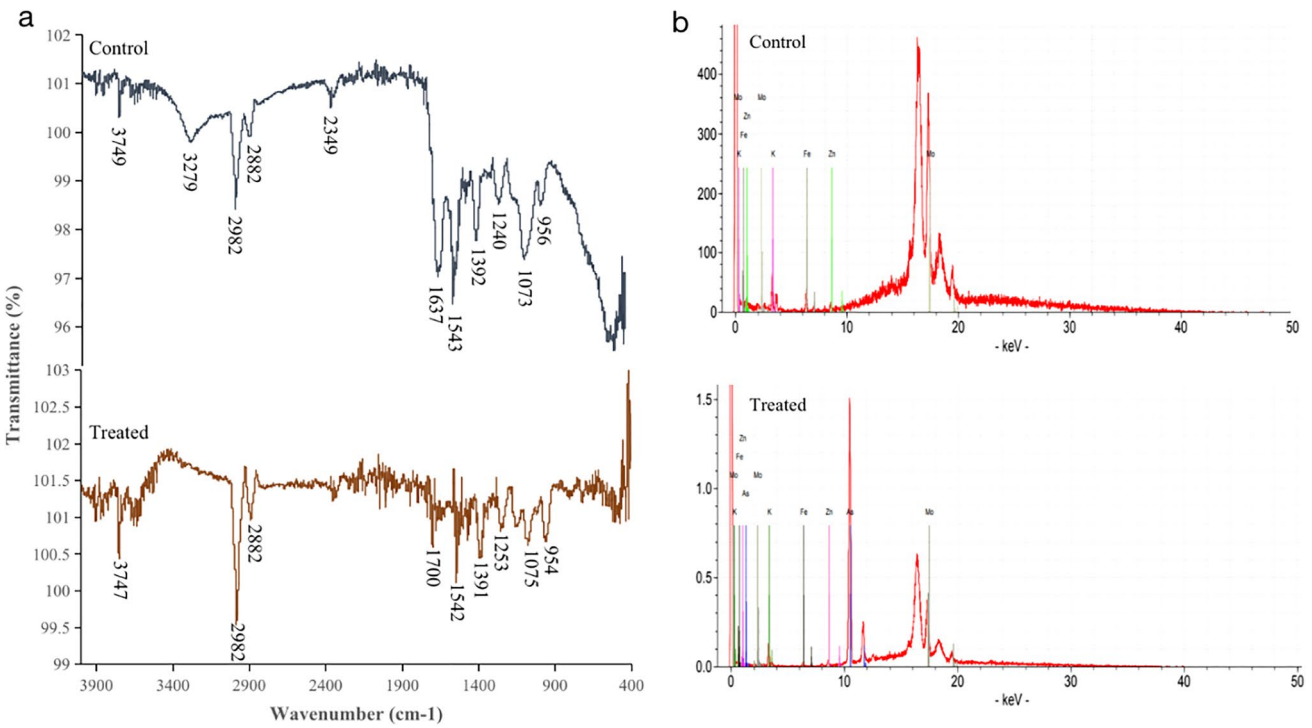

Fig. 4 **a** FTIR spectra indicating the changes in peak in control and arsenic-treated conditions; **b** XRF spectra showing presence of arsenic peak in treated bacterial biomass

carbohydrates (121), etc. (Supplementary file Fig. S2). Membrane transporters are one of the key regulators for metal and antibiotics resistance (Arroyo-Herrera et al. 2022). Total 36 genes responsible for membrane transport have been documented. A few examples of transporters related to metal resistance are MATE family efflux transporter, DHA2 family efflux MFS transporter permease subunit, fluoride efflux transporter CrcB, and ACR3 family protein responsible for arsenic efflux. Arsenic reductase (*arsC*), *ars* operon transcriptional regulator (*arsR*), and flavin-dependent monooxygenase (*arsO*) also have been found to be situated within the genome of the strain AKS4c (Table 1). Bacterial transformation of arsenic is regulated by *ars* operon, and this operon consists of *arsC*, *arsB*, *arsR*, *arsO*, etc. genes. Within the bacterial cell, arsenate is reduced to arsenite by the catalytic activity of the arsenate reductase (ArsC). Then, arsenite is released from the bacteria through the transmembrane arsenic exporter (ArsB). Expression of the genes in the *ars* operon is regulated by ArsR (Kabiraj et al. 2022). Another arsenite exporter (i.e. ACR3) is a common exporter of arsenite which is present around all domains of life. In this current strain, *ACR3* was also found which has role in arsenic resistance.

Other metal-resistant genes like *merA*, *merB*, *merR*, and *cadA* are mainly responsible for cadmium, mercury, cobalt, zinc, etc. resistance. An overview of arsenic, other heavy metal, and antibiotic-resistant genes, genes associated with plant growth regulation, are documented in Supplementary file Table S2.

To survive in arsenic stress, microorganisms have developed various strategies; biotransformation (oxidation, reduction, methylation) of arsenic by using arsenic resistant genes (associated with *ars*, *aio*, *arr*, etc. operons) is one of them. A few putative arsenic-resistant genes were mentioned in the study of Young et al. (2010); *Micrococcus* sp. strain MS-AsIII-49, isolated from metal contaminated area, was also harbouring *arsC*, *arsR*, and *ACR3* (Costa et al. 2015) as we found in the current strain. *M. luteus* strain BPB1 from Bangladesh showed conserved *arsC* genes (Vijay et al. 2017); while lots of arsenic-resistant genes (basically related to arsenate reduction) were found in *M. luteus* strain AS2 (Sher et al. 2020a). These genes detoxify arsenic (convert arsenate to arsenite) and alter its mobility and reduce availability in the environment. Bacteria uplifted with this potentiality can be applied for bioremediation of arsenic through biotechnological approaches.

In context to heavy metal (HM) resistance, there are P-type ATPases related to import and export metallic ions of Cu, Cd, Zn, etc. Genes like *czcD*, *copZ*, and *merA* are common in strain AS2, strain MS-AsIII-49 and strain AKS4c. Beside these, *copA* and mercury-resistant genes (*merB*, *merR*, etc.) are situated in the genome of the current strain.

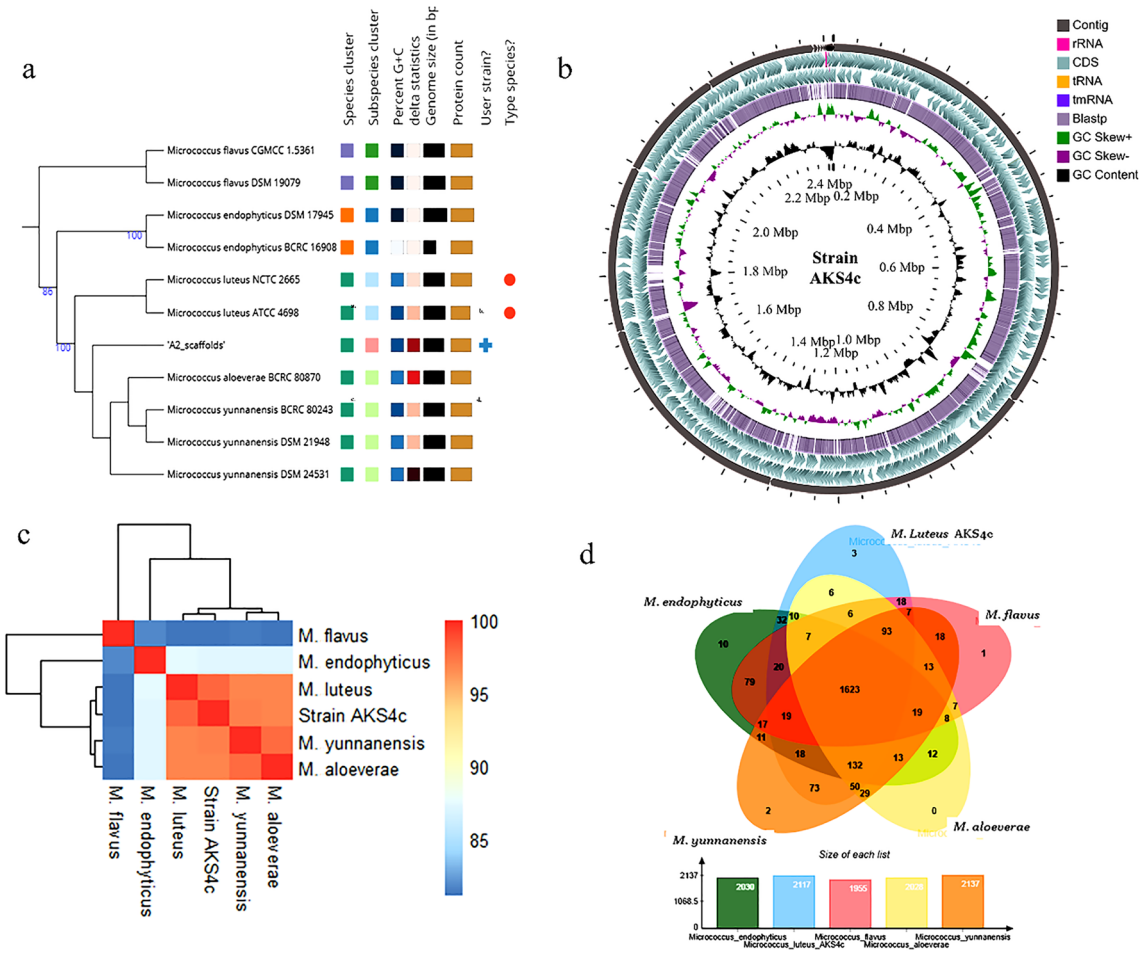

Fig. 5 **a** Phylogenetic tree for identification of the strain by TYGS map; **b** circular view of the genome; **c** ANI heatmap showing relationships of strain AKS4c with other related species of *Micrococcus*; **d** Venn diagram showing orthologous genes shared by different species of *Micrococcus*

Arsenic and other heavy metals exert oxidative stress in cells by producing reactive oxygen species (ROS). Antioxidant enzymes are activated to alleviate the situation. Superoxide dismutase, catalase, and peroxidase are important enzymes for regulating cellular homeostasis. Besides these, glutathione and non-protein thiols are key regulators for reduction of arsenic toxicity (Sher et al. 2020a).

Besides bacterial chromosomes, plasmid encoded genes also provide metal resistant. Usually, ion efflux proteins are encoded by bacterial plasmid associated genes which are easily transferable through plasmid. In the genome of strain

Table 1 Proteins and their functions associated with arsenic resistance

Genes	Proteins name	Functions	Protein length (AA)	References
Arsenic resistant genes (*ars* operon)				
arsC	Arsenate reductase	Arsenate is reduced to arsenite by the catalytic activity of this enzyme	156	Ben Fekih et al. 2018
arsB	Arsenite exporter	Arsenite exporter from the bacterial cell	350	Ben Fekih et al. 2018
arsR	Arsenical resistance operon transcriptional regulator	Negatively regulates the *ars* operon	121	Ben Fekih et al. 2018
arsO	Flavin-dependent monooxygenase ArsO	Functions as flavin dependent monoxygenase and related to arsenic resistance	401	Wang et al. 2006
ACR3	Arsenite efflux transporter	Responsible for arsenite efflux from cell	363	Fu et al. 2009

AKS4c, multiple numbers of cadmium efflux pumps (*cadA*) are present. These pumps may confer high Cd resistance (> 500 mg/L). Interestingly, mercury-resistant genes are present (*merA*, *merB*) in genome, but the bacterium was unable to tolerate > 100 mg/L mercuric chloride. RND driven *czcZ* system in Gram-negative bacteria regulates Cd resistance. Here, being Gram-positive bacteria, strain AKS4c bears both *cadA* and *czcZ* system. However, no such generalized mechanism of HM resistance in bacteria is available. Enzyme-regulated biotransformation, i.e. conversion of HMs (like As, Cu, Hg, and Cd), from one to another oxidative state is well established today. Within the cell, toxic metal ions are sequestered by specific sulphur group containing metallothionein (Nanda et al. 2019).

Hence, the bacterium was resistant to multiple numbers of metals, but susceptible to antibiotics. So, coevolution of multi-metal and multi-drugs concept in bacteria is not supported here. There would be some other selective pressures which might be responsible for such types of results (Biswas et al. 2021). Such type of result also found in the study of Halder et al. (2022), where a strain of *Bordetella petrii* was isolated from hospital soil, and it was multi-drug susceptible but multi-metal resistant.

In the genome of strain AKS4c, tryptophan biosynthesis genes are available (Supplementary file Table S2). Tryptophan is the crucial factor for tryptophan dependent biosynthesis of IAA. Other two components like *trpS* and putative Trp biosynthesis-associated membrane protein have also important functions. Cellular auxin (IAA) might be released from the cell by putative auxin efflux carrier protein, and this protein is usually present in plant. In the section, 'Plant growth promotion attributes screening', we have discussed about IAA production by strain AKS4c. Nitrogen fixation is an important character for microorganisms. Nitrogen metabolism–associated genes are also found in the bacterial genome. Here, putative nitrate reductase converts nitrate to nitrite, and then nitrite is metabolized into ammonia by nitrite reductase. The ammonia transporter then transports ammonia in the outside the cell. This ammonia is taken up by plant root for biosynthesis of nitrogenous compounds, like amino acids and nitrogen bases.

Genomic islands

There are about 22 genomic islands (GIs) associated with > 300 genes (including hypothetical proteins) in the strain AKS4c (Supplementary file Fig. S3). Approximately 12% of the genome is acquired by GIs. The largest GI was found about 43,415 kb long (GI-22), and the smallest was 4637 kb (GI-5). There are some important genes such as ArsR family transcriptional regulator, metalloregulator ArsR/SmtB family transcription factor, glutaredoxin family protein, cation diffusion facilitator family transporter, cadmium resistance transporter, mercury (II) reductase, TetR/AcrR family transcriptional regulator, organomercurial lyase (MerB), and HM-responsive transcriptional regulator. These genes are basically involved in HM resistance. Availabilities of hypothetical proteins open the floor to investigate more genetic features through research. They might play important roles in adaptation of the harsh conditions, but their proper functions are not discovered so far. The presence of transposase enzyme provides the clue to cut out the genomic part from one genome to another for pursuing horizontal gene transfer. GI-associated genes and their probable functions are depicted in Supplementary file Table S3.

To adapt in a specific niche, bacteria use to acquire some specific genetic elements from the environment through horizontal gene transfer (HGT). The role of GIs for providing genotypic plasticity for adaptation in heavy metal, antibiotic contaminated environments, pathogenicity, and bacterial evolution is now well established (Hacker and Carniel 2001; Pathak et al. 2016; Kabiraj et al. 2023c), but proper mechanism of selection and transfer/uptake of genes is not elucidated so far. Total 43 GIs were identified in *Micrococcus* sp. strain 2385 (Pathak et al. 2016); while comparative genomic analyses on different strains of *M. luteus*, Li et al. (2021) got 4–14 GIs. These results indicate rigorous genetic recombination have been occurred in the genome of different strains of *M. luteus*.

GIs are available in the genome, but only one incomplete phage genome was identified by PHASTER to be present (Supplementary file Fig. S4). The sequence of phage genome is about 8.2 kb long and associated with 13 genes. GC content more than 70% is indicating that the bacterial genome might be quite selective in response to the GC content of the accessory genomes. All genes are related to synthesize viral component biosynthesis. So, there might not be direct relationship with bacterial adaptation in a harsh environment.

Beside the genomics study, transcriptomics, proteomics, metabolomics, etc. techniques are now utilized to uncover the actual mechanism of arsenic tolerance by bacteria. Genomics study only can reveal the presence and absence of specific genes for the arsenic detoxification. RNA sequence analyses could discover the transcript level change of gene expression under arsenic stress (Majumdar et al. 2022). So, in future, transcriptome analyses of this strain under arsenic stress will be performed to uncover the actual scenario of arsenic stress management by this strain.

Plant growth promotion attributes screening

IAA, GA, and proline productions were quantitatively analysed. The concentrations of these products were about 187 µg/mL, 143 µg/mL, and 185 µg/mL for IAA, GA,

and proline, respectively, in control condition. Significant enhancement (255 µg/mL) of proline production was documented in arsenic stress. However, concentrations of IAA (172 µg/mL) and GA (135 µg/mL) were slightly reduced in treated sets. These results assured that this bacterium was capable of plant growth hormone production in arsenic stress. Strain AKS4c was able to produce siderophore, ammonia in the media. It was unable to grow in nitrogen free medium and does not solubilize phosphate. However, in Pikovskaya's agar medium, the bacteria were grown properly in 48-h experiment; but, no halo zone was detected. Some authors suggested that only bacterial growth on Pikovskaya's agar medium is enough proof for phosphate solubilizing capability of isolate. In this regard, strain AKS4c can be considered as phosphate solubilizing bacteria. HM-resistant plant growth-promoting bacteria are now highly recommended for sustainable agriculture (Kabiraj et al. 2020). IAA is a plant hormone which enhances cell division, extension of tissues, or cell differentiation. IAA producing abilities of genus *Micrococcus* has been well studied (Shahzad et al. 2017; Mike-Anosike et al. 2018; Boonmahome and Mongkolthanaruk 2023). This genus usually synthesizes IAA by using tryptophan as precursor (i.e. tryptophan-dependent pathway), but Ahmad et al. (2020) reported a tryptophan-independent pathway of IAA biosynthesis. *Micrococcus* sp. strain RSS2 and *M. luteus* strain 4.43 produced 10 µg/mL and > 784 µg/mL IAA, respectively (Mike-Anosike et al. 2018; Boonmahome and Mongkolthanaruk 2023). Ambawade and Pathade (2015) isolated *Bacillus siamensis* strain BE 76 from banana plant which was capable to produce 198.16 mg/L and 254.29 mg/L GAs in the presence and absence of L-tryptophan, respectively. Beside enhancement of seed germination and plant growth promotion, GAs provide potentiality to overcome various abiotic stresses such as, cold, salt, and osmotic stress (Colebrook et al. 2014). Proline has highly beneficial role in plant stress management; it acts as metal chelator, antioxidative defence, and signalling molecule (Hayat et al. 2012). In stressful condition, proline is overproduced; here in this result, in arsenic stress, it showed overproduction than control. Siderophore producing species like *Variovorax paradoxus*, *Microbacterium* sp., and *Methylobacterium mesophilicum* enhanced both plant growth and phytoextraction of HMs from soil. Siderophore not only accumulate irons, but also other essential metallic ions which make available to plant roots, consequently, lead to plant growth promotion (Rajkumar et al. 2010). *Achromobacter xylosoxidans* and *Nitrosomonas europaea* were applied as biofertilizer in the field due to its ability for ammonia production. Ammonia-producing microorganisms supply ammonium ions to plant roots (Khatoon et al. 2020). By uptaking the nitrogen source in the form of ammonium, plants improve their growth.

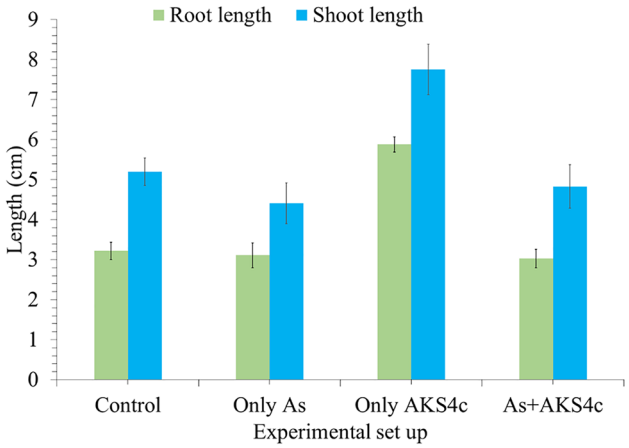

Fig. 6 Role of the bacteria in growth promotion of shoot and root lengths of rice seedlings

Rice seedling growth promotion screening

Arsenic has detrimental impacts on rice seedling which had been proved as we got reduced shoot and root length in arsenic-treated condition (Fig. 6). In respect to length, shoot was much affected than root in arsenic stress. Significant enhancement of lengths of rice seedlings was observed in only AKS4c-treated conditions. Interestingly, root length was shortest in As + AKS4c-treated sets, but length of shoot was increased against only As treated condition. Beneficial plant–microbe interaction enhances the growth of plants. Here, both plant and microbe get benefited to each other. Plant growth-promoting rhizobacteria (free living rhizospheric bacteria) sometimes produce plant hormones, siderophores, fix nitrogen as a result, growth, and development of plants which are enhanced significantly (Afsal et al. 2020). *M. luteus* have been studied as PGPR by applying it on maize (Raza and Faisal 2013), tomato (Badawy et al. 2022), rice (Shahzad et al. 2017), etc. Bacterial strains like *B. aryabhattai* MCC3374, *P. dispersa* AS18, and *B. pacificus* ASK1a were isolated from rice rhizosphere and arsenic-contaminated groundwater and had PGPR activities and alleviated arsenic stress from rice seedlings (Ghosh et al. 2018, 2021; Kabiraj et al. 2023b). Phyto-stimulation of tomato plant by presence of *M. luteus* in Cd and Ni stress was studies by Badawy et al. (2022). But, the role of *M. luteus* in rice seedling growth promotion against arsenic stress might not be elucidated so far. The IR and XRF analyses revealed that this strain had ability to adsorb arsenic. Being a metalloid, arsenic could not be totally eradicated from the environment; we can just reduce its abundance to the living organisms (here rice seedling root) and that could be partially achieved through using bacterial arsenic adsorption mechanism. If bacteria adsorb arsenic, root system of rice seedlings might not be come into contact directly with arsenic ions. But, after death of the bacteria,

adsorbed arsenic molecules may be further released in the environment which is one of the major challenges for the field application of bioremediation technology. Now, in rhizosphere, to overcome this situation, two possibilities can be assumed: after proliferation of applied arsenic tolerable bacterial strain, newly bacterial cells may readsorb the arsenic ions; another way, iron [Fe(III)], the strongest adsorbent of arsenic may help to make complex with arsenic that could change the redox state, solubility of arsenic. So, iron-metabolizing microbes may help in this situation (Darma et al. 2021). In rhizosphere, complex interactions between various microorganisms and plant root take place (Khan et al. 2023). Not only that, plants growth-promoting attributes helps seedlings to grow properly. Genetic analyses, experimental screening on PGP attributes, and finally application on rice seedling growth promotion, this strain can be applied as biofertilizer after robust screening.

Conclusion

Micrococcus luteus is ubiquitously distributed bacterial species, and in this study, it was isolated form arsenic contaminated groundwater. This bacterium was susceptible to multiple numbers of antibiotics and resistant to multi-metals including arsenic. So, theory of co-resistance to antibiotics and heavy metals is not supported properly. The genome is comprised of various HMs and arsenic-resistant genes which may provide it to tolerate stressful conditions. XRF and FTIR confirmed its ability to immobilize arsenic by bacterial biomass. Subsequently, it was able to synthesize plant growth hormones in arsenic stress. Thus, strain AKS4c can reduce arsenic availabilities to plants, additively producing some PGP traits which results rice seedling growth promotion in arsenic stress. So, this strain might be applicable in arsenic bioremediation from rice field after further studies. In future, transcriptome and proteome profiling of both bacteria and rice seedling can provide a better picture of plant–microbe interactions.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11356-023-30361-7.

Acknowledgements We are thankful to UGC-Centre for Advanced Study, Department of Botany and DST-FIST, The University of Burdwan, Burdwan, for pursuing all the research facilities. We also acknowledge University Science and Instrumentation Centre (USIC), BU. RB and AK are thankful to DHESTBT (WB-DBT) [Memo. No. 819(Sanc.)/STBT-11012(11)/11/2020-ST SEC], and UH is thankful to SRF (State Fund) for the financial assistance [Fc (Sc.)/RS/SF/BOT./2017-18/22].

Author contribution Idea: RB. Experiment: AK and UH. Manuscript writing: AK. Genome sequencing: AC and RKV. Genome analyses and editing: AK, UH, and RB. All authors read the full manuscript and approved for submission.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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