Remodulation of central carbon metabolic pathway in response to arsenite exposure in *Rhodococcus* sp. strain NAU-1

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**Summary**

Arsenite-tolerant bacteria were isolated from an organic farm of Navsari Agricultural University (NAU), Gujarat, India (Latitude: 20°55’39.04” N; Longitude: 72°54’6.34” E). One of the isolates, NAU-1 (aerobic, Gram-positive, non-motile, coccobacilli), was hyper-tolerant to arsenite (AsIII, 23 mM) and arsenate (AsV, 180 mM). 16S rRNA gene of NAU-1 was 99% similar to the 16S rRNA genes of *Rhodococcus* (Accession No. HQ659188). Assays confirmed the presence of membrane bound arsenite oxidase and cytoplasmic arsenate reductase in NAU-1. Genes for arsenite transporters (arsB and ACR3(1)) and arsenite oxidase gene (aoxB) were confirmed by PCR. Arsenite oxidation and arsenite efflux genes help the bacteria to tolerate arsenite. Specific activities of antioxidant enzymes (catalase, ascorbate peroxidase, superoxide dismutase and glutathione S-transferase) increased in dose-dependent manner with arsenite, whereas glutathione reductase activity decreased with increase in AsIII concentration. Metabolic studies revealed that *Rhodococcus* NAU-1 produces excess of gluconic and succinic acids, and also activities of glucose dehydrogenase, phosphoenol pyruvate carboxylase and isocitrate lyase were increased, to cope with the inhibited activities of glucose-6-phosphate dehydrogenase, pyruvate dehydrogenase and α-ketoglutarate dehydrogenase enzymes respectively, in the presence of AsIII. Enzyme assays revealed the increase in direct oxidative and glyoxylate pathway in *Rhodococcus* NAU-1 in the presence of AsIII.

**Introduction**

Microorganisms play an essential role in shaping the natural environment. They have evolved specific metabolic pathways allowing them to utilize a wide range of substrates, many of which are toxic to higher organisms. Through the conversion of both anthropogenic and naturally occurring pollutants to less toxic products, such microorganisms affect widespread natural bioremediation. An important toxic compound is arsenic, a metalloid that primarily exists in two redox states: the reduced form, arsenite (AsIII), and the oxidized form, arsenate (AsV). AsIII is more toxic to most of the organisms, as it is more soluble and mobile than arsenate (Jackson *et al.*, 2003). Inorganic arsenic species are classified as potent human carcinogens. The US Environmental Protection Agency (EPA) has reduced the maximum contaminant level (MCL) for arsenic in drinking water to 10 μg l⁻¹ (Agency USEP, 2001); however, the groundwater arsenic concentration in some areas of India and Bangladesh has exceeded to an alarming level of 2000 μg l⁻¹ (Tripathi *et al.*, 2007).

AsIII interferes with sulfhydryl groups in amino acids and dithiols (glutaredoxin). The enzymes which generate cellular energy in glycolysis [phosphofructokinase (PFK), hexokinase and glyceraldehyde 3-phosphate] and citric acid cycle [pyruvate dehydrogenase (PDH)] are also severely affected by AsIII (Mandal and Suzuki, 2002; Ralph, 2008). AsV, a phosphate analogue, can interfere with phosphate uptake and oxidative phosphorylation by binding to the Fo/F1 ATP synthase, thereby inhibiting ATP production. Exposure to arsenicals either *in vitro* or *in vivo* in model organisms caused the induction of heat shock proteins (Hsp), superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione peroxidase enzymes (Hughes, 2002).

Microorganisms cope with the toxic effects of arsenic by: (i) minimizing the uptake of arsenate through the system for phosphate uptake, (ii) increasing the level of antioxidants to reduce the effect of reactive oxygen...
species and (iii) using arsenic detoxification pathway, the
ars operon (Ahmann et al., 1994; Ji and Silver, 1995;
Mukhopadhyay et al., 2002). All these studies suggest that arsenite resistance in bacteria involves multiple factors. Therefore, the aim of this study was to isolate an arsenite-tolerant microorganism and understand the metabolic perturbations involved during As\textsuperscript{III} tolerance. Our results clearly demonstrated that in Rhodococcus sp. strain NAU-1, ars operon, antioxidant system and remodulation of central carbon metabolic pathway play an important role during arsenite exposure.

Results

Isolation and characterization of arsenite-tolerant bacteria

Twenty isolates of arsenite-resistant bacteria were isolated from organic farm of Navsari Agricultural University (NAU), Gujarat, India. Out of these 20 isolates, one isolate (NAU-1) showed hyper-tolerance to both As\textsuperscript{III} (Arsenite, 23 mM) and As\textsuperscript{V} (Arsenate, 180 mM). Microscopic observation of the isolate (NAU-1) was found to be Gram-positive, non-motile and coccobacilli. Analysis of the partial 16S rRNA sequence of NAU-1 showed 99% identity to 16S rRNA genes of Rhodococcus sp. (Accession No. HQ659188). Growth kinetics of Rhodococcus sp. NAU-1 showed increased lag phase of growth and achieved stationary phase at 21 h in the presence of As\textsuperscript{III}-amended M9 minimal media containing 100 mM glucose as carbon source (Fig. 1). Rhodococcus sp. strain NAU-1 could also grow in the presence of other heavy metals (data not shown) like CuSO\textsubscript{4} (1.5 mM), CoCl\textsubscript{2} (2 mM), CdCl\textsubscript{2} (1 mM), NiCl\textsubscript{2} (1 mM) and HgCl\textsubscript{2} (0.1 mM).

DNA fragments from different genes associated with arsenite resistance were amplified by PCR using Rhodococcus sp. strain NAU-1 genomic DNA and gene-specific primers: arsenite oxidase (aoxB, ~ 450 bp), arsenite efflux pump (arsB, ~ 700 bp) and arsenite transporter (ACR3(1), ~ 750 bp) (Fig. 2).

Biotransformation of arsenate and arsenite

Biotransformation potential of arsenic-tolerant Rhodococcus sp. strain NAU-1 was analysed by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES). In intracellular samples the G6PDH enzyme activity was found to be 4.94 ± 0.38 whereas in extracellular sample only 0.04 ± 0.00 μM min\textsuperscript{-1} mg\textsuperscript{-1} total protein. In the study, intracellular and extracellular As\textsuperscript{III} concentrations were found to be 0.054 ± 0.003 mM and 1.14 ± 0.12 mM respectively. Similarly, intracellular and extracellular concentrations of As\textsuperscript{V} were 0.051 ± 0.007 mM and 2.56 ± 0.09 mM respectively. Arsenite oxidase activity was localized mainly in the membrane fraction compared with cytosolic fraction. Also, a positive correlation between membrane bound specific arsenite oxidase activity and As\textsuperscript{III} concentration was observed (Fig. 3). Unlike arsenite oxidase, arsenate reductase activity was more prominent in cytoplasmic fraction (10.5 ± 0.13 μM min\textsuperscript{-1} mg\textsuperscript{-1} total protein) than the membrane fraction (0.12 ± 0.01 μM min\textsuperscript{-1} mg\textsuperscript{-1} total protein) at 5 mM As\textsuperscript{V} (Fig. 3). No arsenite oxidase and arsenate reductase activities were detected in any of heat-killed controls.

Biochemical alterations during arsenic tolerance

To understand the metabolic adaptations during arsenite stress in Rhodococcus sp. strain NAU-1, changes in the level of the following parameters were studied.

Carbon metabolism. Carbon metabolic pathway of Rhodococcus sp. strain NAU-1 was studied by performing assay of some important enzymes and analysis of organic acid concentrations. GDH (glucose dehydrogenase)
Activities of PDH, α-KGDH, MDH, ICL, G6PDH, CS, GDH, ICDH and PPC in *Rhodococcus* sp. NAU-1.

Table 1. Activities of PDH, α-KGDH, MDH, ICL, G6PDH, CS, GDH, ICDH and PPC in *Rhodococcus* sp. NAU-1.

| Metabolic enzymes | As(III): 0 mM | As(III): 1 mM | As(III): 5 mM | As(III): 10 mM |
|-------------------|---------------|---------------|---------------|---------------|
| PDH               | 11.78 ± 0.81  | 5.53 ± 0.50***| 3.58 ± 0.086***| 2.13 ± 0.14***|
| α-KGDH            | 18.18 ± 0.39  | 8.37 ± 0.44***| 4.42 ± 0.20***| 1.36 ± 0.13***|
| MDH               | 5.16 ± 0.27   | 4.34 ± 0.28**| 3.75 ± 0.20   | 0.86 ± 0.10***|
| ICL               | 13.66 ± 0.087 | 15.00 ± 0.02* | 19.43 ± 2.32**| 26.1 ± 0.79** |
| G6PDH             | 9.39 ± 0.29   | 6.57 ± 0.17***| 4.94 ± 0.38***| 2.63 ± 0.13***|
| CS                | 17.71 ± 0.28  | 56.80 ± 1.36***| 60.68 ± 0.37***| 75.68 ± 0.84***|
| GDH               | 27.27 ± 1.53  | 57.17 ± 0.15***| 78.13 ± 5.25***| 121.91 ± 2.57***|
| ICDH              | 71.85 ± 2.41  | 92.26 ± 0.89***| 93.85 ± 1.27***| 95.23 ± 2.95***|
| PPC               | 17.87 ± 1.64  | 104 ± 2.65*** | 371.65 ± 1.02**| 410.63 ± 8.86**|

a. MDH is expressed in μM min⁻¹ mg⁻¹ total protein.

All the enzyme activities were estimated from mid log phase to late log phase cultures except for CS and ICDH which were estimated in stationary phase. All enzyme activities are expressed in μM min⁻¹ mg⁻¹ total protein except for MDH which is depicted in μM min⁻¹ mg⁻¹ total protein. The values are depicted as mean ± SEM of three independent observations. ***P < 0.001; **P < 0.01; *P < 0.05; ns, non-significant. All parameters are compared with the control, i.e. culture without arsenite in medium.
Table 2. Intracellular and extracellular organic acid production from arsenite-treated (5 mM) and untreated samples of Rhodococcus sp. NAU-1 (expressed in mM).

| Organic acids (mM) | Intracellular concentrations | Extracellular concentrations |
|-------------------|-----------------------------|-----------------------------|
|                   | AsIII: 0 mM                  | AsIII: 5 mM                  | AsIII: 0 mM                  | AsIII: 5 mM                  |
| Gluconic acid     | 2.79 ± 0.26                 | 10.7 ± 0.7***               | 4.01 ± 0.03                  | 48.57 ± 0.48***              |
| Pyruvic acid      | 2.83 ± 0.23                 | 5.06 ± 1.01***              | 0.66 ± 0.01                  | 0.95 ± 0.00***               |
| Succinic acid     | 0.99 ± 0.14                 | 2.92 ± 0.11***              | 0.36 ± 0.04                  | 0.10 ± 0.10***               |
| Citric acid       | 0.62 ± 0.04                 | 0.79 ± 0.03*                | 0.72 ± 0.03                  | 0.58 ± 0.00*                 |
| α-KG              | 0.50 ± 0.02                 | 1.02 ± 0.05***              | 1.62 ± 0.05                  | 1.05 ± 0.1*                  |
| Fumaric acid      | 0.51 ± 0.01                 | 0.21 ± 0.02***              | 0.10 ± 0.01                  | 0.08 ± 0.01**                |
| Malic acid        | 0.6 ± 0.02                  | 0.19 ± 0.01***              | 0.63 ± 0.01                  | 0.38 ± 0.00**                |
| Oxaloacetic acid  | 0.39 ± 0.02                 | 0.86 ± 0.01***              | 0.11 ± 0.01                  | 0.28 ± 0.04***               |

Organic acid yields were estimated by comparing the retention time of sample with standards. Stationary phase cultures grown on amended M9 medium were collected. Results are expressed as mean ± SEM of three independent observations.

*P < 0.05; **P < 0.01; ***P < 0.001; ns, non-significant, after comparing treated samples with untreated controls.

2006). ICP-OES analysis of AsIII-treated Rhodococcus sp. strain NAU-1 revealed the presence of extracellular and intracellular AsIII and AsV. Remarkably, in Rhodococcus sp. strain NAU-1, arsenite oxidase is localized in the inner plasma membrane whereas arsenate reductase is localized in the cytosol and arsenite oxidase activity is ~10-fold more than arsenate reductase. Thus, predominant membrane-bound arsenite oxidase converts the initial arsenite supplied to the bacterium to less toxic arsenate, and then the arsenate enters the bacterial cells via phosphate transport membrane systems (Cervantes et al., 1994; Rosen, 2002). Inside the cytoplasm of cells, Arsenated tolerance was observed in Rhodococcus sp. strain NAU-1, which involves AsV reduction again to AsIII via cytoplasmic AsV reductase (ArsC) enzyme. AsIII inside the cytoplasm could be extruded out via arsenite efflux pump (arsB) and arsenite transporter (ACR3(1)). Their presence was confirmed by positive PCR amplification of respective arsB and ACR3(1) genes (Accession No. HQ659194).

Non-efflux-based mechanisms of arsenic detoxification were examined in Rhodococcus sp. strain NAU-1. Exposure to arsenic results in increased rates of H2O2 production and membrane lipid peroxidation as a mechanism of tolerance (Kowaltowski et al., 1996; Liu et al., 2001). It was suggested that this process leads to the generation of organic hydroperoxides and oxygen radicals, which in turn induces major components of the oxidative stress response, including SOD and catalase. Interestingly, with increased concentration of AsIII, increased catalase, APOX, SOD and GST activities were observed in NAU-1. The AsIII could bind with glutathione to form arsenite–glutathione complex (AsIII–GS3) by the action of GST enzyme. This complex is then extruded out of the bacterial cells via ABC transporters located on the membrane (Ghosh et al., 1999). Notably, AsIII–GS3 complex is an inhibitor of GR activity (Styblo et al., 1997; Kala et al., 2000). AsIII-treated Rhodococcus sp. strain NAU-1 showed dose-dependent increase in GST- and decrease in GR-specific activity, suggesting the formation of arsenite–glutathione complex (AsIII–GS3) for extrusion by ABC transporters. However, these transporters are not capable of removing the entire amount of AsIII as revealed by intracellular ICP-OES studies. Effects of leftover intracellular arsenic were further analysed. AsIII-treated NAU-1 cells showed a decrease in G6PDH activity (the enzyme mediating the intracellular glucose oxidative phosphorylation) and an increase in GDH activity, followed by an increase in gluconic acid concentration, suggesting that Rhodococcus sp. strain NAU-1 diverts glucose towards

Table 3. Activity of antioxidant enzymes in Rhodococcus sp. NAU-1.

| Antioxidant enzymes (µM min^-1 mg^-1 total protein) | AsIII: 0 mM | AsIII: 1 mM | AsIII: 5 mM | AsIII: 10 mM |
|---------------------------------------------------|-------------|-------------|-------------|--------------|
| CAT                                               | 6.90 ± 0.39 | 8.66 ± 0.10*| 11.59 ± 0.08***| 18.21 ± 0.49***|
| GST                                               | 2.10 ± 0.22 | 5.19 ± 0.19 | 5.07 ± 0.01* | 9.74 ± 0.8*** |
| SOD                                               | 138.45 ± 1.47 | 459.58 ± 7.24** | 1328.21 ± 91.15*** | 3355.35 ± 87.31*** |
| APX                                               | 34.30 ± 1.68 | 45.54 ± 0.08** | 43.30 ± 1.23** | 74.17 ± 1.87*** |
| GR                                                | 39.22 ± 2.92 | 27.24 ± 0.17* | 25.38 ± 0.09** | 14.88 ± 3.82** |

a. SOD is expressed in U mg^-1 total protein.

All enzyme activities were estimated in late log phase. All enzyme activities are expressed in µM min^-1 mg^-1 total protein except for SOD which is depicted in U mg^-1 total protein. The values are depicted as mean ± SEM of three independent observations.

***P < 0.001; **P < 0.01; *P < 0.05. All parameters are compared with the control, i.e. culture without arsenite in medium.
periplasmic-directed oxidative pathway, mediated by GDH. The PEP-pyruvate-OAA node is an important metabolic link between ED (Entner–Doudoroff) pathway and citric acid cycle. Arsenite-treated NAU-1 showed PDH enzyme inhibition, leading to the accumulation of intracellular pyruvic acid. Also, 20.8-fold increased PPC activity was observed, suggesting that the *Rhodococcus* sp. strain NAU-1 could adopt an alternative pathway for oxaloacetic acid (OAA) production. Additionally, arsenite-treated *Rhodococcus* sp. strain NAU-1 showed an increase in CS and ICDH activities and inhibition of α-KGDH complex followed by significant increase in ICL activity and succinic acid concentration (Tables 1 and 2). This suggests the metabolic shift from citric acid pathway to glyoxylate pathway. It will be interesting to investigate the role of GDH and metabolic shift during arsenite tolerance in *Rhodococcus* sp. strain NAU-1. Also, NAU-1 was able to tolerate different concentrations of copper, cobalt, cadmium, nickel and mercury (data not shown). This suggests that multimetal tolerance could be associated with adaptability of central metabolic pathway. It will be interesting to know the nature of metabolic adaptations of *Rhodococcus* sp. strain NAU-1 to AsIII exposure could account for the broad metal tolerance ability.

In conclusion, apart from arsenite oxidation, arsenite efflux mechanism and antioxidant enzymes, central carbon metabolic pathway remodulation by increasing the activity of GDH, PPC and ICL and inhibited activities of G6PDH, PDH and α-KGDH enzymes contribute towards tolerance to arsenite in *Rhodococcus* sp. strain NAU-1.

**Experimental procedures**

**Isolation of arsenite-tolerant bacteria**

The sample was collected from organic soil of Navsari Agricultural University, Gujarat, India (Latitude: 20°55'39.04"N; Longitude: 72°54'6.34"E). Arsenic-tolerant bacteria were isolated by enrichment method. Soil sample was inoculated into amended M9 minimal medium (P > 100 μM) at pH 7.2 (Sambrook and Russell, 2001), supplemented with 100 mM glucose as carbon source (Buch *et al.*, 2009) and 1 mM of sodium arsenite (NaAsO₂) and incubated at 28°C on a rotary shaker for 48 h. The grown culture was harvested and transferred to fresh arsenite-amended medium. After 5–6 subculturing with increasing concentrations of arsenite from 1 to
18 mM, an enrichment culture was established. The culture was sequentially purified by streaking on As\textsuperscript{III}-supplemented amended M9 agar plates, isolated, and morphologically different colonies which could tolerate maximum amount of arsenite were purified by subculturing 5–6 times. Only one strain Rhodococcus NAU-1 showed hyper-tolerance to arsenite from examined bacterial strains; hence, this strain was used for further study; isolate was stored at −20°C as 50% glycerol stock. Prior to use, the strain was grown to mid exponential phase in amended M9 minimal medium at 28°C with shaking.

Identification of bacteria

DNA was isolated from cells grown on amended M9 medium for 18–48 h by NaCl–CTAB method (Sambrook and Russell, 2001). PCR amplification of 16S rRNA gene was carried out using degenerated universal primer pair 27f and 1492r (Biogene) (Aksornchu et al., 2008). The thermal cycle was performed in a Master cycler (Eppendorf, Germany) and consisted of an initial denaturation step at 94°C for 5 min, then 30 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 1.5 min, with a final extension of 72°C for 10 min. Ten microlitres of PCR products were examined by agarose gel electrophoresis and then remaining 40 µl of product was purified using Big dye terminator v3.1 clean up method described in Sambrook and Russell (2001) for sequencing. The purified 16S rRNA fragments were used as templates for DNA sequencing with an ABI Prism 3130 automatic sequencer (Applied Biosystems, USA) using same reverse and forward primers. The derived sequence was analysed by performing online BLAST sequence homology test.

Biochemical and phenotypic characterization

Rhodococcus sp. strain NAU-1 was grown in amended M9 medium at 28°C in 150 ml Erlenmeyer flasks, with 0, 10, 12 and 15 mM NaAsO\textsubscript{2} with continuous shaking at 180 r.p.m. in the orbital shaker. Culture broth of 2 ml was collected at different time intervals till it reached stationary phase; bacterial growth was monitored by measuring optical density (OD) of the cultures at 600 nm using spectrophotometer (CARY 50 UV, Australia). Gram staining of cells of Rhodococcus NAU-1 was carried out and cells were observed by compound microscopy using oil immersion objective to ascertain cell shape. Biochemical tests were performed on cultures grown at 28°C in amended M9 broth as described by Lanyi (1987). Maximum tolerable concentration of Rhodococcus NAU-1 for As\textsuperscript{III} was also checked in Luria–Broth (LB) medium (Sambrook and Russell, 2001) by adding increasing concentrations of As\textsuperscript{III} from 2 to 30 mM. The method of Drewniak and colleagues (2008) was used for determining the minimal inhibitory concentrations (MIC) of other metal elements. Medium supplemented with the respective metal compounds was inoculated with cells from fresh overnight cultures to a final density of ~10\textsuperscript{8} cells ml\textsuperscript{−1} and then incubated for 24 h. The metals and their compounds used for MIC determination are as follows: As\textsuperscript{V} 0.0–250 mM; Cd, Ni, Co, Cu 0.0–3.0 mM; and Hg 0.0–0.5 mM.

Biotransformation of arsenite and arsenate

Samples were inoculated in amended M9 minimal medium with 5 mM arsenite, and allowed to grow till late log phase (OD 1.3, at 600 nm). Twenty millilitres of grown culture was collected and centrifuged at 7168 g for 3 min. Supernatant was collected as extracellular sample. Intracellular samples were then prepared by washing the pellet twice with 100 mM phosphate buffer (pH 7.0), and then resuspending the cells in the same buffer. Cells were then lysed using ultrasonic probe (Sonics Vibra cell 500, USA) with amplitude of 50% at 50 W with 30 s pulses and 15 s off mode for 5–10 min. Intracellular and extracellular samples were confirmed by performing assay of cytosolic marker enzyme G6PDH, as described below. Arsenic in samples was converted to different forms by method described by Cummings and colleagues (1999). As\textsuperscript{V} and As\textsuperscript{III} amounts were determined by use of ICP-OES equipped with hydride generator. Standard solutions of total arsenic supplied by Merck (India) were used. Blanks were HCl (for As\textsuperscript{V}) or mixture of KIO\textsubscript{3} and HCl (for As\textsuperscript{III}), as used in preparation. Controls were heat-killied samples for both the arsenic species. Heat-killied samples were prepared by heating the bacterial grown cultures at 98°C for 10 min.

Amplification and sequencing of arsenic tolerance-related genes

The amplification of aoxB, arsB, ACR3(1) and ACR3(2) genes was performed using four pairs of degenerate primers (#1F and #1R for aoxB, darsB1F and darsB1R for arsB, dacr1F and dacr1R for ACR3(1), dacr5F and dacr4R for ACR3(2)) as described by Cai and colleagues (2009). The PCR products were purified, sequenced and analysed as described above for 16S rRNA gene.

Organic acid analysis

Isolates were grown in amended M9 minimal medium without and with 5 mM of NaAsO\textsubscript{2}. Five millilitres of late log phase cultures with pH below 5.5 was collected and centrifuged at 12 000 g (Eppendorf centrifuge, 5804R) for 5 min. The supernatant was used as extracellular fraction and pellet obtained was washed twice with 50 mM Tris buffer (pH 7.0) and then resuspended in 2 ml of the same buffer. These cell suspensions were sonicated as described above; the sonicate thus obtained was then centrifuged at 14 000 g for 30 min at 4°C and used as intracellular fractions. Extracellular and intracellular fractions were filtered through 0.22 µm filters and stored in −20°C for high-performance liquid chromatogram (HPLC) analysis. Detection and quantification of organic acids was carried out on Knauer advanced HPLC equipped with PDA detector 2800, Knauer plus auto sampler 3800, Knauer manager 5000, Knauer smartline pump 1000, Knauer inline degasser, and Eurospher 100-5 C-18 column 250 mm × 4.6 mm with precolumn and 5 µm particle size (Merck, Germany). The mobile phase was 0.02% orthophosphoric acid (Merck, Germany) in the gradient of flow rate as described by Vyas and Gulati (2009). Eluates were detected at λ 210 nm and identified by retention time and co-chromatographed by spiking the sample with the authentic organic acids. Pure organic acid standards prepared in
double distilled water were filtered using 0.22 μm nylon membranes and their retention times were determined under similar conditions. Comparison of peak areas with external standards was used for quantification.

**Preparation of cells/cell-free extracts and enzyme assays**

Overnight grown cells under amended M9 minimal media conditions were harvested in mid log to late log growth phase from 30 ml of cell culture by centrifugation at 9200 g for 2 min at 4°C. The preparation of cell-free extracts for PPC and G6PDH assays was carried out according to Kodaki and colleagues (1985). The cell pellet was washed once with 80 mM phosphate buffer (pH 7.5) followed by resuspension in the same buffer containing 20% glycerol and 1 mM DTT. The cells were then subjected to lysis by sonicating for maximum 1–1.5 min in an ice bath, followed by centrifugation at 9200 g at 4°C for 30 min to remove the cell debris. The supernatant was used as cell-free extract for the enzyme assays. In case of arsenite oxidase and arsenate reductase enzymes, 50 mM Tris-Cl (pH 7.0) buffer was used and sonicated pellets were also analysed as membrane fractions, to localize the activity of respective enzymes. The whole-cell preparation for GDH assay was carried out by washing the harvested cells thrice with normal saline and resuspending in 0.01 M phosphate buffer (pH 6.0) with 5 mM MgCl₂.

Arsenite reductase activity was measured using a coupled assay system that measures the arsenite-dependent oxidation of NADPH (Mukhopadhyay et al., 2000). Reductase activity was measured as a change in absorbance at 340 nm. The quantity of NADPH oxidized was calculated using an extinction coefficient of 6200 M⁻¹ cm⁻¹. The assay of arsenite oxidase enzyme was carried out by following the method of Anderson and colleagues (1992). The reduction of the artificial electron acceptor 2,4-dichlorophenolindophenol was monitored at 600 nm in the presence of 1.5 and 10 mM As³⁻.

Total catalase (EC 1.11.1.6) activity was determined in the homogenates by measuring the decrease in absorption at 240 nm as the consumption of H₂O₂ (ε = 39.4 mM⁻¹ cm⁻¹) according to the method of Aebl (1984). APOX (EC 1.11.1.11) activity was measured immediately in fresh extract and was assayed by following the decrease in absorbance at 290 nm due to ascorbate oxidation (ε = 2.8 mM⁻¹ cm⁻¹) according to the method of Nakano and Asada (1981). Enzyme activity of APOX and catalase were expressed as the consumption of H₂O₂ (ε = 40 mM⁻¹ cm⁻¹) as the consumption of H₂O₂ (ε = 40 mM⁻¹ cm⁻¹) and the extinction coefficient of 6200 M⁻¹ cm⁻¹ at 340 nm. The molar extinction of CDNB was taken as 9.6 mM⁻¹ cm⁻¹ at 340 nm.

CS (EC 2.3.3.1) activity was estimated by following the absorbance of 5,5′-dithiobis(2-nitrobenzoic acid) at 412 nm, which would change due to its reaction with the thiol group of CoA (Serre, 1969). The assay mixture contained the following in 1.0 ml: Tris/HCl (pH 8.0), 93 mM; acetyl-CoA, 0.16 mM; oxaloacetate, 0.2 mM; 5,5′-dithiobis(2-nitrobenzoic acid), 0.1 mM and cell lysate. The reaction was started by addition of oxaloacetate. The molar absorption coefficient was taken as 13.6 mM⁻¹ cm⁻¹ at 412 nm. PPC (EC 1.1.1.39) activity was monitored by following NADPH oxidation at 340 nm in an assay with NADP and malate dehydrogenase; G6PDH (EC 1.1.1.49) and ICDH (1.1.1.42) activities were determined by following the reduction of NADP at 340 nm; ICL (1.1.1.49) activity was monitored by measuring glyoxylate formation at 324 nm with the aid of phenylhydrazine-1HCl; and GDH (EC 1.1.5.2) was assayed by following the coupled reduction of 2,6-dichlorophenolindophenol at 600 nm, as described by Buch and colleagues (2008). All enzyme activities were determined at 28 ± 2°C, against appropriate controls lacking the substrate or the enzyme source in the reaction mixture. One unit of specific enzyme activity was defined as the amount of protein required to convert 1 mM substrate min⁻¹ mg⁻¹ total protein, unless specified otherwise. Total protein concentration of crude extracts and whole-cell suspensions was measured by a modified Lowry method (Peterson, 1979) using BSA as standard, with corrections made for Tris buffer. All enzyme activities are expressed in nM min⁻¹ mg⁻¹ total protein except that catalase, GST, APOX, GR, MDH, arsenite oxidase and arsenate reductase are depicted in μM min⁻¹ mg⁻¹ total protein and SOD is expressed in U mg⁻¹ total protein.

**Nucleotide sequence accession numbers**

The nucleotide sequences are posted in the NCBI GenBank database. Their Accession Numbers are: HQ659188 for 16S rRNA gene and HQ659194 for ACR3(1).

**Authors’ contributions**

R. J. carried out sample collection and bacterial isolation, drafted the manuscript and participated in molecular genetic studies. H. A. carried out biochemical studies. A. J. participated in the design of the experiments and helped to draft the manuscript. S. J. and N. K. conceived the idea. S. J. coordinated the study and helped to draft the manuscript. All authors read and approved the final manuscript.

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