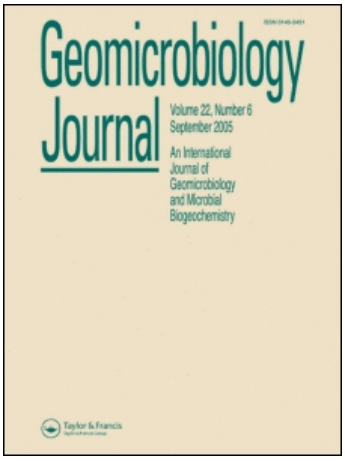

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Saurabh Gupta [a]; Ranjana Prakash [a]; N. Tejo Prakash [a]; Carolyn Pearce [b]; Richard Pattrick [b]; Marina Hery [b]; Jonathan Lloyd [b]
[a] Department of Biotechnology and Environmental Sciences; and School of Chemistry and Biochemistry, Thapar University, Patiala, India [b] Williamson Centre for Molecular Environmental Science, School of Earth, Atmosphere and Environmental Science, University of Manchester, Manchester, United Kingdom

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Selenium Mobilization by *Pseudomonas aeruginosa* (SNT-SG1) Isolated from Seleniferous Soils from India

Saurabh Gupta,[1] Ranjana Prakash,[1] N. Tejo Prakash,[1] Carolyn Pearce,[2] Richard Pattrick,[2] Marina Hery,[2] and Jonathan Lloyd[2]

[1]*Department of Biotechnology and Environmental Sciences; and School of Chemistry and Biochemistry, Thapar University, Patiala 147004, India*

[2]*Williamson Centre for Molecular Environmental Science, School of Earth, Atmosphere and Environmental Science, University of Manchester, Manchester M13 9PL, United Kingdom*

Selenium (Se) is a metalloid required at trace concentrations for normal metabolic activities of the cell. The bioavailable forms viz., selenate and selenite have been found in localized high concentrations in seleniferous environments. Studies are in progress on bacterial strains that were isolated from one such location in the North-West region of Punjab, India. A facultative anaerobe, identified as *Pseudomonas aeruginosa* by 16S rRNA gene homology, was isolated from the rhizosphere of crop plants from this region and was examined for selenium mobilization potential in the presence of selenium oxyanions. The isolate was observed to reduce 53 and 21% of sodium selenite and selenate to elemental selenium, respectively, and volatilize 4.7 and 5.1% within 72-hour duration. This is one of the few selenium tolerant aerobic bacteria isolated and reported from tropical seleniferous soils from India, and the first to show volatilization potential. These organisms are being considered for bioaugmenting Se-impacted soils for enhanced Se mobilization and removal.

Keywords Selenium; bacteria, biotransformation, rhizosphere, soil

INTRODUCTION

Selenium (Se) is of particular scientific interest as it is both essential and toxic to animals and humans with a very fine line drawn between biological need and toxicity. Selenium exists in atmospheric, marine, and terrestrial environments in nature and the heterogeneity in its distribution results in movement of selenium between these different environments (Nriagu 1989). Microorganisms play an important role in the cycling of Se, and transform Se through oxidation, reduction and methylation reactions. Selenate and selenite, the two most toxic and bioavailable forms of selenium present in alkaline and neutral environments can be transformed to a variety of less toxic, non-mobile and gaseous forms, which subsequently alter its bioavailability and behavior in the environment. The biological transformation of nonvolatile Se to volatile compounds is a major pathway in the global flux of Se from terrestrial and marine environments to the atmosphere (Haygarth 1994). The biomethylation of Se in seleniferous environments is readily carried out by bacteria and plants, and is thought to be an important detoxification mechanism in these organisms (Dungan and Frankenberger 1999).

Selenium occurs in natural waters in trace amounts as a result of geochemical processes, such as the weathering of rocks and the erosion of soils, and is usually present in the water as selenate (SeO_4^{2-}), or selenite (SeO_3^{2-}); however, the elemental, mineral or sorbed forms may be carried in suspension (Bauer 1997). Selenium is particularly concentrated in the soils of drier regions where soils tend to be more alkaline (Sharma and Singh 1983). Concentrations in soils are quite variable, ranging from <0.1 to as high as 8000 mg/kg (Berrow and Ure 1989). Ure and Berrow (1982) reported an average Se concentration of 0.40 mg/kg for 1623 soils from throughout the world. Pockets of soils with anomalously high selenium content have been identified in India especially in north-eastern parts of Punjab (Dhillon and Dhillon 2003). In the Nawanshahr-Hoshiarpur region in Punjab, concentrations of 1.02 to 6.79 mg/kg in soil, and 194-297 μg/l in ground water have been detected (Sharma et al. 2009).

There are limited reports of aerobic facultative bacteria involved in the reduction of selenium oxyanions (Lortie et al. 1992; Garibasu et al. 1996; Losi and Frankenberger 1997). We have isolated a number of aerobic strains from Punjab soils with potential to metabolize selenium. This manuscript describes the characterization of one of the most promising strains for

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Address correspondence to N. Tejo Prakash, Assistant Professor, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala 147004 India. E-mail: tejo.nagaraja@manchester.ac.uk

TABLE 1
Profile of physicochemical parameters of the soil from Barwa-Jainpur villages of Seleniferous belt

	pH	Conductivity ($mS.cm^{-1)}$	Org. C (wt %)	Ava. N (wt %)	Ava. K ($mg.kg^{-1}$)	Ava. S ($mg.kg^{-1}$)	Ava. P ($mg.kg^{-1}$)	Total Se ($mg.kg^{-1}$)
Mean	8.563	695	0.41	0.0103	187	6.23	0.21	1.449
SEM	0.044	71.39	0.015	0.0005	11.26	0.71	0.02	0.1282

bioremediation of Se contaminated sites(a Pseudomonad, most closely related to *P. aeruginosa*), including the ability to reduce and volatilize selenium oxyanions (selenate and selenate), and its phylogenetic placement by 16S rRNA gene homology This strain was selected for further studies because *Pseudomonads* are known root colonizers, reported to promote growth and protect the plants from pathogens (Lugtenberg and Dekkers 1999). Selenium tolerant bacteria and their potential to reduce and/or volatilize selenium have hitherto not been reported from tropical seleniferous soils.

METHODS

Collection of Soil Samples and its Characterization

Soil samples were collected from a seleniferous site bordering the Nawanshahr-Hoshiarpur region in Northwest India (75° 55 E; 31° 56 N). The soil from which the isolates were obtained was characterized for selected physicochemical parameters (Table 1). The available sulfur (turbidometric method), Available phosphorous (Olsen et al. 1954), Available nitrogen (Subbiah and Asija 1956) and Available potassium were measured using a flame photometer. Soil pH, conductivity were measured using a pH and conductivity meter respectively. Soil organic carbon was determined as described by Walkley and Black (1934).

Selenium levels were quantified using a graphite furnace-atomic absorption spectrometer (GFAAS-AAnalyst 600, Perkin-Elmer) by loading 20 μL of acid digested sample , using AS60 autosampler in the presence of a palladium and magnesium modifier to minimize the interferences (USEPA 1996). The software, WinLab 32 was used to program the analysis profile, which included drying at 110°C for 30 sec, matrix evaporation at 130°C for 30 sec followed by pyrolysis at 1300°C for 45 sec, in inert conditions provided by a continuous flow of argon. Finally, atomization was carried out under normal atmosphere at 1900°C for 5 sec and 2400°C for 3 sec for cleaning the remaining residue.

Enrichment and Isolation of Bacterial Isolates

Fresh rhizospheric soil samples, from agricultural fields with wheat crop, were dried at room temperature and sieved. Soil samples (10 g) were suspended in tryptone soya broth (TSB; Hi-Media, India). Each suspension was serially diluted and plated in tryptone soya agar (TSA; Hi-Media, India) containing added Se as 0.210 gm of sodium selenite, Na_2SeO_3(Merck, Bangalore, India) and 0.119 gm of sodium selenate, Na_2SeO_4 (SD Fine, Mumbai, India) representing 50 $mg.l^{-1}$ of Se in the growth medium. Plates were incubated in aerobic chambers at 37°C for 24 h. Red colonies, indicating reduction of Se oxyanions to Se (0), were re-streaked on TSA without Se to confirm that the colour was not due to pigmentation. Pure cultures were re-streaked, microscopically examined and maintained on Se supplemented plates. Out of strains isolated on the basis of red-colony formation, one strain was picked, based on its potential to tolerate up to 100 $mg.l^{-1}$ of selenium as Na_2SeO_4 and Na_2SeO_3 in TSB, as well as in minimal salt medium ($g.l^{-1}$: $K_2HPO_4.3H_2O$, 4.8; KH_2PO_4, 1.5; $(NH_4)_2SO_4$, 1.0; $Na_3(C_6H_5O_7).2H_2O$, 0.5; $MgSO_4.7H_2O$, 0.2; yeast extract, 0.1).

Biochemical and Molecular Characterization of Selected Strain

Biochemical characterization of this strain, henceforth referred as SNT-SG1, was carried out at the Microbial Type Culture Collection (MTCC-IMTECH), Chandigarh, India following standard protocols (Kreig et al. 1984) (Table 2). Molecular characterization was performed by isolating genomic DNA using standard protocols (Sambrook et al. 1989) and amplifying and sequencing the almost complete 16S rRNA gene. PCR amplification was carried out using universal primers (27F and 1492R). Initial denaturation was carried at 95°C for 1 min for 1 cycle followed by 35 cycles (denaturation 94°C for 30 sec, annealing 55°C for 30 sec, extension 72°C for 30 sec) and final extension at 72°C for 5 min. The amplification of a 1.5 Kb fraction was confirmed by agrose gel electrophoresis. The PCR product was eluted, purified and ligated to a linearized vector pTZ57 R/T (Qiagen) as per the manufacturer's recommendations and transformed in competent *Escherichia coli* DH5-α cells. The cloned genes were then amplified and sequenced using M13 and M14 primers, at the DNA sequencing facility, University of Delhi, Delhi, India. Multiple sequence alignment was carried out using BLAST (Altschul et al. 1997) followed by classification using (RDP II Classifier tools) and phylogenetic analysis using MEGA 4 (Dudley et al. 2007).

Growth Profile

Strain SNT-SG1 was exposed to 25, 50, 75 and 100 mg Se as contained in Na_2SeO_4 and Na_2SeO_3 per liter of culture medium to examine the growth profile of the organism with

TABLE 2
Morphological and biochemical characteristics of *Pseudomonas* sp.

Colony morphology			
Configuration	Irregular		
Margin	Rhizoid		
Elevations	Raised		
Surface	Smooth, moist		
Pigment	Light yellow		
Opacity	Opaque		
Gram's reaction	negative		
Cell shape	Irregular rod		
Size (μm)	Non-uniform		
Biochemical parameters			
Growth on MacConkey agar	NLF	Acid Production from carbohydrates	
Indole test	–	Cellobiose	–
Methyl red test	+	Dextrose	–
Voges Proskauer test	–	i-Inositol	–
Citrate utilization	+	Lactose	–
Gas production from glucose	-	Raffinose	–
Esculin hydrolysis	–	Sucrose	–
Gelatin hydrolysis	+	Xylose	–
Starch hydrolysis	–		
Urea hydrolysis	–	Sugar utilization from carbon source	
Nitrate reduction	+(W)	Glycerol	+
H_2S production (TSI agar)	–	Lactate	–
Catalase test	+	Fumarate	+
Oxidase test	+		

+W= weak positive, NLF=Non-lactose fermenters.

different oxyanions of Se. Cells were inoculated into tryptone soya broth supplemented with selenium and incubated on an orbital shaker at 120 rpm and 37°C, along with a control lacking added selenium. Growth was monitored by regular measurements of optical density, at 600 nm, every 4 h for 24 h. The optical density was not recorded beyond 24 h due to interference of red Se(0) precipitation in the observations.

Se Mobilization Studies

To check the Se mobilization potential of the SNT-SG1 strain, in terms of reduction and volatilization of selenium oxyanions, the isolate was inoculated in TSB (Hi-media) supplemented with 100 mg Se as sodium selenate and sodium selenite per liter of culture medium. The organism was grown in round bottom flasks. The outlet of each flask was connected to a 250-mL gas-washing glass bottle filled with 150 ml of alkaline peroxide trapping solution and a steady supply of fresh sterile air (produced by bubbler pumps), set at approximately 150 ml.h^{-1}, was introduced through an inlet valve and a pre-sterilized 0.22 μ membrane filter (Whatman, USA); an exit valve allowed spent air to be collected. This spent air, containing volatilized selenium components, was trapped in alkaline hydrogen peroxide (Merck, USA).

The efficiency of this method to trap volatile selenium compounds is reported to be greater than 90% (Terry et al. 1992). The trapping solution was replaced with fresh solution after every 24 h. All experiments were conducted in triplicate. Abiotic controls were maintained under similar conditions in tryptic soya broth supplemented with selenium oxyanions. Simultaneously, Se uptake by the isolate was determined after separation of the cells by centrifugation (8000 rpm at 4°C for 10 min) from the spent medium followed by acid digestion and subsequent dilution. The residual Se in culture supernatants was also determined after acid digestion and subsequent dilution. Selenium in the H_2O_2 trapping solution was also quantified after acid digestion using GF-AAS using the same program conditions as for soil analysis.

RESULTS

Characterization of SNT-SG1

Characterization of the morphological, physiological and biochemical characteristics of isolate SNT-SG1 was carried out (Table 2). Physiologically, it can tolerate a wide range of pH values (4.0–9.0), a temperature range of 15°C–42°C and a high salt concentration (to at least 10% w/v) (Figure 1). The culture can

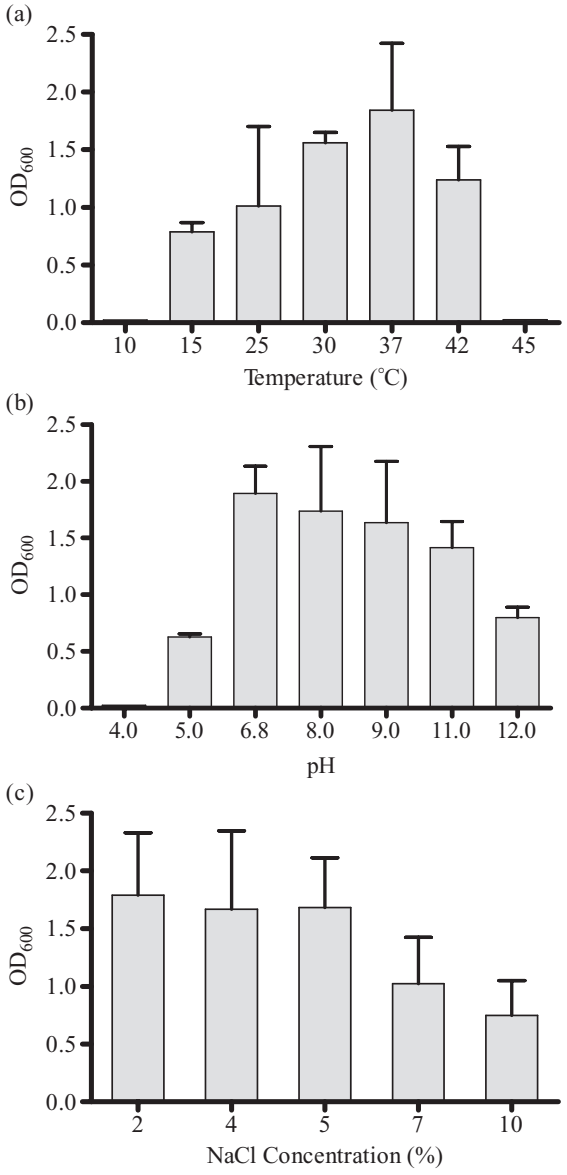

FIG. 1. Tolerance profile of *Pseudomonas* strain to different (a) temperatures; (b) pH; and (c) NaCl concentrations.

be maintained and grown under aerobic, microaerophilic (3% oxygen) and anerobic conditions, hence it can be considered as a facultative organism. Analysis of the 16S rRNA gene sequence by multiple sequence alignment (BLAST) indicated 99% homology to *Pseudomonas aeruginosa* with 98% alignment coverage over 1.5kb. The sequence was assigned the GenBank accession number EU391389 and the strain is deposited at the Microbial Type Culture Collection (MTCC-IMTECH, India).

Classification of SNT-SG1, as belonging to the genera *Pseudomonas*, was confirmed using the RDP II Classifier (Wang et al. 2007). For phylogenetic profiling, additional sequences were obtained from GenBank and the Ribosomal Database Project (RDP II - release 9.58) (Benson et al. 2006; Cole et al. 2007). Sequences were aligned using Clustal W (Thomson et al. 1996) and phylogenetic analysis carried out using MEGA 4.0.1 (Dudley et al. 2007) by generating a boot-strap corrected maximum parsimonious tree. Sequences were selected based on certain commonly found *Pseudomonads* in rhizospheric soil (Figure 2).

Selenium Enrichments

All soil samples examined in this study were found to be alkaline and to contain between 0.79 and 2.9 mg selenium per kg of selenium (Table 1). Although, the range observed is within the world average of 0.4 $mg.kg^{-1}$ (Ure and Berrow 1982) and below the estimated threshold level of 4 $mg.kg^{-1}$ (Engberg et al. 1998), the selenium levels in soil as estimated by this group using neutron activation indicated total Se concentration of 6.79 $mg.kg^{-1}$ in certain pockets of the region (data not shown). Neutron activation results have also shown Se >640 $mg.kg^{-1}$ in seeds of Indian mustard (*Brassica juncea*) grown in this region and similar observations of Se hyperaccumulation were noted in other plants such as wheat, maize and rice (Sharma et al. 2009). The high Se zone extends to about 1500 acres across the Nawanshahr-Hoshiarpur region and, as this region is rich in agricultural activity, the fate of the selenium through the water-soil-plant-food/fodder system is of major concern.

Se-Challenged SNT-SG1

When exposed to selenite, the isolate, SNT-SG1 produced a persistent red colour while the culture exposed to selenate produced an initial red colour, while color intensity due to Se faded slightly with time (after 72 h). At log phase, a substantial decrease in the growth of isolate was observed in the presence of selenate (Figure 3), whereas, exposure to selenite did not affect the growth of the organism at any of the Se concentrations tested, as compared to the control without added selenium (Figure 4). The growth profile was observed only up to 24 h as further optical density observations were influenced by the formation of red selenium.

Loss of selenium through reduction and volatilization by *P. aeruginosa* was also quantified. More than 50% of the selenium added was transformed/reduced by the isolate after 24 hours, when exposed to 100 mg of Se in selenite per liter as compared to only slightly more than 10% in 48 hrs when exposed to 100 mg of Se in selenate per liter. However, the overall pattern of selenium volatilization of selenite and selenate exposed cultures followed similar trends because after reaching the stationary phase, a substantially increased volatilization was observed (4.7% in selenite- and 5.1% in selenate-supplemented medium) in the trapping solution, with trapping efficiency of $>90\%$, in the time course of 72 hours. More than half of the selenium supplemented as selenite was accumulated in the cell pellet in the first 24 hours as compared to only one-tenth in the case of selenate. At the end of the experiment approximately 5–7% of the selenium was found volatilized in both selenate and selenite spiked media (Figures 5, 6). A complete mass

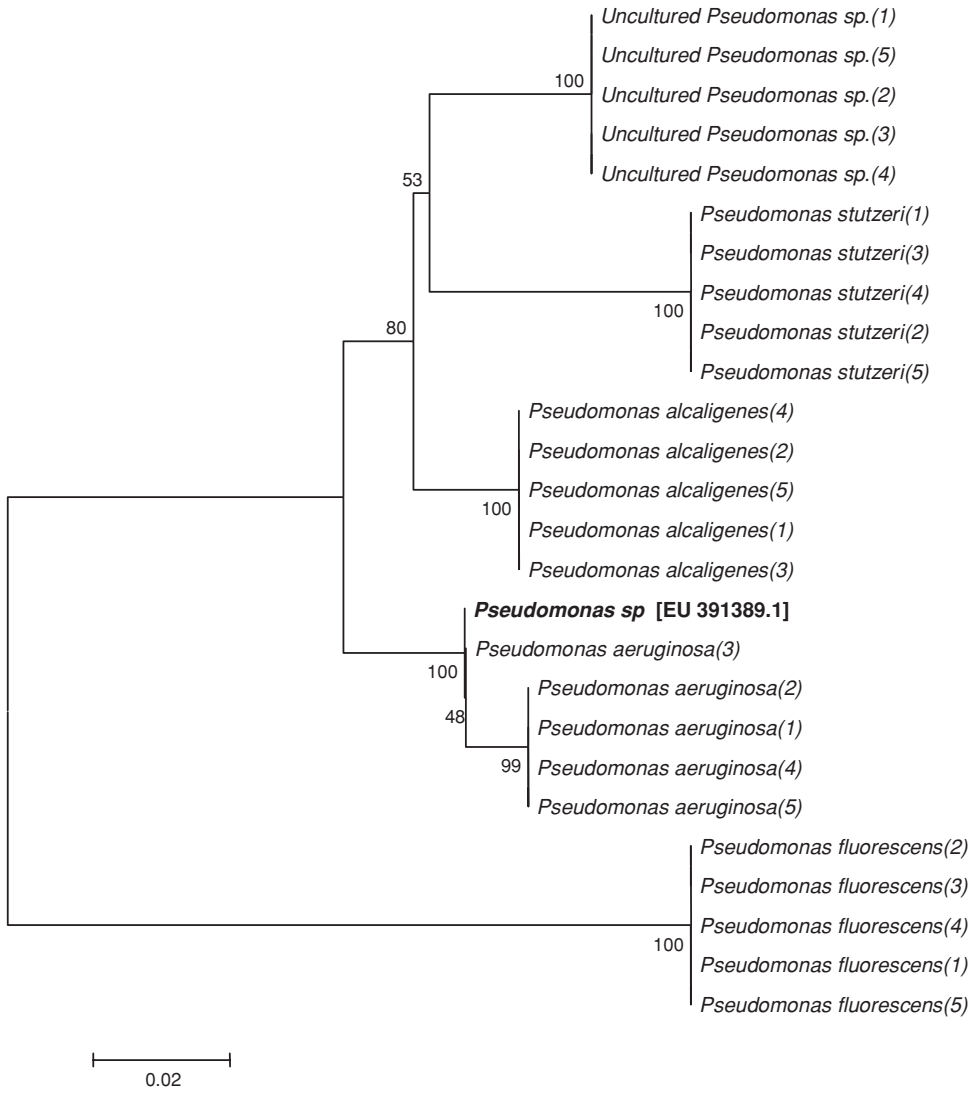

FIG. 2. Phyogenetic tree of *Pseudomonads* showing the affiliation of SNT-SG1 based on 16S rRNA gene sequence using maximum parsimony analysis. The species used for the tree (with their serial order) are given along with their accession number *Uncultured Pseudomonas sp* .- (1) 489671, (2) 489670, (3) 375162, (4) 167324, (5)167178; *P.aeruginosa* – (1) 344794, (2) 359042, (3) 090892, (4) 375847, (5) 364810; *P.floresence* – (1) 048842, (2) 048841, (3) 969089, (4) 360313, (5) 178447; *P.alcaligenes* – (1) 249591, (2) 240201, (3) 13270, 13269, 41365; *P.stutzeri*- (1) 189751, (2) 331404, (2) 888887, (3) 327524, (4) 327995.

balance of the total selenium could not be achieved due to the adherence of the biomass to containers and the loss of some Se during the digestion and graphite furnace treatments.

DISCUSSION

Pockets of seleniferous soils have been identified in north eastern parts of Punjab, India, by examining the Se content of soils, irrigation water, plants and animal tissues. The selenium concentration in the surface and sub surface layers of soils were 2.12 ± 1.13 mg.kg^{-1}and 1.16 ± 0.51mg.kg^{-1}, respectively (Dhillon and Dhillon 2003). However, as indicated earlier, on-going studies with nuclear analytical techniques indicate concentrations up to 6.79 mg.kg^{-1}in this region. It seems that the deposition of seleniferous materials transported by seasonal rivulets from higher reaches of the Shiwalik hills and, particularly, the use of groundwater (with >290 μg Se per liter) for frequent irrigation of crops like lowland rice in this relatively arid region, leads to the development of pockets of particularly high selenium (Dhillon and Dhillon 2003; Sharma et al. 2009).

Parent material of the soils is derived from Upper Shiwalik rocks comprised mainly of polymictic conglomerates of variable composition containing fragments of granite, basalt and limestone that can produce a range of weathering products (Dhillon and Dhillon 2003). These lithologies can have a range of Se-concentrations but do not normally have high concentrations unless they are sulfide-rich. It is clear that some process, probably associated with sub-surface water-rock-microbe interactions

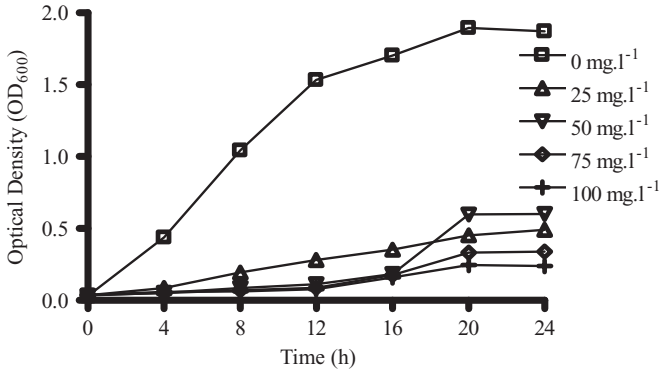

FIG. 3. Growth (OD_{600}) profile of *Pseudomonas* sp. grown at various concentrations of Se added as selenate.

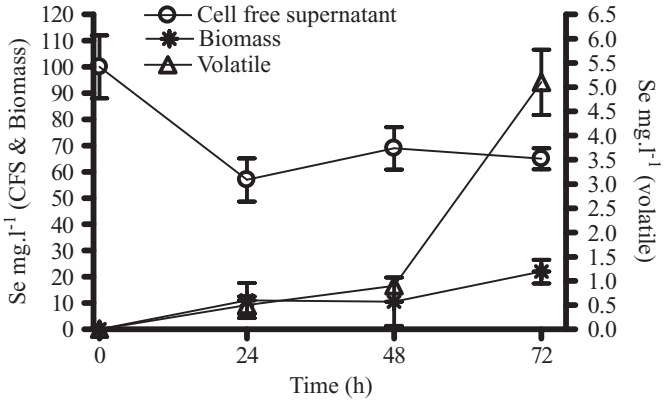

FIG. 5. Concentration of total selenium in biomass, cell free supernatant (CFS) and trapping solution analyzed after exposure of *Pseudomonas* sp. to 100 mg.l^{-1} selenium (as selenate) to 72 h in growth medium.

and the subsequent cycle of irrigation and evaporation, is responsible for continuing Se-enrichment. The resultant selenium accumulation in food and fodder that has caused a deleterious effect on the environment with the consequences for health and socioeconomic conditions in this region (Hira et al. 2004), could be attributed to these biogeochemical processes underlying the mobilization of selenium into aquifers.

'One of our longer-term research aims is to exploit the *in-situ* organisms for the volatilization of selenium from soils, and thus reduce the accumulation in food and fodder crops. As a part of this study, we have isolated a number of bacterial strains from rhizospheric soils and are in the process of understanding the mechanism(s) of selenium transformation by these organisms. The results presented here constitute the first findings on the bacterial isolates from this seleniferous region. Rhizospheric bacteria play an important role in tropical seleniferous soils through their role in selenium mobilization by reduction and/or volatilization.

Studies on mobilization of selenium by facultative bacteria have been reported for a diverse variety of species such as *Rhodobacter sphaeroides* (Fleet-Stalder et al. 2000), *Enterobacter cloacae* (Losi and Frankenberger 1997; Dungan and Frankenberger 2000), *Pseudomonas fluorescens* (Garibasu et al. 1996; Hapuarachchi et al. 2004) and *Bacillus subtilis* (Garibasu et al. 1996). However, there have been no previous studies on isolates from tropical seleniferous regions as reported here. The isolate, SNT-SG1 in the present study has been observed to grow in significantly high concentrations of selenite (>100 mM Se) with potential to reduce and volatilize selenium. Although, the growth in the presence of selenate is relatively slow, the organism is still observed to reduce and volatilize Se.

The bacteria of genera *Pseudomonads*, to which the present isolate belongs, have been observed to be rhizospheric colonizers with the potential to facilitate plant growth. In addition to playing a significant role in phytostimulation, biofertilization and pathogen resistance, this group of bacterial species is also know to bio-transform toxic soil contaminants, such as organics and metals, facilitating bioremediation (Lugtenberg and Dekkers 1999). The ability of SNT-SG1 to reduce, as well as volatilize selenium is further evidence of this characteristic of *pseudomonads*. The data show various growth profiles and

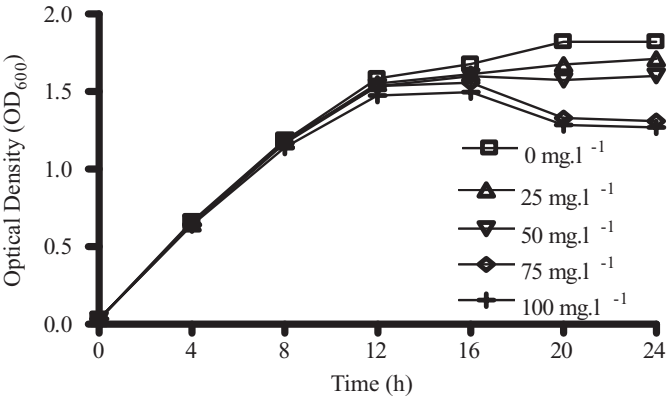

FIG. 4. Growth (OD_{600}) profile of *Pseudomonas* sp. grown at various concentrations of Se added as selenite.

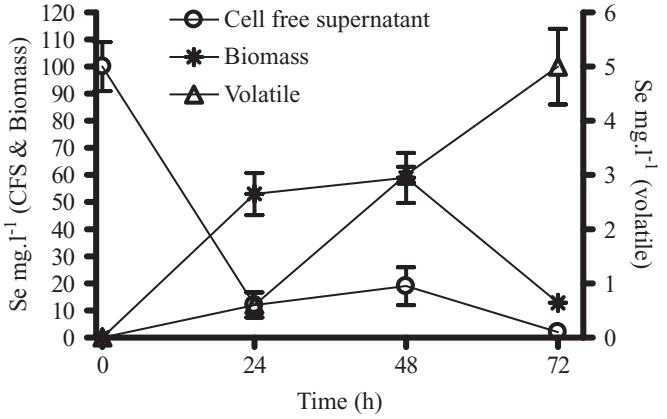

FIG. 6. Concentration of total selenium in biomass, cell free supernatant (CFS) and trapping solution analyzed after exposure of *Pseudomonas* sp. to 100mg.l^{-1} selenium (as selenite) to 72 h in growth medium.

affinity in the presence of both selenium oxyanions (selenate and selenite), with better growth and selenium reduction in the presence of selenite when compared to selenate.

Approximately 60% of the selenite and 30% of the selenate was reduced or volatilized from the oxyanion state to organic (seleno-amino acids, selenium containing enzymes, etc.) or elemental forms (Se^0), on or within the biomass. Volatilization only accounted for a small component of this process using this strain and under the conditions used here. These amounts were in addition to the selenides, which were excreted into the bulk phase and could not be quantified separately. However, the results indicate the organisms have efficient detoxification and transformation mechanisms for selenium oxyanions, especially selenite.

Observations of comparably good growth on exposure to selenite and reduced growth in the presence of selenate have been reported in certain aerobic bacteria (Garibasu et al. 1996; Fleet-Stalder et al. 2000). However, SNT-SG1 was observed to exhibit tolerance to selenium as selenite notably higher than the organisms reported earlier. Under the conditions described here, as indicated by Combs et al. (1996), even the lowest additions of selenite or selenate seem to exceed the requirements for growth, with the system probably initially assimilating the excess into seleno-amino acids and further excess being detoxified as the red elemental form, which has a very low bioavailability.

Although oxyanion speciation was not quantified in the present study, selenium uptake and assimilation was evident in the case of both the oxyanions. Over 72 h, the profile of total selenium in various fractions viz., cell-free supernatant, biomass and the trapping solution indicated that the organism diverted further selenium assimilation towards volatilization although the volatilized fraction was always significantly less than the accumulated fraction. Although Se volatilization is not commonly observed in aerobic bacteria, results indicating biomethylation of selenium oxyanions has been reported in these systems (Dungan and Frankenberger 2000; Fleet-Stalder et al. 2000).

Dimethylselenide (DMSe) has been identified as the major biological metabolite of Se methylation (Dungan and Frankenberger 2001). The methylation of Se is a biological process and is thought to be a protective mechanism used by microorganisms to detoxify their surrounding environment (Frankenberger and Karlson 1994). The biotransformation of Se to volatile Se compounds is considered a major process in the movement of Se in the environment (Haygarth 1994). Although the biological significance of Se methylation has yet to be elucidated, once volatile Se compounds are released to the atmosphere, Se loses its hazardous potential.

CONCLUSIONS

This study presents one of the few results in Se mobilizing rhizosphere bacteria from tropical seleniferous soils of India. Our results indicate that exploiting *in situ* rhizospheric bacteria in the transformation of selenite and selenate into the much less bioavailable red elemental form of selenium and volatile selenides or methylated selenium compounds may provide a useful approach for remediating selenium contamination in the agricultural soils of the seleniferous belt in India.

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