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Isolation and characterization of an arsenate-reducing bacterium and its application for arsenic extraction from contaminated soil

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Abstract

A gram-negative anaerobic bacterium, *Citrobacter* sp. NC-1, was isolated from soil contaminated with arsenic at levels as high as 5000 mg As kg^{-1}. Strain NC-1 completely reduced 20 mM arsenate within 24 h and exhibited arsenate-reducing activity at concentrations as high as 60 mM. These results indicate that strain NC-1 is superior to other dissimilatory arsenate-reducing bacteria with respect to arsenate reduction, particularly at high concentrations. Strain NC-1 was also able to effectively extract arsenic from contaminated soils via the reduction of solid-phase arsenate to arsenite, which is much less adsorptive than arsenate. To characterize the reductase systems in strain NC-1, arsenate and nitrate reduction activities were investigated using washed-cell suspensions and crude cell extracts from cells grown on arsenate or nitrate. These reductase activities were induced individually by the two electron acceptors. This may be advantageous during bioremediation processes in which both contaminants are present.

Keywords: Arsenate, Dissimilatory arsenate-reducing bacteria, Arsenic extraction, Arsenate-reducing bacterium, Terminal electron acceptor

Introduction

Arsenic (a combination of arsenate and arsenite) is toxic to bacteria, as well as to most other forms of life. Arsenic has been identified as a major risk for human health in northeast India, Bangladesh, the northwest United States, and other parts of the world [4, 25]. Arsenic forms a very small percentage of the earth's crust, but can become enriched in soil and aquatic environments as a result of dissolution and weathering [12]. This toxic element has a complex biogeochemical cycle that is partially mediated by microorganisms, including both oxidation and reduction reactions involving arsenite and arsenate [31, 35].

In Japan, soil contamination by arsenic from anthropogenic sources in urban areas has become a serious problem. To address this soil contamination, which is typically caused by industrial sites that use harmful substances, the Japanese Ministry of Environment enacted the Soil Contamination Countermeasure Law in 2003 [33]. This law sets a soil concentration standard for arsenic of 150 mg kg^{-1}. Remediation methods for arsenic contamination include containment, solidification, and stabilization; however, these all require appropriate controls and long-term monitoring because the arsenic is retained in the treated soil and continues to pose a leaching risk. Soil washing techniques using chemical agents have also been developed, but these involve the risk of depleting valuable minerals from the soil [3, 36]. Consequently, a cost-effective remediation method that readily reduces the environmental risk posed by arsenic with less damage to the soil must be developed.

In the subsurface environment, arsenic primarily exists in inorganic forms as oxyanions of As(III) (arsenite) or As(V) (arsenate). Under oxidizing conditions in the surface soil, the predominant form of arsenic is As(V). Bacterial reduction of arsenic in surface soil from As(V) to As(III) can cause the transfer of arsenic from the solid to the liquid phase because As(III) is much less strongly adsorbed to soil than As(V) [20, 29, 32]. Once the As(III) is present in the liquid phase, it can easily be removed from the liquid phase through precipitation or complexation with sulfide or sulfide-containing materials or adsorption to Fe(II)-based solids [21, 27, 28].

Lovley reported that microorganisms can remove a number of metals and metalloids from the environment or waste streams by reducing them to a lower oxidation state [18]. Microbial arsenic mobilization has bioremediation potential for the removal of arsenic from contaminated soils [8, 17] because it converts the arsenic into arsenite, which is more mobile than arsenate.

Dissimilatory arsenate-reducing bacteria (DARB) are able to reduce As(V) to As(III) and can use this toxic metalloid as a terminal electron acceptor in anaerobic respiration [2]. Since the first report of an anaerobic bacterium capable of using arsenate as an electron acceptor for growth, at least 11 other phylogenetically diverse prokaryotes that can achieve growth via dissimilatory arsenate reduction (DAsR) to As(III) have been identified [11]. DARB are agents with the potential for cost-effective bioremediation [38] of As(V), but only one attempt has been made to develop a biological treatment process that uses these organisms [38]. Yamamura et al. reported that a DARB, facultatively anaerobic *Bacillus* sp. SF-1, effectively extracted arsenic from various arsenic-contaminated solids via the reduction of solid phase arsenate to arsenite [38], indicating that DARB could be useful in arsenic contaminated sites as an arsenic extraction agent. However, little is currently known about the reducing reactions of other DARB on arsenic contaminated sites; thus, additional experiments using other DARB are required to further investigate their potential use.

In this study, we describe isolation of a novel arsenate-reducing bacterium, *Citrobacter* sp. NC-1, which was capable of using arsenate as an electron acceptor. In addition, the isolate was characterized during the reduction of arsenate. Arsenic extraction was also investigated experimentally to determine if strain NC-1 could efficiently remove arsenate from As(V)-containing soils.

Materials and methods

Media and Enrichment

Bacterial enrichment cultures were set up in 50 mL serum bottles containing 20 mL of a basal salt medium. The basal salt medium used in this study contained 0.05 g of K_2HPO_4, 0.05 g of KH_2PO_4, 0.1 g of NaCl, 0.3 g of $MgSO_4{\cdot}7H_2O$, 0.2 g of $CaCl_2{\cdot}2H_2O$, 0.6 mg of H_3BO_3, 0.169 mg of $CoCl_2{\cdot}6H_2O$, 0.085 mg of $CuCl_2{\cdot}2H_2O$, 0.099 mg of $MnCl_2{\cdot}4H_2O$, and 0.22 mg of $ZnCl_2$, and was supplemented with 0.1 g (0.01%) of yeast extract (BSMY) in 1000 mL of Tris-HCl buffer (pH 8.0). *L*-cysteine (1.5 g/L) and either 10 mM or 100 mM $Na_2HAsO_4{\cdot}7H_2O$ were added separately from sterile, anaerobic stocks. Unless otherwise stated, 2.0 g/L of glucose (glucose medium, GM) was added as the sole carbon source.

Soil samples collected from an old industrial site located in Hyogo Prefecture, Japan were used as the source of the inoculums for the enrichment cultures. The representative soil sample contained 5,000 mg As kg^{-1} soil. The enrichment cultures were maintained with a weekly subculture using the medium described above for six months. A yellow color indicated a positive arsenate reduction reaction (the formation of As(III)). After approximately twenty enrichment cultures at 28°C, the arsenate-reducing bacterium was successfully isolated using the traditional serial dilution method. To isolate the colonies, a 10-fold dilution of the enrichment culture was spread on Petri plates containing glucose (2.0 g/L), BSMY, and arsenate (2 mM) with 1.5% agar. The plate was then incubated under anaerobic conditions using an Anaerobic Gas Generation Kit (Oxoid Ltd, Hants, UK). The procedure was repeated twice to ensure a pure culture. The purity of the isolated culture was confirmed using an inverted microscope (Diaphot TMD300, Nikon, Tokyo, Japan) equipped for simultaneous recording of cell length.

Growth experiments

The ability of the isolated strain to reduce and grow on arsenate and other oxyanions was investigated by several growth experiments. In liquid culture, 20 mL of medium was used in 50 mL serum bottles. Cells of the isolated strain were cultivated anaerobically in glucose-BSMY and *L*-cysteine (1.5 g/L) for 24 h, then harvested by centrifugation (6,000×g, 10 min, 4°C) and washed twice with Tris-HCl buffer (pH 8.0). Next, 200 μL of cell suspension was used to inoculate the medium to give an optical density of 0.03 at 600 nm (OD_{600}). For anaerobic cultivation, the bottles were sealed with a butyl rubber septum and aluminum crimp seals. The headspace above the liquid phase was replaced with N_2 gas and cultivation was conducted by rotary shaking. The cultures were incubated in the dark at 28°C and periodically sacrificed, at which time the cell density was determined. The population of strain NC-1 was monitored using the plate-count technique with CGY medium (casitone 5.0 g/L, glycerin 5.0 g/L, yeast extract 1.0 g/L, and agar 15 g/L). The plate was then incubated under anaerobic conditions using an Anaerobic Gas Generation Kit (Oxoid Ltd, Hants, UK). Portions of the samples were filtered (0.45 µm, DISMIC-25cs; Advantec, Tokyo) and frozen until analysis. All experiments were performed in duplicate and the results shown are the mean values.

Electron donors and electron acceptors used for growth

Several electron acceptors were tested for their ability to support growth when glucose was present as the electron donor, including arsenate (5 mM), nitrate (5 mM), nitrite (5 mM), sulfate (5 mM), thiosulfate (5 mM), Fe(III) (as described by Lovley and Phillips [18]), and selenate (5 mM). The electron donors tested for their ability to support growth when arsenate was present as the electron acceptor included formate, molecular hydrogen, acetate, pyruvate, lactate, malate, fumarate, citrate, glycerol, phenol, ethanol, methanol, benzoate, fructose, sucrose, ribose, and xylose (all at 5 mM, except molecular hydrogen, for which 10 mL was added). The initial NC-1 inoculum used for these experiments was grown in minimal medium containing glucose (2.0 g/L), *L*-cysteine (1.5 g/L), and arsenate (5 mM). Growth with a given electron acceptor was only considered positive if a minimum of 90% of the electron acceptor was reduced after at least three subsequent subcultures. Since good growth (i.e., an increase in the number of bacteria from about 5×10^6 mL^{-1} to at least 10^8 mL^{-1} in non-pH controlled cultures) was only observed in cultures where arsenate was the terminal electron acceptor, the ability of NC-1 to grow with various electron donors was only determined using arsenate as the electron acceptor. Growth with a given electron donor was only considered positive if the numbers of motile organisms had increased from about 5×10^6 mL^{-1} to at least 10^8 mL^{-1} after at least three subsequent subcultures, and if at least 90% of the arsenate initially present in the culture was reduced to arsenite. In cultures in which the arsenate was reduced to arsenite, the total amount of arsenic in the culture remained constant throughout the experiment.

Experiments with washed cell suspensions

The objective of these investigations was to determine whether arsenate reduction is catalyzed by an enzyme specific for arsenate or by other reductases in strain NC-1, for example nitrate reductase, which are active nonspecifically for arsenate. Log-phase cells of the isolated strain were grown anaerobically with arsenate (10 mM) or nitrate (10 mM) in glucose-BSMY and then harvested by centrifugation (6,000×g, 10 min, 4°C). The harvested cells were washed twice in Tris–HCl buffer (pH 8.0) and then suspended in the same buffer containing glucose (2.0 g/L) and arsenate (1 mM) or nitrate (1 mM). Cell suspensions (20 mL) were incubated in 50 mL serum bottles with a headspace of N_2 gas on a rotary shaker (120 rpm, 28°C). The arsenate or nitrate concentration in the suspensions was monitored to confirm which oxyanion induced the reducing activity.

Effect of pH and electron donors on arsenate reduction

To evaluate the effect of pH on arsenate reduction, cell suspensions grown on arsenate were prepared with ultrapure water (pH adjusted to 6.5 with HCl), Tris–HCl buffer (pH 7.2–9.0) or glycine–NaOH buffer (pH 9.4–10.0). To investigate the effect of the electron donors, various electron donors instead of glucose were added to cell suspensions to give final concentrations of 5 mM.

Oxygen sensitivity

Strain NC-1 was grown to the mid-log phase on 10 mM lactate and 10 mM As(V), and a 10% inoculum was used to inoculate the experimental tubes in triplicate. Sterile air was added to give final concentrations of 0, 1, 2, 5, and 10% air by volume in the Balch tube headspace, and no reductant was added to the experimental tubes. To determine if strain NC-1 could resume growth after being exposed to 10% air, cells from the 10% air treatment were subsampled after 24 h of incubation and reinoculated into the 0% air tubes. The cultures were then shaken at 120 rpm and 28°C. Growth was monitored spectrophotometrically, and the accumulation of As(III) was quantified once growth was evident. Controls consisted of autoclaved cells.

Extraction of As from forest soil

To confirm that the isolated strain could extract As from natural soil systems,, we investigated the reductive extraction of As from a soil artificially contaminated with As(V), simulating soil contamination by As discharges or emissions. A forest soil was collected from the nearby countryside in Muroran (pH, 5.3; ignition loss, 13.4%) and used to make a model of contaminated soil. The soil was dried at 60°C for two days, after which it was sieved through a 2 mm mesh sieve. Next, 1.5 mL of 1 M As(V) solution was added to 100 g portions of the soil, followed by vigorous shaking at room temperature for 12 h. After drying, the soil was used as a model contaminated soil. The concentration of As in each model soil was calculated at approximately 1,200 mg kg^{-1}. One gram of the model contaminated soil was added to each 50 mL serum bottle. The bottle was then autoclaved (1 h, 121°C), and 20 mL of glucose-BSMY was added (for comparison of results, the amount of As(V) contained in the 5% [w/v] soil-medium mixture, if completely extracted, would equate to 0.76 mM dissolved As) [38]. An anaerobically grown cell suspension was then inoculated into each bottle, because As(V)-reducing activity can be readily induced under anaerobic conditions in the presence of As(V).

Analytical procedures

The arsenate and selenate concentrations in filtered samples were quantified by ion chromatography (IC, DX-300 system; Dionex, CA, USA) using a conductivity detector [9, 13]. The levels of arsenite were indirectly determined by measuring the difference in arsenate concentration between oxidized samples (oxidized by 9.1 mM H_2O_2) [50] and untreated samples [9, 13]. Nitrate and nitrite were determined using an ion chromatography system equipped with an IonPac AS4A-SC column, an IonPac AG4S-SC guard column (Dionex) and a SPD-10AV UV-VIS detector (Shimadzu, Kyoto, Japan) at 215 nm. The total arsenic in the filtrates was measured using a Hitachi Z6100 polarization Zeeman atomic adsorption spectrophotometer (Hitachi, Ibaraki, Japan).

An assay for dissimilatory arsenate reductase and nitrate reductase was conducted as previously described [14, 34] by measuring the oxidation of reduced benzyl viologen as an artificial electron donor, with the activity being calculated as one μmol of benzyl viologen oxidized per min using an extinction coefficient of 19.5 cm^{-1} mM^{-1}.

Nucleotide sequence accession number

The sequence determined in this study for strain NC-1 has been deposited in the DNA Data Bank of Japan (DDBJ) under accession number AB602381. Strain NC-1 (NBRC 107886) has been deposited in the NITE Biological Resource Center (NBRC) in Japan.

Results

Taxonomy of the isolated organism

The isolated organism was named strain NC-1. The arsenate-reducing organism is an anaerobic, gram-negative, rod-shaped bacterium. NC-1 colonies were white when cultured on glucose-BSMY agar with arsenate (2 mM). Strain NC-1 is able to produce *β*-galactosidase, but not indole, arginine dihydrolase, lysine decarboxylase, or ornithine decarboxylases (data not shown). The strains were positive for H_2S production and citrate utilization, but did not produce urease. As shown in the phylogenetic tree (Supplementary Fig. 1), strain NC-1 was identified as a *Citrobacter* sp. Strain NC-1 is a member of the γ-Proteobacteria family and is most closely related to *Citrobacter freundii* AB210978 (99.9% sequence identity), but also shares significant identity (99.7%) with *Citrobacter braakii* NR02868.

Growth characteristics

When NC-1 was grown in minimal medium with arsenate (5 mM) as the terminal electron acceptor, the following electron donors and carbon sources supported its growth: glucose, fructose, sucrose, ribose, xylose, acetate, pyruvate, lactate, formate, citrate, hydrogen, fumarate, glycerol, and malate (data not shown). No growth occurred on phenol, ethanol, methanol, benzoate, hydrogen, or fumarate when arsenate was absent. Phenol, ethanol, methanol, and benzoate also did not support growth in the presence of arsenate, but slight growth (from 5×10^6 to 9×10^6 cells mL^{-1}) was observed when hydrogen was added. When NC-1 was grown with glucose (2.0 g/L) as the electron donor and carbon source, only nitrate (5 mM) was able to replace arsenate as the terminal electron acceptor (data not shown). The electron acceptors sulfate, thiosulfate, Fe(III), selenate, and oxygen did not support its growth.

Arsenate reduction by strain NC-1

Figure 1 shows the timing of the growth of strain NC-1 during arsenate reduction. In cultures containing 5 mM, 10 mM, and 20 mM arsenate, strain NC-1 began to reduce arsenate within 12 h, and the arsenate was completely reduced within 20, 24 h, and 48 h, respectively (Fig. 1).

Cell growth occurred concurrently with arsenate reduction. However, in cultures containing 60 mM arsenate, the growth of strain NC-1 was significantly inhibited and the cell density decreased after about 15 mM of arsenate was reduced, although the arsenate reduction proceeded further (Fig. 1). During cell growth, lactic acid and pyruvic acid accumulation was observed as a result of glucose consumption (data not shown). However, yeast extract (0.1 g/L) did not serve as a carbon and energy source, as no growth or arsenate reduction occurred in the absence of glucose (data not shown).

The growth of strain NC-1 in the presence and absence of arsenate under anaerobic conditions was compared (data not shown). The growth of strain NC-1 was observed under both conditions, but more significant growth was observed in the presence of arsenate, indicating that arsenate can act as the terminal electron acceptor for anaerobic respiration (dissimilatory arsenate reduction). Arsenate reduction was not observed in the control experiments without NC-1 cells (data not shown).

When about 10 mM of arsenite was present with 10 mM of arsenate, cell growth inhibition was observed, suggesting that high concentrations of arsenite are toxic to strain NC-1 (data not shown).

Effect of other electron acceptors on arsenate reduction

Strain NC-1 can use nitrate as a terminal electron acceptor for anaerobic respiration in addition to arsenate (data not shown). When nitrate was present with arsenate, arsenate reduction proceeded concomitantly with nitrate reduction, although a slight inhibitory effect was observed (data not shown). These findings indicate that nitrate did not significantly inhibit the arsenate-reducing activity of strain NC-1.

Effect of pH and electron donors on arsenate reduction

The effect of pH on arsenate reduction by strain NC-1 was studied using washed cells grown on arsenate. The NC-1 cell suspension showed arsenate reducing activity across a pH range of 7.2–9.0, with an optimal pH of approximately 8.5 (data not shown). In a previous study, the optimal growth of strain NC-1 occurred at pH 8.0 [6].

Various carbon sources that can be used for the growth of strain NC-1 promoted arsenate reduction. Lactate and glucose were particularly effective substrates, while fumarate was not very effective when compared with the other carbon sources (data not shown). Phenol, methanol, ethanol, and benzoate, which are not growth substrates for strain NC-1, did not promote arsenate reduction. Hydrogen enhanced the reduction of arsenate, but the degradation rate was much lower than when lactate or glucose was used, possibly because of poor growth of NC-1. Pyruvate and fumarate can be used for good growth substrates similar to lactate or glucose, but their reducing activity was lower than those of lactate or glucose (data not shown). These results indicate that strain NC-1 can use various carbon sources as electron donors for arsenate reduction, although a degree of substrate specificity was observed.

Oxygen sensitivity

Strain NC-1 was capable of growth and As(V) respiration when 0 or 1% air was present in the headspace of the culture tubes. However, no cell growth occurred in cultures containing 2, 5 or 10% air (data not shown), and no As(V) respiration occurred when 2, 5, or 10% air was present (data not shown).

Arsenate and nitrate reduction by washed cell suspensions

Washed cells of strain NC-1 grown on either arsenate or nitrate as the electron acceptor were examined for their ability to reduce arsenate. Cells of strain NC-1 grown on arsenate actively reduced arsenate, with l mM being almost completely reduced within 10 hours. However, cells grown on nitrate could not significantly reduce arsenate (Table 1). No activity was shown in control experiments without any electron donor (data not shown).

The nitrate reducing activity was also investigated using washed-cell suspensions. In suspensions containing nitrate, cells grown on arsenate did not reduce nitrate, with only cells grown on nitrate being able to reduce nitrate (Table 1).

Reductase activities in crude cell extracts

To determine the dissimilatory arsenate and nitrate reductase activities in strain NC-1, crude extracts from cells grown on arsenate or nitrate as the sole electron acceptor were tested for the ability to couple the oxidation of benzyl viologen with the reduction of each electron acceptor. Crude extracts from cells grown on arsenate exhibited the highest arsenate reductase activity. Similarly, crude cell extracts grown on nitrate showed the highest reductase activity for nitrate. The maximum reductase activity in a given crude cell extract was obtained against the substrate on which the cells were grown (data not shown).

Inhibition of arsenate and nitrate reduction by tungstate

In the absence of tungstate, strain NC-1 actively reduced l mM arsenate and nitrate, with the arsenate and nitrate being completely reduced within 12 h and 8 h, respectively (data not shown). However, the addition of tungstate (1 mM) lowered the arsenate and nitrate reduction activities. The inhibition ratios for arsenate and nitrate reduction were 55.7% and 47.3%, respectively, indicating that tungstate inhibited both reduction activities.

Extraction of As from contaminated forest soil

In the experiment using the model contaminated forest soil, after 100 h in the presence of NC-1, the concentration of dissolved As increased to 80% of the total As initially added to the soil, and most of the dissolved As was present as As(III) (Fig. 2). In the control (no NC-1) experiment, although a slight increase in the dissolved As concentration was followed by a plateau, the dissolved As concentration was much lower than that observed in the experiment with NC-1, and the majority of the As was detected as As(V). These findings indicated that the dissolution of As observed in the control experiment was caused by the desorption of excess As(V) from the soil.

Discussion

Citrobacter sp. NC-1, which was isolated from arsenic contaminated soil, was characterized as a DARB. Although a number of DARBs have been reported (Supplementary Table 1), only *Citrobacter* sp. TSA-1 is from the *Citrobacter* genus [11]. However, Herbel et al. assumed that *Citrobacter* sp. capable of reducing arsenate may also exist in nature, because *Citrobacter* sp. TSA-1 was isolated from the termite hindgut rather than from nature. Thus, the isolation of *Citrobacter* sp. NC-1 from arsenic-contaminated soils strongly support their suggestion.

Strain NC-1 could grow on glucose as an electron donor and arsenate as an electron acceptor. Arsenate reduction by strain NC-1 was significantly inhibited by aerobic conditions. Although arsenate reduction can also be catalyzed by arsenic-resistant microbes, this can occur in the presence of oxygen [19]. Thus, this inhibition by oxygen is evidence that strain NC-1 is a DARB. The toxic effect of arsenite may explain the growth inhibition of strain NC-1 at high concentrations (60 mM) of arsenate (Fig. 1). These results suggest that arsenate reduction by strain NC-1 does not occur via the arsenic resistance system, which does not appear to be involved in energy conservation [5, 15], but via dissimilatory reduction.

DARB are considered to be attractive agents for the bioremediation of arsenic contaminated soils and sediments [8, 17] because they can mobilize arsenic from the solid phase into the liquid phase [1, 39]. The experimental results reported here indicate that this strain has several properties making it advantageous for bioremediation. The arsenate-reducing activity of strain NC-1 is comparable or superior to that of previously reported DARB, and even occurred at an extremely high concentration of arsenate (~60 mM). This report presents data that reveal, for the first time, that bacterial reduction of arsenate at high concentrations (~60 mM) may be possible. The presence of other electron acceptors, such as nitrate, did not inhibit the arsenate reduction, and various electron donors supported the arsenate reduction. Strain NC-1 has separate pathways for the dissimilatory reduction of arsenate and nitrate. Interestingly, there seem to be significantly different reductase systems between strain NC-1 and other prokaryotes that can reduce arsenate, selenate and nitrate. Washed-cell suspensions of both selenate-and nitrate-grown cells of *Sulfurospirillum barnesii* had a constitutive ability to reduce arsenate, and the arsenate-grown cells catalyzed selenate reduction [16, 23]. Thus, controlling the expression of the reductases may lead to effective removal of target contaminants, even in the presence of alternative electron acceptors.

Tungstate, which is known to block a number of molybdoenzymes, including nitrate reductase, by substituting tungsten for molybdenum at the active site, [7, 10, 26] had strong inhibitory effects against arsenate, selenate and nitrate reduction under anaerobic conditions.

Therefore, the dissimilatory arsenate and nitrate reductases in strain NC-1 may contain molybdenum as a cofactor as well as the dissimilatory arsenate reductase of *C. arsenatis* [30] and *B. selenitireducens* [24].

Strain NC-1 was capable of extracting As from a model soil artificially contaminated with As(V) to a greatly improved extent when compared with the abiotic control. The amount of As extracted by NC-1 considerably exceeded the levels reported in a study conducted by Yamamura et al. [38], where the extraction rate reached 56% of the total As initially added to the soil (1,124 mg kg^{-1}) after 120 h in the presence of *Bacillus* sp. SF-1. The soil conditions (i.e., pH and ignition loss) were similar to those of the soil used in the experiments with NC-1. Thus, these results indicate that strain NC-1 is more effective than *Bacillus* sp. SF-1 for the extraction of arsenate from contaminated soils. Taken together, these results confirmed that NC-1 possesses the potential to efficiently extract As from soil via the reduction of As(V) to As(III), and demonstrated that NC-1 can be used for the extraction of As from diverse As(V) contaminated soils.

A study to develop a soil cleanup process using a slurry-phase bioreactor and strain NC-1 is currently underway.

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Figure legends

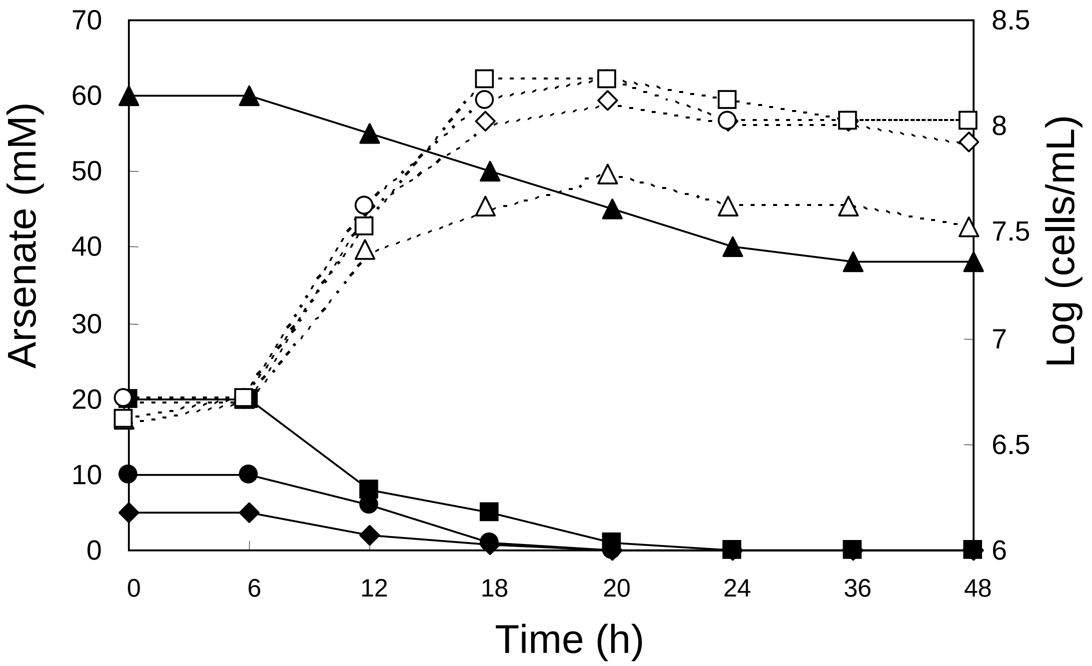
Fig. 1. Arsenate reduction by strain NC-1 and cell growth. Cultures were incubated with glucose (2.0 g/L) and 5, 10, 20, 60 mM arsenate. Solid symbols represent arsenate concentrations (diamonds are 5 mM; circles are 10 mM; squares are 20 mM; triangles are 60 mM); open symbols represent the number of cells in the corresponding cultures. Each value represents an average of two analyses (the difference between the data obtained in the two analyses was within 5%).

Fig. 2. Extraction of As from forest soil artificially contaminated with As(V). Cultures were incubated for 100 h with 2.0 g/L glucose. The pre-cultivated cultures of strain NC-1 (about 1.2×10^{8} mL^{-1}) were added to the soil-medium mixture. The concentration of As in each model soil was calculated to be approximately 1,200 mg kg^{-1}. Data represent the averages of two separate experiments (the difference between the data obtained in the two experiments was within 5%).

Supplementary Figure Legends

Fig. 1. Phylogenic tree based on comparison of the 16S rRNA gene sequence. The phylogenic tree was generated using the neighbor-joining method. Bootstrap values shown are based on 100 replications. Scale bar represents 0.005% sequence difference.

Fig. 1



70
60
50
40
30
20
10
0
Arsenate (mM)
8.5
8
7.5
7
6.5
6
Log (cells/mL)
0
6
12
18
20
24
36
48
Time (h)

Fig. 2

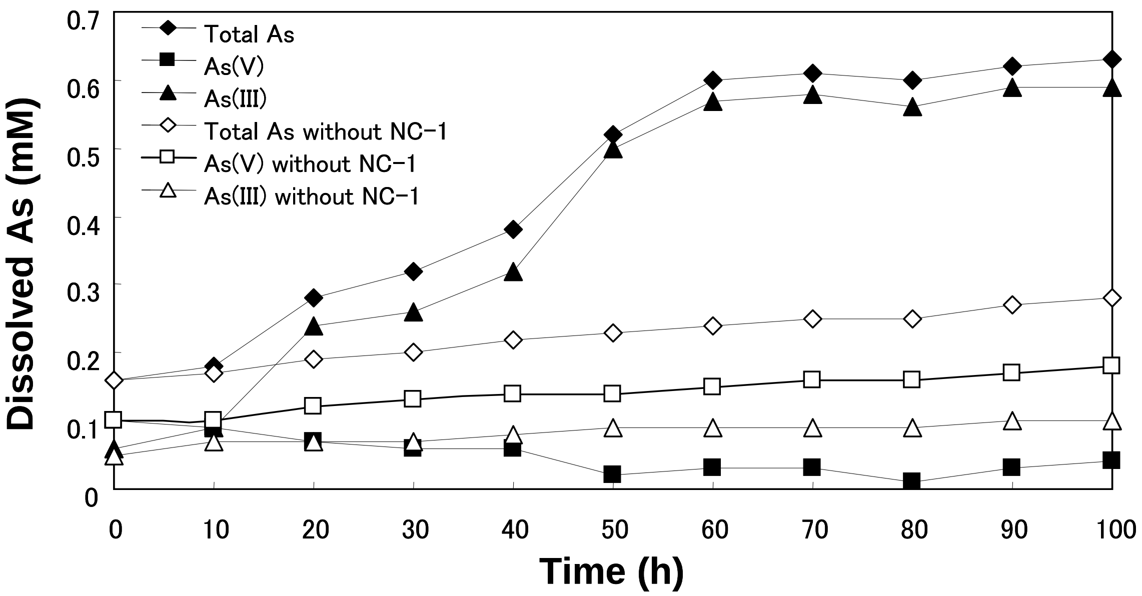

Total As
As(V)
As(III)
Total As without NC-1
As(V) without NC-1
As(III) without NC-1
Dissolved As (mM)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
50
60
70
80
90
100
Time (h)

Table 1 Arsenate and nitrate-reducing activity in washed cell suspensions of strain NC-1 grown in arsenate or nitrate with glucose as the electron donor[a]

Cell suspensions	% Arsenate reduced[b] after		Cell density[c] ($\times 10^7$ cells/mL)
	6 h	10 h	
Arsenate-grown	28	98.8	6.2
Nitrate-grown	0	9.2	2.9
Cell suspensions	% Nitrate reduced[b] after		Cell density ($\times 10^7$ cells/mL)
	6 h	12 h	
Nitrate-grown	32	52	5.9
Arsenate-grown	1.8	2.2	3.9

[a]The initial glucose concentration was 2.0 g/l, the arsenate and nitrate concentration was 1.0 mM.

[b]Results are expressed as a percentage of reduced arsenate or nitrate after 6 h or 10 h of incubation. The initial percentage before incubation was considered to be 0%.

[c]Cell density indicates the number of cells per mL in the suspension. Cell growth was not observed during incubation. Each value represents an average of two analyses (the difference of the data obtained in the two analyses was within 2.5%).

Supplementary methods

Preparation of crude cell extracts and enzyme assay

Cells of the isolated strain were grown anaerobically in 1.5 L cultures (three 500 mL Erlenmeyer flasks) with arsenate (10 mM) as the sole electron acceptor until the late log phase. The cells were harvested by centrifugation and washed twice with 50 mL of ice-cold 50 mM Tris-HCl buffer (pH 8.0) containing l mM dithiothreitol (DTT) (Buffer A). The cells were then resuspended in 10 mL ice-cold Buffer A containing l mM phenylmethane sulfonyl fluoride and then disrupted using a micro homogenizing system (Micro Smash™ Ms-100, Tomy Seiko Corp. Ltd, Japan). After DNase and RNase treatment, unbroken cells were removed by centrifugation at 2,600×g for 5 min at 4°C and the supernatants were used as crude cell extracts.

Inhibition experiment with tungstate

To determine if the arsenate reductases in the isolated strain contain a molybdenum cofactor, the effect of tungstate on arsenate reduction was tested in anaerobic growth cultures. Log phase cells of the isolated strain were cultivated anaerobically and inoculated into glucose-BSMY containing arsenate (1 mM). The cultures (20 mL) were then incubated with l mM Na2WO4 in anaerobic (N_2 atmosphere) serum bottles and the reducing activities were measured after 6 or 10 h.

DNA sequencing and phylogenetic analysis

For phylogenetic identification of the two isolates, the 16S rRNA gene fragment was amplified by polymerase chain reaction (PCR) with a pair of universal primers, 27f (5'-GAGTTTGATCMTGGCTCAG-3') and 1392r (5'-ACGGGCGGTGTGTRC-3'), under standard conditions. The PCR mixture consisted of 1 μL containing 10 pmol of each primer, 5 μL of 10×Ex Taq buffer, 4 μL of 2.5 mM each dNTP, 0.25 μL of Takara Ex Taq HS (Takara Bio, Shiga, Japan), and 2 μL of DNA extract in a final volume of 50 μL. After initial denaturation at 94°C for 3 min, 30 cycles of denaturation at 94°C for 30 s followed by primer annealing at 55°C for 1 min and extension at 72°C for 1 min were performed, after which the samples were subjected to a final extension at 72°C for 7 min. The PCR product was then purified with an ExoSAP-IT (GE Healthcare) PCR purification kit and sequenced using a BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems).

The sequence determined in this study was compared with other gene sequences in the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/). The sequence determined in this study and data retrieved from the GeneBank database were aligned using ClustalW. The alignments were refined by visual inspection. A neighbor-joining tree was constructed using the TreeView software package. A total of 1,297 bases were analyzed and bootstrap values were generated from 1,000 trees.

The physiological characteristics of the isolates were also determined using commercially available identification systems (API 20 A; bioMérieux, Japan).

Supplementary Table 1 Dissimilatory arsenate-reducing bacteria (DARB)

Species	Phylogeny	Electron acceptors	References
Thermus sp. HR13	Thermus	Arsenate, O_2	Gihring et al. 2001
Deferribacter desulfuricans SSM1	Deferribacter	Arsenate, Nitrate, S(0)	Takai et. 2003
Chrysiogenes arsenatis BAL-1 [T]	Chrysiogenes	Arsenate, Nitrate, Nitrate	Macy et al. 1996; Krafft and Macy 1998
Bacillus arsenicoselenatis E1H [T]	Low G+C Gram-positive	Arsenate, Nitrate, Selenate	Blum et al. 1998
Bacillus selenitireducens MLS10 [T]	Low G+C Gram-positive	Arsenate, Nitrate, Nitrate, Selenate, Trimethylamine oxide, low- O_2	Blum et al. 1998; Afkar et al. 2003
Bacillus sp. JMM-4	Low G+C Gram-positive	Arsenate, Nitrate	Santini et al. 2002
Bacillus sp. HT-1	Low G+C Gram-positive	Arsenate	Herbel et al. 2002
Bacillus sp. SF-1	Low G+C Gram-positive	Arsenate, Selenate, Nitrate	Fujita et al. 1997; Yamamura et al. 2003
Desulfitobacterium sp. GBFH	Low G+C Gram-positive	Arsenate, Selenate, Thiosulfate, Sulfite, S(0), Fe(III), Mu(IV), Fumarate	Niggemyer et al. 2001
Desulfitobacterium frappieri PCP-1 [T]	Low G+C Gram-positive	Arsenate, Nitrate, Selenate, Thiosulfate, Sulfite, S(0), Fe(III), Mu(IV), Fumarate	Bouchard et al. 1996; Niggemyer et al. 2001
Desulfitobacterium hafniense DCB-2 [T]	Low G+C Gram-positive	Arsenate, Nitrate, Selenate, Thiosulfate, Sulfite, S(0), Fe(III), Mu(IV), Fumarate	Christiansen and Ahring 1996[9]; Niggemyer et al. 2001[30]
Desulfosporosinus auripigmenti OREX-4 [T]	Low G+C Gram-positive	Arsenate, Sulfite, Thiosulfate, Sulfite, Fumarate	Newman et al. 1997a, 1997b; Stackebrandt et al. 2003
Strain Y5	Low G+C Gram-positive	Arsenate, Nitrate, Sulfite, Thiosulfate, Fe(III)	Liu et al. 2004
Citrobacter sp. TSA-1	Gamma Proteobacteria	Arsenate	Herbel et al. 2002
Strain GFAJ-1	Gamma Proteobacteria	Arsenate	Felisa et al. 2010
Shewanella sp.ANA-3	Gamma Proteobacteria	Arsenate, Nitrate, Thiosulfate, Fumarate, O_2, Mn O_2 $Fe(OH)_3$,AQDS	Saltikov et al. 2003
Strain MLMS-1	Delta Proteobacteria	Arsenate	Hoeft et al. 2004
Desulfomicrobium sp. BEN-RB	Delta Proteobacteria	Arsenate, Sulfite	Macy et al. 2000
Wolinella succinogenes BSA-1	Epsilon Proteobacteria	Arsenate	Herbel et al. 2002
Sulfrospirillum arsenophilum MIT-13 [T]	Epsilon Proteobacteria	Arsenate, Nitrate, Fumarate	Ahmann et al. 1994, 1997; Stolz et al. 1999
Sulfrospirillum barnesii SES-3 [T]	Epsilon Proteobacteria	Arsenate, Nitrate, Nitrite, Selenate, Thiosulfate, S(0), Fe(III), Mn(IV), Fumarate, aspartate, Trimethylamine oxide	Oremland et al. 1994, 1999; Laverman et al. 1995; Stolz et al. 1997, 1999; Zobrist et al. 2000

Supplementary Fig. 1