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Diversity of arsenite-resistant cocci isolated from Hutti Gold Mine and bioreactor sample

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Arsenite, cobalt, chromium, copper, nickel, molybdenum, selenate and selenite-resistant cocci were isolated from Hutti Gold Mine reactor and processed water samples having extreme physico-chemical characteristics of pH 1.2–8.20, conductivity 3.2–58 mS and arsenic 11.0–1443.0 mg/l. Cultural, morphological, biochemical characters and antibiotic sensitivity of all the four cocci were studied. On the basis of biochemical and Biolog® test, one isolate was identified as *Staphylococcus aureus*, and two of these were identified as *Citricoccus* sp. SRHGAs38 and *Staphylococcus* sp. SRDAs32 using 16S rRNA gene sequence. These isolates could be used for metal removal.

Keywords: Arsenite resistant, Biolog®, *Citricoccus*, *Staphylococcus*, substrate utilization profile.

ARSENIC is widely distributed in the environment as a consequence of natural phenomena and anthropogenic activity[1]. It is frequently found as a mineral in combination with sulphur as orpinment (As_2S_3) and realgar (AsS) and especially with iron and sulphur as arsenopyrite (FeAsS)[2]. In acid mine drainage (AMD), arsenite concentration reaches as high as 2–13 mg/l (ref. 2). All the soluble forms of arsenic, arsenite (As^{3+}) and arsenate (As^{5+}) are toxic to living organisms, but arsenite is more toxic than arsenate. This toxicity is due to its interference in normal phosphorylation processes[1,3]. Other than arsenic pollution, AMD or acid rock drainage (ARD) represents very high dissolved solids and many a time with pH as low as 2.0 (ref. 4). In spite of such extreme conditions, iron and sulphur oxidizing autotrophic organisms are the most prevalent microbes in such ecosystems, but several heterotrophic organisms comprising bacteria, fungi and actinomycetes have also been reported[1,3].

Hutti Gold Mine (HGM), Karnataka, India, represents refractory gold minerals interlocked in arsenopyrite network[5,6]. This mine is one of the main sources for gold production in India. The presence of arsenic in the mineral, mine drainage and processed water is reported at HGM site (http://www.minesofindia.com), which makes the ecosystem non-conducive for the growth of microorganisms. As microorganisms present in normal soil are not usually resistant to high concentration of heavy metals[7]. But, they are capable of developing tolerance or resistance towards the toxic effect of metals. Species of *Bacillus*, *Achromobacter* and *Pseudomonas* are reported from arsenical ecosystems[3]. *P. putida* and *Alcaligenes eutrophus* were isolated from gold arsenic deposits of Kazakisthan[3]. *P. arseniteoxidans* was able to grow through chemoautotrophic oxidation of arsenite whereas, arsenite oxidizer Nt-26 isolate is reported to grow heterotrophically[2,3].

Till date, no reports are available on diversity of arsenite-resistant heterotrophic cocci community from Indian mines. Thus, the aim of the present work was to study the diversity of arsenite-resistant heterotrophic cocci from arsenopyrite HGM and refractory gold concentrate bioreactor ecosystem.

Water samples were collected from refractory gold concentrate biooxidation reactor, storage tank of the bioreactor effluent and gold mine processed water of HGM situated in Raichur district, Karnataka, India.

Physico-chemical parameters such as colour, pH, redox potential, conductivity, salinity and total dissolved solids (TDS) were analysed by standard methods using respective portable meters (model – Eutech, Singapore). Ferrous iron was determined by diphenylamine titrimetric method[8], total iron by 1,10-phenanthroline method and total arsenic with silver diethyldithiocarbamate and atomic absorption spectrophotometric method[9].

Collected water samples were diluted to 1 : 100 and 1 : 1000 times and 0.1 ml of the diluted samples were innoculated in triplicate on high plate count (HPC) agar and nutrient agar (Himedia, India) plates with and with-

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out 20 mM of arsenite. Colony forming units (CFU) of arsenite sensitive and resistant bacteria were counted after 48 h of incubation.

Arsenite concentration from 0 to 200 mM was studied in liquid media by inoculating 10% (v/v) actively growing inoculum having 10^8 cells/ml for each isolate. For positive control, a medium broth of each isolate without arsenite and for negative control, an uninoculated broth medium was used. All the flasks were incubated on environmental orbital shaker having 150 rpm at 35 ± 2°C temperature and growth was measured at 48 and 72 h from the flask having arsenic concentration between 0–60 mM and 80–200 mM respectively at 560 nm using microprocessor-based UV visible spectrophotometer (Systronics, 119).

Identification of the isolates by Biolog® microplate was carried out according to the procedure provided along with the identification system[10]. Actively growing cultures were harvested and as per the protocol, 150 µl of the inoculum was inoculated in Biolog® GP plates. Plates were incubated at 35 ± 2°C. Results were recorded between 4–6 h and 20–24 h of incubation time and identification was done using Biolog® software (Biolog Inc., USA).

All the isolated bacteria that grew on 20 mM of arsenite ($NaAsO_2$) containing HPC agar were studied for their morphological, colony and selected biochemical characters[11]. Substrate utilization profile of Biolog® GP plate was also studied at 620 nm by GDV-Programmable Microplate Reader (GIO DE VITA E.C. S.R.L., Rome) with GDV 990 BV6 software.

Metal resistance study was carried out by inoculating 10 ml of actively grown cultures (10^8 cells/ml) harvested from HPC broth containing 20 mM of sodium arsenite in 250 ml Erlenmeyer flask containing 90 ml of nutrient broth with 0–300 mM of Co^{2+}, Cr^{6+}, Cu^{2+}, Ni^{2+}, Sb^{3+}, Se^{3+}, Se^{4+} and Zn^{2+} as their sulphate salts whereas Mo^{2+} was added as ammonium molybdate. Flasks were incubated at 35 ± 2°C on an environmental orbital shaker at 150 rpm. Bacterial growth was measured spectrophotometrically in terms of absorbance at 560 nm against blank of respective metal containing uninoculated broth as mentioned earlier.

Sugar utilization profile and functional diversity of the cocci were assessed on the basis of their ability to utilize 21 different sugars for which phenol red basal broth medium supplemented with 0.5% w/v of various sugars (Himedia, India) containing inverted Durham's tube was used. The result in terms of acid or alkali with gas production in Durham's tube was recorded in the medium. These data were also used to differentiate cocci based on sugar utilization profile.

All the four arsenite-resistant cocci were screened for their antibiogram using multiple antibiotic discs containing 12 different antibiotics (PBL Bio-Disc-12, India).

Diversity among isolates was determined by carrying out principal component analysis (PCA) using statistical package SPSS for windows (version 7.5, SPSS 1996) considering morphological, colony and antibiotic sensitivity characteristic of the isolates.

Physico-chemical analysis of water samples collected from biooxidation demonstration plant set up at HGM and processed water of the mine showed extreme diversity in pH, redox potential, conductivity, salinity, TDS, total iron, ferrous iron and arsenic in the range of 1.24–8.20, 86–574 mV, 3.2–58 mS, 1.9–8.7 ppt, 1.8–30.9 ppt, 11.0–1443.0 mg/l, 5.6–6.0 mg/l and 0.32–2854.5 mg/l respectively[12]. High acidic pH, redox potential and arsenic in reactor leachate compared to the mine processed water sample were obviously due to the biological oxidation of arsenopyrite and ferrous iron by *Acidithiobacillus* and *Leptospirillum* group of organisms that resulted in solubilization of arsenic from the concentrate and increased ferric iron in the leachate[12]. The processed water showed alkaline pH, low redox potential and high ferrous iron concentration. This could be due to cyanide treatment, influence of the gangue material present in the minerals and low biological ferrous iron oxidation activity. The dissolved arsenic concentration in all the samples was higher than the permissible limit of 50 µg/l (ref. 1).

Microbial count of heterotrophic bacteria in the presence and absence of arsenite was studied. Bacterial counts were in the range of 10^3–10^5 in the absence of arsenite and 10^3–10^4 in its presence. Based on colony morphology, nine varieties of isolates were observed. HPC agar and nutrient agar media showed almost equal number of CFU and varieties of the colonies (Table 1). Total cultivable bacterial counts and diversity were low in these samples compared to unpolluted water. In uncontaminated water samples, bacterial counts range between 10^6 and 10^8 CFU/ml (ref. 13). The obtained low bacterial count and low diversity could be due to the extreme low pH, presence of arsenic and other conditions of the samples. Microorganisms possess great adaptability towards adverse conditions and this could be the reason for observed arsenite-resistant heterotrophic population in the collected samples. Amongst the samples studied, processed water of HGM showed higher count and more varieties of arsenite-resistant bacteria compared to leachate of refractory gold concentrate biooxidation reactor and storage tank of the bioreactor effluent samples.

Arsenite-resistant organisms that are able to grow on 20 mM of arsenite were further tested for their growth on higher arsenite concentration. Out of the eight isolates studied, isolate HGM 1, HGM 28, HGM 32 and HGM 38 grew up to 120 mM of arsenite concentration, the detailed results of growth at various arsenite concentrations are shown in Figure 1. HGM 1 and HGM 28 were isolated from refractory gold concentrate biooxidation reactor and storage tank of the bioreactor effluent respectively, whereas HGM 32 and HGM 38 were isolated from processed water of HGM. The observed growth of all the four isolates in the absence of arsenite and in the presence of

Table 1. Quantitative and qualitative data of heterotrophic isolates obtained from the sample on different media

	Total number of isolates ($\times 10^3$) (CFU)			
	High plate count agar arsenite concentration (mM)		Nutrient agar arsenite concentration (mM)	
Sample no.	0	20	0	20
1	8 ± 0.3 (3)	8 ± 0.4 (2)	8 ± 0.3 (3)	–
2	79 ± 0.4 (9)	35 ± 0.3 (3)	89 ± 0.6 (9)	32 ± 0.3 (3)
3	188 ± 0.6 (7)	38 ± 0.5 (4)	160 ± 0.7 (8)	36 ± 0.2 (4)

Figure in parenthesis indicates variety in isolates.

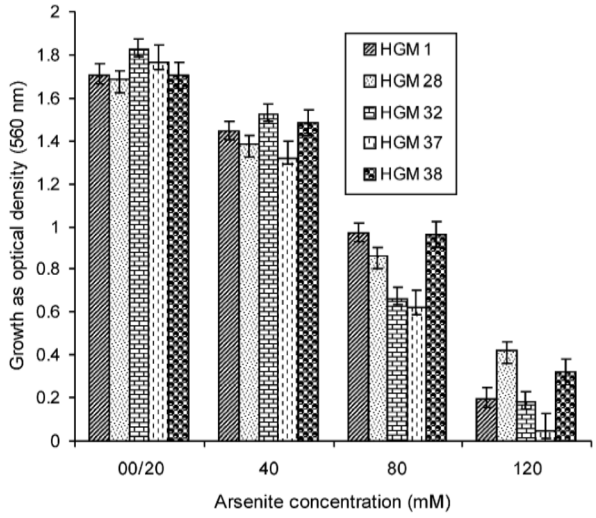

Figure 1. Comparative growth of isolates in terms of absorbance (560 nm) at various arsenite concentrations.

20 mM of arsenite was identical and indicated no inhibition in the presence of this concentration of arsenite. This could be because cultures selected for these studies were from a plate containing 20 mM of arsenite. The generation time of these isolates in the presence of 80 mM of arsenite in the medium was 18.72, 30.13, 6.02 and 14.14 h respectively. Isolate HGM 37 grew up to 80 mM of arsenite concentration (Figure 1). On the other hand HGM 21, HGM 25 and HGM 34 were also isolated from 20 mm arsenite but they could not grow even in the presence of 40 mM of arsenite (data not shown). Salmassi *et al.*[14] cited published value of the highest level of arsenic tolerance of 100 and 60 mM arsenite reported by Green in 1918 and Turner in 1949 respectively. As these reported data are quite old, present findings are important. Suzuki *et al.*[15], Patel *et al.*[16] and Mandal *et al.*[17] have studied arsenic resistance in the last 15 years, but it is for Gram negative bacteria and highest resistance observed is 30 mM arsenite and 65 mM arsenate. Suresh *et al.*[18] have reported *Deinococcus indicus* sp. nov. growing in presence of 0.2 mM of arsenite and 10 mM of arsenate only. Study on isolation of arsenic-tolerant organisms from contaminated site was carried out by Aksornchu *et al*[19], who isolated 45 cultures resistant to 5 mM arsenite and 24 were able to tolerate as high as 40 mM sodium arsenite. Among these isolates, six were Gram positive coccoid. However, hypertolerance to arsenate in biofilm bacteria is reported by Drewniak *et al.*[20] but it does not include *Staphylococcus* or *Citricoccus* sp. Arsenite resistance of 120 mM in *Staphylococcus* sp. and *Citricoccus* sp. is the first report.

On the basis of morphological, colony, biochemical and antibiotic sensitivity, the isolates were linked with each other using PCA (Figure 2). As can be seen from Figure 2, these eight isolates can be distinctly separated in five clusters, viz. HGM 1, HGM 28 and HGM 38 first cluster, HGM 21 and HGM 25 the second cluster and the remaining three showed no considerable relationship and were scattered in three distinct locations. HGM 1, HGM 28 and HGM 38 were Gram positive cocci having yellow pigmentation, whereas HGM 32 was Gram positive cocci with orange pigment. HGM 37 is a Gram negative rod, thus it is placed at a quite distinct place.

Among these four isolates, HGM 38 was the biggest with 1.4 μm cell diameter, whereas HGM 1 was the smallest with cell diameter of 1 μm and HGM 28 and HGM 32 showed medium cell diameter of 1.2 μm. In terms of cell arrangement also, they showed diversity as HGM 32 had large clusters, while HGM 38 was found with small rare clusters and most of the cells were arranged singly or in pairs. On the basis of morphological and some of the routine biochemical characteristics, the four isolates that grew in presence of 120 mM arsenite were tentatively identified. Isolates HGM 1 and HGM 28 were found to show similarity to *Staphylococcus* sp., isolate HGM 32 to *S. aureus* and HGM 38 to *Micrococcus* sp. (results not shown). All the isolates were catalase positive and except HGM 32, they were oxidase test negative. All the four isolates showed yellow or yellowish orange pigmentation. This indicates that yellow pigmentation could be playing some role in arsenite resistance development.

Sugar fermentation was studied for all the four isolates. HGM 32 showed either acid or alkali reaction in all the

21 sugars studied. Whereas acid or alkali production was not detected in adonitol and dulcitol by isolate HGM 1, rhamnose and salicin sugar by isolate HGM 28 and arabinose sugar by HGM 38. All other results of 21 sugars studied were the same for all the four isolates (data not shown). On the basis of substrate utilization profile of 95 carbon sources of nine different groups in Biolog® GP plates, HGM 1 was found to be the most versatile with the highest activity in all the substrate groups except carboxylic acids (Figure 3). Based on substrate utilization pattern obtained from the Biolog® plate, the isolates could be arranged in order of decreasing efficiency in terms of total number of substrates utilized, as isolate HGM 1 > HGM 38 > HGM 28 > HGM 32.

Results of the Biolog® GP plate confirm the identity of isolate HGM 32 as *S. aureus*. Whereas identification of HGM 1, HGM 28 and HGM 38 was not confirmed even by Biolog® identification system and showed only 22.5%, 30% and 33% similarity to *S. gallinarum*, *S. arlettae* and *S. arlettae* respectively. The sugars and substrates of Biolog® GP plate utilization profiles clearly indicate the diversity amongst these four isolates.

Out of these four isolates, two isolates were identified with 16S rRNA gene sequence. HGM 38 is identified as *Citricoccus* sp. SRHGAs38 (accession no. EF375486) (812 nucleotides). This isolate showed 93% similarity with *Citricoccus* sp. (accession no. EU305672 and AB094464) and also showed 93% similarity with *Micrococcus* sp. (accession no. EU394442 and EU330348). Whereas isolate HGM 32 is identified as *Staphylococcus* sp. SRDAs32 (accession no. EU419887) (570 nucleotides) showing 93% similarity with *S. aureus* (accession no. AJ536434).

Antibiotic sensitivity profile of the isolates was also studied and from the obtained data no co-relationship can be established between arsenite resistance and antibiotic resistance. However, this finding showed wide variation among the isolates in terms of their growth response towards the selected antibiotics. HGM 1, HGM 28 and HGM 38 were resistant towards ofloxacin, whereas HGM 28 and HGM 32 were resistant to peftoxacin and ampicillin respectively. All the four isolates were equally sensitive to lincomycin. HGM 1 and HGM 32 were found to be highly sensitive to roxythromycin showing inhibition zone size above 30 mm, but HGM 28 and HGM 38 showed minimum inhibition with zone size of less than 15 mm (data not shown).

Minimum inhibitory concentration of cobalt, chromium, copper, nickel, molybdenum, selenate and selenite in the presence of 20 mM of arsenite was studied for the four selected isolates. It was found that cobalt and nickel were the most toxic metal ions amongst the metals studied, in spite of this; all the four isolates grew in presence of 30 mM of these metals and also grew in presence of 40 and 100 mM of zinc and antimony respectively. Whereas selenite was found to be least toxic among the metals studied and all the isolates showed growth even in the presence of 300 mM of selenite. All the four cocci showed considerable diversity in terms of resistance towards chromium (60–100 mM), copper (40–50 mM),

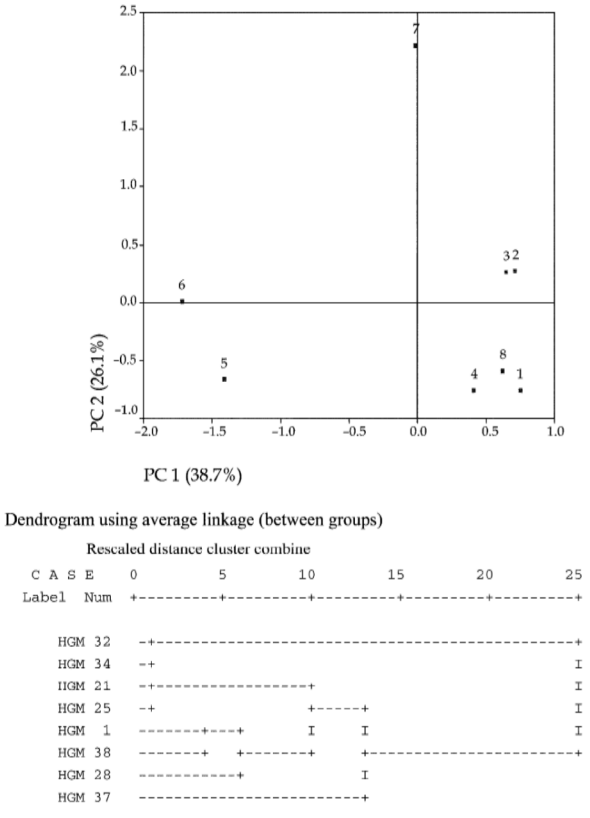

Figure 2. Principal component analysis based on all variables studied for all isolates (1–8 isolate nos: HGM 1, HGM 21, HGM 25, HGM 28, HGM 32, HGM 34, HGM 37 and HGM 38).

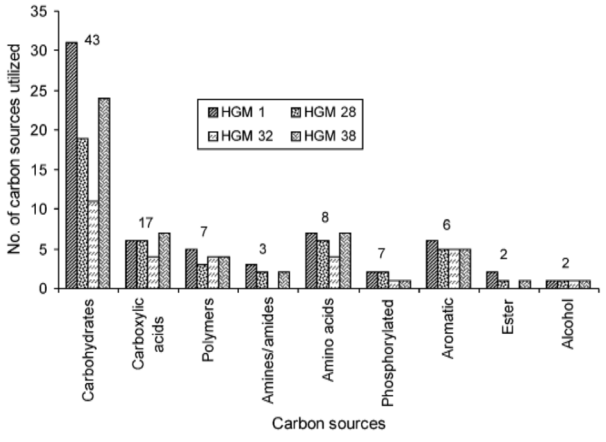

Figure 3. Comparison of utilization of carbon substrates of Biolog® plate by isolates (figures on the bar indicate total number of the substrate present in the Biolog® GP plate).

molybdate (40 mM) and nickel (30 mM). Non-pigmented *Serratia marcescens* isolated from serpentine deposit of Cuba is reported for minimum inhibitory concentration of 20 and 25 mM for cobalt and nickel respectively[21]. Polymetallic resistance of these isolates is a unique characteristic, no such data are available for cocci. As these organisms were isolated from extreme ecosystems in terms of pH, redox potential, conductivity, salinity, TDS, total iron, ferrous iron and arsenic and they were also found to precipitate iron when grown on citrate agar medium (data not shown), these properties could be used for bioremediation of metallic as well as organic pollutants from AMD.

The following conclusions can be drawn from the present study: Yellow-pigmented cocci showed the highest arsenite resistance; Wide diversity in terms of substrate utilization profile and antibiotic sensitivity was observed in arsenite resistant cocci; Substrate utilizing profile could be a useful approach for differentiating heterotrophic community prevailing even in extreme mining environment; This is the first report of *Staphylococcus* and *Citricoccus* species resistant to such a high concentration of different metals in general and arsenite in particular; These isolates could be used for bioremediation of metallic and organic pollutants from acid mine drainage.

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