microbial biotechnology

Microbial Biotechnology (2012) **5**(6), 764–772 doi:10.1111/j.1751-7915.2012.00368.x

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

Raina Jain,[1] Hemanta Adhikary,[2] Sanjay Jha,[1]* Anamika Jha[3] and G. Naresh Kumar[2]

[1]*Department of Biotechnology, ASPEE College of Horticulture and Forestry, Navsari Agricultural University, Navsari, Gujarat 396456, India.*

[2]*Department of Biochemistry, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat 390002, India.*

[3]*Department of Biotechnology, Ashok & Rita Patel Institute of Integrated Study & Research in Biotechnology & Allied Sciences, New Vidyanagar, Gujarat 388121, India.*

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 hypertolerant to arsenite (As^{III}, 23 mM) and arsenate (As^{V}, 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 As^{III} 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 As^{III}. Enzyme assays revealed the increase in direct oxidative and glyoxylate pathway in *Rhodococcus* NAU-1 in the presence of As^{III}.

Received 25 April, 2012; revised 26 August, 2012; accepted 5 September, 2012. *For correspondence. E-mail sanjayjha14@nau.in; Tel. (+91) 2637 282745 ext 659; Fax (+91) 2637 282145.

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 (As^{III}), and the oxidized form, arsenate (As^{V}). As^{III} 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).

As^{III} 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 As^{III} (Mandal and Suzuki, 2002; Ralph, 2008). As^{V}, 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

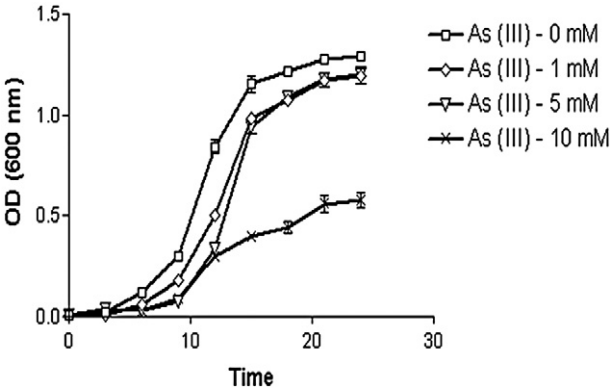

Fig. 1. Growth curve of *Rhodococcus* sp. NAU-1 on amended M9 minimal medium. The values are plotted as mean ± SD of three independent observations.

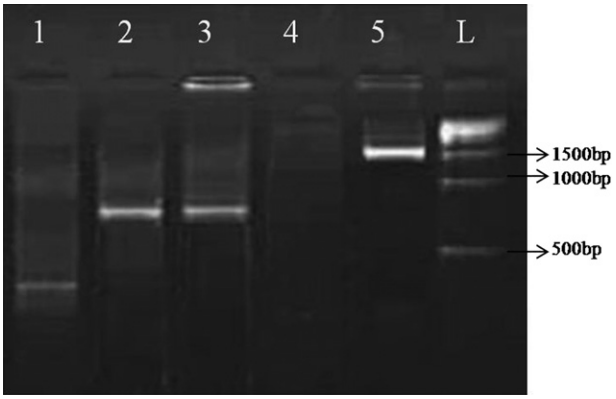

Fig. 2. PCR amplification results of arsenic-related and 16S rRNA genes of *Rhodococcus* sp. NAU-1. Lane 1: *aoxB*; Lane 2: *arsB*; Lane 3: *ACR3(2)* (not present in *Rhodococcus* sp. NAU-1); Lane 4: *ACR3(1)*; Lane 5: 16S rRNA gene segments; Lane L: ladder.

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^{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^{III} (Arsenite, 23 mM) and As^{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^{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 $CuSO_4$ (1.5 mM), $CoCl_2$ (2 mM), $CdCl_2$ (1 mM), $NiCl_2$ (1 mM) and $HgCl_2$ (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^{-1} mg^{-1} total protein. In the study, intracellular and extracellular As^{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^{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^{III} concentration was observed (Fig. 3). Unlike arsenite oxidase, arsenate reductase activity was more prominent in cytoplasmic fraction (10.5 ± 0.13 μM min^{-1} mg^{-1} total protein) than the membrane fraction (0.12 ± 0.01 μM min^{-1} mg^{-1} total protein) at 5 mM As^{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)

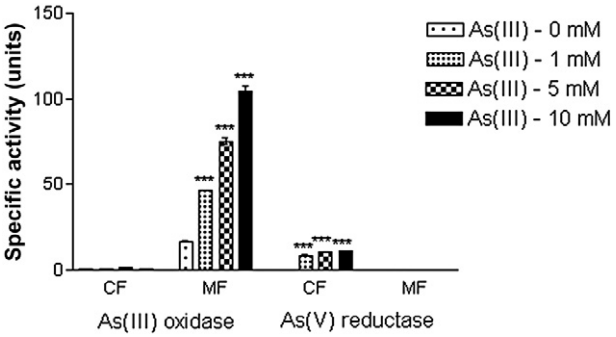

Fig. 3. Arsenite oxidase and arsenate reductase activity in *Rhodococcus* sp. NAU-1. CF, cytoplasmic fraction; MF, membrane fraction. The activities have been estimated in late log phase, without adding arsenite and after adding 1, 5 and 10 mM arsenite in the amended M9 minimal medium. All enzyme activities are expressed in μM min^{-1} mg^{-1} total protein. The values are depicted as mean ± SEM of three independent observations. ***$P < 0.001$, **$P < 0.01$. All parameters are compared with the control, i.e. culture without arsenite in medium.

activity of *Rhodococcus* sp. strain NAU-1 was found to be increased by 2.9-fold when arsenite concentration was enhanced from 1 mM to 5 mM, whereas the activity of G6PDH declined to about 1.9-fold. The enzymes activity of phosphoenol pyruvate carboxylase (PPC) increased to 20.8-fold and PDH decreased to 3.3-fold respectively. Activity of TCA cycle enzymes such as CS and ICDH increased to 3.4- and 1.3-fold respectively, whereas α-KGDH decreased to 4.1-fold. On the other hand, ICL activity of glyoxylate pathway increased by 1.4-fold (Table 1).

At 5 mM As^{III} concentration, intracellular concentrations of gluconic (3.8-fold), succinic (2.54-fold), pyruvic (1.8-fold), citric (1.3-fold), α-ketoglutaric (2.1-fold) and oxaloacetic (1.7-fold) acids increased, whereas fumaric (2.4-fold) and malic (3.2-fold) acids decreased as compared with As^{III}-untreated cells (Table 2). Also, extracellular concentrations of gluconic acid remarkably increased (12.1-fold) as compared with the untreated cells (Table 2).

Antioxidant enzymes. Activities of antioxidant enzymes, viz. catalase, ascorbate peroxidase (APOX), glutathione S-transferase (GST) and SOD, increased with increasing As^{III} levels in the medium. At 5 mM As^{III} a 1.7-fold increase in catalase activity, 1.3-fold in APOX, 2.4-fold in GST and 9.6-fold in SOD was observed in comparison with control. Glutathione reductase activity decreased by 1.4, 1.5 and 2.6 times in the presence of 1, 5 and 10 mM As^{III} respectively (Table 3).

Discussion

Tolerance to the toxic arsenite by microorganisms is extensively documented (Oremland *et al.*, 2004; Achour *et al.*, 2007; Chang *et al.*, 2008; Lioa *et al.*, 2011). Microorganisms cope up with the toxic effects of arsenite either by restricting its entry inside the cell or by converting it to less toxic form and immobilizing (Hughes, 2002). *ars* operon, one of the arsenic detoxification mechanisms, is mainly attributed to tolerance of arsenite in microorganisms. The present study is an attempt to isolate arsenite-tolerant microorganisms, and to extend our knowledge beyond current *ars* gene-mediated resistance models. The relative contribution of multiple gene products, and central carbon metabolic pathways were analysed in arsenite-tolerant *Rhodococcus* sp. strain NAU-1. Interestingly, the present study revealed remodulation of central metabolism in favour of arsenite tolerance (Fig. 4).

Ars-mediated tolerance involves the reduction of arsenate (As^{V}) to arsenite (As^{III}) via cytoplasmic As^{V} reductase (ArsC), followed by the extrusion of As^{III} by a membrane-associated ArsB efflux pump that is efficient at removing As^{III} (Mobley and Rosen, 1982). Most arsenite-resistant bacteria possess both arsenate reductase and arsenite oxidase activities (Silver and Phung, 2005; Mateos *et al.*,

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

Metabolic enzymes (nM min^{-1} mg^{-1} total protein)	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[a]	5.16 ± 0.27	4.34 ± 0.28[ns]	3.75 ± 0.20*	0.86 ± 0.10***
ICL	13.66 ± 0.087	15.00 ± 0.02[ns]	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 nM 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	
	As^{III}: 0 mM	As^{III}: 5 mM	As^{III}: 0 mM	As^{III}: 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	2.52 ± 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^{ns}
Malic acid	0.6 ± 0.02	0.19 ± 0.01***	0.63 ± 0.01	0.38 ± 0.00**
Oxaloacetic acid	0.39 ± 0.02	0.66 ± 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 As^{III}-treated *Rhodococcus* sp. strain NAU-1 revealed the presence of extracellular and intracellular As^{III} and As^{V}. 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, Ars-mediated tolerance was observed in *Rhodococcus* sp. strain NAU-1, which involves As^{V} reduction again to As^{III} via cytoplasmic As^{V} reductase (ArsC) enzyme. As^{III} 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 H_2O_2 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 As^{III}, increased catalase, APOX, SOD and GST activities were observed in NAU-1. The As^{III} could bind with glutathione to form arsenite–glutathione complex (As^{III}–GS_3) 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, As^{III}–GS_3 complex is an inhibitor of GR activity (Styblo *et al*., 1997; Kala *et al*., 2000). As^{III}-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 (As^{III}–GS_3) for extrusion by ABC transporters. However, these transporters are not capable of removing the entire amount of As^{III} as revealed by intracellular ICP-OES studies. Effects of leftover intracellular arsenic were further analysed. As^{III}-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)	As^{III}: 0 mM	As^{III}: 1 mM	As^{III}: 5 mM	As^{III}: 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[a]	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.

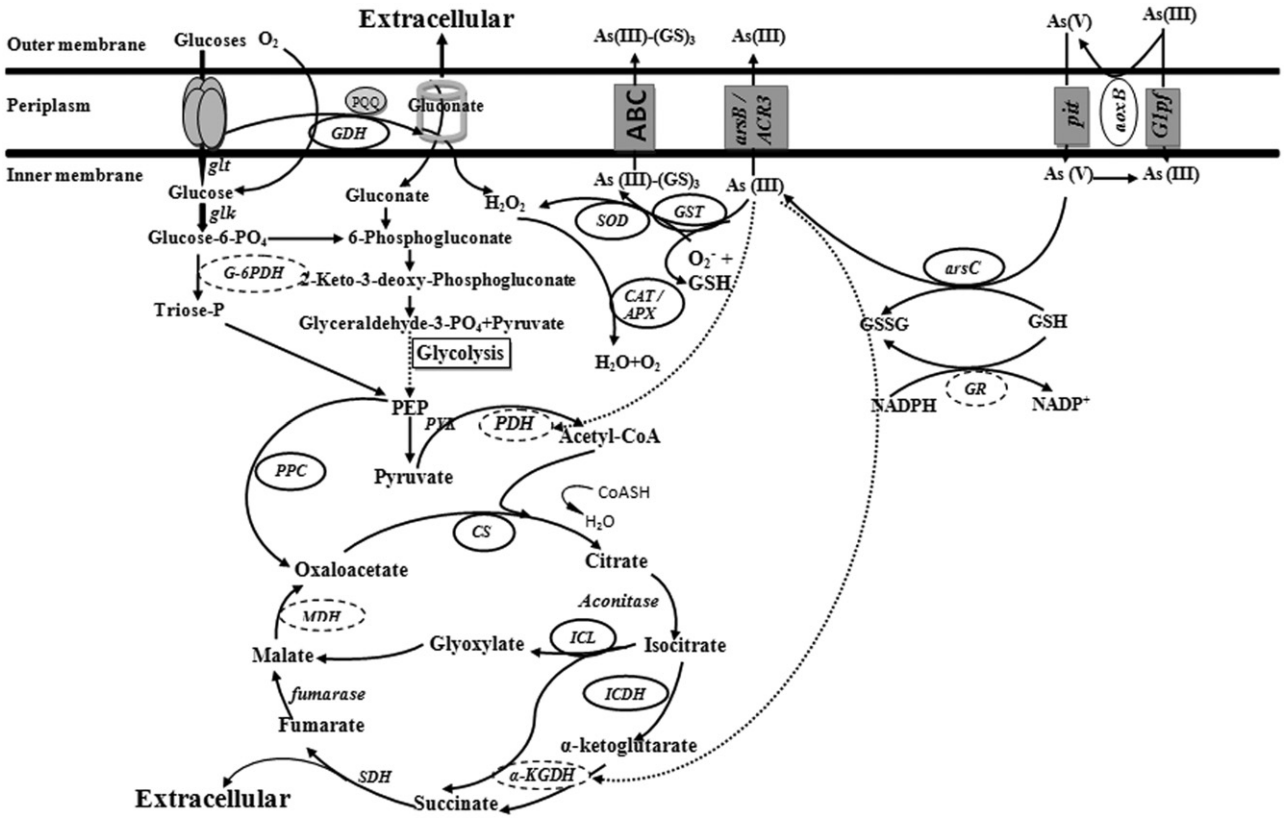

Fig. 4. Metabolic processes of *Rhodococcus* sp. strain NAU-1 in the presence of As^{III}. Complete ovals around enzyme names indicate the enzymes which are induced in the presence of arsenite. Dashed ovals around enzyme names indicate repressed enzymes in the presence of arsenite.

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 As^{III} 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 ($NaAsO_2$) 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^{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 $NaAsO_2$ 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^{III} was also checked in Luria–Broth (LB) medium (Sambrook and Russell, 2001) by adding increasing concentrations of As^{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 $\sim 10^6$ cells ml^{-1} and then incubated for 24 h. The metals and their compounds used for MIC determination are as follows: As^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^V and As^{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^V) or mixture of KIO_3 and HCl (for As^{III}), as used in preparation. Controls were heat-killed samples for both the arsenic species. Heat-killed 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 $NaAsO_2$. 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 $MgCl_2$.

Arsenate reductase activity was measured using a coupled assay system that measures the arsenate-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 As^{III}.

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 H_2O_2 ($\varepsilon = 39.4$ mM^{-1} cm^{-1}) according to the method of Aebi (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 ($\varepsilon = 2.8$ mM^{-1} cm^{-1}) according to the method of Nakano and Asada (1981). Enzyme activity of APOX and catalase were expressed as μmol substrate oxidized min^{-1} g^{-1} protein. Total SOD (EC 1.15.1.1) activity was measured spectrophotometrically based on inhibition in the photochemical reduction of nitro blue tetrazolium (NBT) using the method of Van Rossun and colleagues (1997). Enzyme unit of SOD was calculated according to formula given by Constantine and Stanley (1977). GR (EC 1.6.4.2) activity was measured by following NADPH reduction at 340 nm following the method of Schaedle and Bassham (1977). GST (EC 2.5.1.18) activity was measured by observing the conjugation of 1-chloro, 2, 4-dinitrobenzene (CDNB) with GSH (Boyland and Chasseaud, 1969). 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 4.1.1.31) activity was monitored by following NADH oxidation at 340 nm in an assay combined with 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 (4.1.3.1) activity was monitored by measuring glyoxylate formation at 324 nm with the aid of phenylhydrazine-HCl; 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 nM 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.

Acknowledgements

The authors extend sincere thanks to Prof. Datta Madamwar, Biosciences Department, S. P. University, V. V. Nagar, Anand, India for helping in arsenite oxidase and arsenate reductase assays. This work was supported by the Department of Biotechnology, Ministry of Science and Technology, Government of India and Gujarat State Biotechnology Mission (GSBTM), Gujarat. No Author has any conflict of interest.

References

Achour, A.R., Bauda, P., and Billard, P. (2007) Diversity of arsenite transporter genes from arsenic-resistant soil bacteria. *Res Microbiol* **158:** 128–137.

Aebi, H. (1984) Catalase *in vitro*. *Methods Enzymol* **105:** 121–126.

Agency USEP (2001) *Arsenic and Clarifications to Compliance and New Source Contaminant Monitoring*. Washington, DC, USA: National Drinking Water Regulations. 40 CFR part 141 and 142.

Ahmann, D., Roberts, A.L., Krumholz, L.R., and Morel, F.M.M. (1994) Microbes grow by reducing arsenic. *Nature* **371:** 750.

Aksornchu, P., Prasertsan, P., and Sobhon, V. (2008) Isolation of arsenic-tolerant bacteria from arsenic-contaminated soil. *Songklanakarin J Sci Technol* **30:** 95–102.

Anderson, G.L., Williams, J., and Hille, R. (1992) The purification and characterization of arsenite oxidase from *Alcaligenes faecalis*, a molybdenum-containing hydroxylase. *J Biol Chem* **267:** 23674–23682.

Boyland, E., and Chasseaud, L.F. (1969) The role of glutathione and glutathione S transferase in mercapturic acid biosynthesis. *Adv Enzymol Relat Areas Mol Biol* **32:** 173–219.

Buch, A., Archana, G., and Naresh Kumar, G. (2008) Metabolic channeling of glucose towards gluconate in phosphatesolubilizing *Pseudomonas aeruginosa* P4 under phosphorus deficiency. *Res Microbiol* **159:** 635–642.

Buch, A., Archana, G., and Naresh Kumar, G. (2009) Enhanced citric acid biosynthesis in *Pseudomonas fluorescens* ATCC 13525 by overexpression of the *Escherichia coli* citrate synthase gene. *Microbiology* **155:** 2620–2629.

Cai, L., Liu, G., Rensing, C., and Wang, G. (2009) Genes involved in arsenic transformation and resistance associated with different levels of arsenic contaminated soils. *BMC Microbiol* **9:** 4–16.

Cervantes, C., Ji, G., Ramirez, J., and Silver, S. (1994) Resistance to arsenic compounds in microorganisms. *FEMS Microbiol Rev* **15:** 355–367.

Chang, J.S., Kim, Y.H., and Kim, K.W. (2008) The *ars* genotype characterization of arsenic-resistant bacteria from arsenic-contaminated gold–silver mines in the Republic of Korea. *Appl Microbiol Biotechnol* **80:** 155–165.

Constantine, N., and Stanley, K.R. (1977) Superoxide dismutases. Occurrence in higher plants. *Plant Physiol* **59:** 309–314.

Cummings, D.E., Frank, C., Jr, Scott, F., and Rosenzweig, R.F. (1999) Arsenic mobilization by the dissimilatory Fe(III)-reducing bacterium *Shewanella alga* BrY. *Environ Sci Technol* **33:** 723–729.

Drewniak, L., Styczek, A., Majder-Lopatka, M., and Sklodowska, A. (2008) Bacteria, hypertolerant to arsenic in the rocks of an ancient gold mine, and their potential role in dissemination of arsenic pollution. *Environ Pollut* **156:** 1069–1074.

Ghosh, M., Shen, J., and Rosen, B.P. (1999) Pathways of As(III) detoxification in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA* **96:** 5001–5006.

Hughes, M.F. (2002) Arsenic toxicity and potential mechanisms of action. *Toxicol Lett* **133:** 1–16.

Jackson, C.R., Jackson, E.F., Dugas, S.L., Gamble, K., and Williams, S.E. (2003) Microbial transformations of arsenite and arsenate in natural environments. *Recent Res Dev Microbiol* **7:** 103–118.

Ji, G., and Silver, S. (1995) Bacterial resistance mechanisms for heavy metals of environmental concern. *J Ind Microbiol* **14:** 61–75.

Kala, S.V., Neely, M.W., Kala, G., Prater, C.I., Atwood, D.W., Rice, J.S., and Lieberman, M.W. (2000) The MRP2/cMOAT transporter and arsenic–glutathione complex formation are required for biliary excretion of arsenic. *J Biol Chem* **275:** 33404–33408.

Kodaki, T., Katagiri, F., Asano, M., Izui, K., and Katsuki, H. (1985) Cloning of phosphoenolpyruvate carboxylase gene from a cyanobacterium, *Anacystis nidulans*, in *Escherichia coli*. *J Biochem* **97:** 533–539.

Kowaltowski, A.J., Castilho, R.F., Grijalba, M.T., Bechara, E.J.H., and Vercesi, A.E. (1996) Effect of inorganic phosphate concentration on the nature of inner mitochondrial membrane alterations mediated by Ca^{2+} ions: a proposed model for phosphate-stimulated lipid peroxidation. *J Biol Chem* **271:** 2929–2934.

Lanyi, B. (1987) Classical and rapid identification methods for medically important bacteria. *Methods Microbiol* **19:** 1–67.

Lioa, V.H., Chu, Y., Su, Y., Hsiao, S., Wei, C., Liu, C., *et al*. (2011) Arsenite-oxidizing and arsenate-reducing bacteria associated with arsenic-rich groundwater in Taiwan. *J Contam Hydrol* **123:** 20–29.

Liu, S.X., Athar, M., Lippai, I., Waldren, C., and Hei, T.K. (2001) Induction of oxyradicals by arsenic: implication for mechanism of genotoxicity. *Proc Natl Acad Sci USA* **98:** 1643–1648.

Mandal, B.K., and Suzuki, K.T. (2002) Arsenic round the world: a review. *Talanta* **58:** 201–235.

Mateos, L.M., Ordonez, E., Latek, M., and Gill, J.A. (2006) *Corynebacterium glutamicum* as a model bacterium for bioremediation of arsenic. *Intrnl Microbiol* **9:** 207–215.

Mobley, H.L., and Rosen, B.P. (1982) Energetics of plasmid mediated arsenate resistance in *Escherichia coli*. *Proc Natl Acad Sci USA* **79:** 6119–6122.

Mukhopadhyay, R., Shi, J., and Rosen, B.P. (2000) Purification and characterization of ACR2p, the *Saccharomyces cerevisiae* arsenate reductase. *J Biol Chem* **275:** 21149–21157.

Mukhopadhyay, R., Rosen, B.P., Phung, T., and Silver, S. (2002) Microbial arsenic: from geocycles to genes and enzymes. *FEMS Microbiol Rev* **26:** 311–325.

Nakano, Y., and Asada, K. (1981) Hydrogen peroxide is scavenged by ascorbate specific peroxidase in spinach chloroplasts. *Plant Cell Physiol* **22:** 868–880.

Oremland, R.S., Stolz, J.F., and Hollibaugh, J.T. (2004) The microbial arsenic cycle in monolake, California. *FEMS Microbiol Ecol* **48:** 15–27.

Peterson, G.L. (1979) Review of the Folin phenol quantitation method of Lowry, Rosenberg, Farr and Randall. *Anal Biochem* **100:** 201–220.

Ralph, S.J. (2008) Arsenic-based antineoplastic drugs and their mechanisms of action. *Met Base Drugs* **10:** 1155–260146.

Rosen, B.P. (2002) Biochemistry of arsenic detoxification. *FEBS Lett* **529:** 86–92.

Sambrook, J., and Russell, D. (2001) *Molecular Cloning: A Laboratory Manual*, 3rd edn. New York, USA: Cold Spring Harbor Laboratory Press.

Schaedle, M., and Bassham, J.A. (1977) Chloroplast glutathione reductase. *Plant Physiol* **59:** 1011–1012.

Serre, P.A. (1969) Citrate synthase. *Methods Enzymol* **13:** 3–11.

Silver, S., and Phung, L.T. (2005) Genes and enzymes involved in bacterial oxidation and reduction of inorganic arsenic. *Appl Environ Microbiol* **71:** 599–608.

Styblo, M., Seves, S.V., Cullen, W.R., and Thomas, D.J. (1997) Comparative inhibition of yeast glutathione reductase by arsenicals and arsenothiols. *Chem Res Toxicol* **10:** 27–33.

Tripathi, R.D., Srivastav, S., Mishra, S., Singh, N., Tuli, R., Gupta, D.K., and Maathuis, F.J.M. (2007) Arsenic hazards: strategies for tolerance and remediation by plants. *Trends Biotechnol* **25:** 158–165.

Van Rossun, M.N.P., Alberda, M., and Van Der Plas, L.H.W. (1997) Role of oxidative damage in tulip bulb scale micropropagation. *Plant Sci* **130:** 207–216.

Vyas, P., and Gulati, A. (2009) Organic acid production *in vitro* and plant growth promotion in maize under controlled environment by phosphate-solubilizing fluorescent *Pseudomonas*. *BMC Microbiol* **9:** 174–189.

