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

3

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12

13 **Abstract**

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

26

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

29

30 **Introduction**

31

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

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

50 In the subsurface environment, arsenic primarily exists in inorganic forms as oxyanions of
51 As(III) (arsenite) or As(V) (arsenate). Under oxidizing conditions in the surface soil, the
52 predominant form of arsenic is As(V). Bacterial reduction of arsenic in surface soil from
53 As(V) to As(III) can cause the transfer of arsenic from the solid to the liquid phase because
54 As(III) is much less strongly adsorbed to soil than As(V) [20, 29, 32]. Once the As(III) is

55 present in the liquid phase, it can easily be removed from the liquid phase through
56 precipitation or complexation with sulfide or sulfide-containing materials or adsorption to
57 Fe(II)-based solids [21, 27, 28].

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

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

76 In this study, we describe isolation of a novel arsenate-reducing bacterium, *Citrobacter* sp.
77 NC-1, which was capable of using arsenate as an electron acceptor. In addition, the isolate
78 was characterized during the reduction of arsenate. Arsenic extraction was also investigated

79 experimentally to determine if strain NC-1 could efficiently remove arsenate from
80 As(V)-containing soils.

81

82 **Materials and methods**

83

84 Media and Enrichment

85

86 Bacterial enrichment cultures were set up in 50 mL serum bottles containing 20 mL of a basal
87 salt medium. The basal salt medium used in this study contained 0.05 g of K_2HPO_4 , 0.05 g of
88 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
89 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
90 $ZnCl_2$, and was supplemented with 0.1 g (0.01%) of yeast extract (BSMY) in 1000 mL of
91 Tris-HCl buffer (pH 8.0). *L*-cysteine (1.5 g/L) and either 10 mM or 100 mM
92 $Na_2HAsO_4 \cdot 7H_2O$ were added separately from sterile, anaerobic stocks. Unless otherwise
93 stated, 2.0 g/L of glucose (glucose medium, GM) was added as the sole carbon source.

94 Soil samples collected from an old industrial site located in Hyogo Prefecture, Japan were
95 used as the source of the inoculums for the enrichment cultures. The representative soil
96 sample contained 5,000 mg As kg^{-1} soil. The enrichment cultures were maintained with a
97 weekly subculture using the medium described above for six months. A yellow color
98 indicated a positive arsenate reduction reaction (the formation of As(III)). After
99 approximately twenty enrichment cultures at 28°C, the arsenate-reducing bacterium was
100 successfully isolated using the traditional serial dilution method. To isolate the colonies, a
101 10-fold dilution of the enrichment culture was spread on Petri plates containing glucose (2.0
102 g/L), BSMY, and arsenate (2 mM) with 1.5% agar. The plate was then incubated under
103 anaerobic conditions using an Anaerobic Gas Generation Kit (Oxoid Ltd, Hants, UK). The

104 procedure was repeated twice to ensure a pure culture. The purity of the isolated culture was
105 confirmed using an inverted microscope (Diaphot TMD300, Nikon, Tokyo, Japan) equipped
106 for simultaneous recording of cell length.

107

108 Growth experiments

109

110 The ability of the isolated strain to reduce and grow on arsenate and other oxyanions was
111 investigated by several growth experiments. In liquid culture, 20 mL of medium was used in
112 50 mL serum bottles. Cells of the isolated strain were cultivated anaerobically in
113 glucose-BSMY and *L*-cysteine (1.5 g/L) for 24 h, then harvested by centrifugation (6,000×g,
114 10 min, 4°C) and washed twice with Tris-HCl buffer (pH 8.0). Next, 200 µL of cell
115 suspension was used to inoculate the medium to give an optical density of 0.03 at 600 nm
116 (OD₆₀₀). For anaerobic cultivation, the bottles were sealed with a butyl rubber septum and
117 aluminum crimp seals. The headspace above the liquid phase was replaced with N₂ gas and
118 cultivation was conducted by rotary shaking. The cultures were incubated in the dark at 28°C
119 and periodically sacrificed, at which time the cell density was determined. The population of
120 strain NC-1 was monitored using the plate-count technique with CGY medium (casitone 5.0
121 g/L, glycerin 5.0 g/L, yeast extract 1.0 g/L, and agar 15 g/L). The plate was then incubated
122 under anaerobic conditions using an Anaerobic Gas Generation Kit (Oxoid Ltd, Hants, UK).
123 Portions of the samples were filtered (0.45 µm, DISMIC-25cs; Advantec, Tokyo) and frozen
124 until analysis. All experiments were performed in duplicate and the results shown are the
125 mean values.

126

127 Electron donors and electron acceptors used for growth

128

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

148

149 Experiments with washed cell suspensions

150

151 The objective of these investigations was to determine whether arsenate reduction is catalyzed
152 by an enzyme specific for arsenate or by other reductases in strain NC-1, for example nitrate
153 reductase, which are active nonspecifically for arsenate. Log-phase cells of the isolated strain

154 were grown anaerobically with arsenate (10 mM) or nitrate (10 mM) in glucose-BSMY and
155 then harvested by centrifugation (6,000×g, 10 min, 4°C). The harvested cells were washed
156 twice in Tris–HCl buffer (pH 8.0) and then suspended in the same buffer containing glucose
157 (2.0 g/L) and arsenate (1 mM) or nitrate (1 mM). Cell suspensions (20 mL) were incubated in
158 50 mL serum bottles with a headspace of N₂ gas on a rotary shaker (120 rpm, 28°C). The
159 arsenate or nitrate concentration in the suspensions was monitored to confirm which oxyanion
160 induced the reducing activity.

161

162 Effect of pH and electron donors on arsenate reduction

163

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

169

170 Oxygen sensitivity

171

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

179 accumulation of As(III) was quantified once growth was evident. Controls consisted of
180 autoclaved cells.

181

182 Extraction of As from forest soil

183

184 To confirm that the isolated strain could extract As from natural soil systems,, we investigated
185 the reductive extraction of As from a soil artificially contaminated with As(V), simulating soil
186 contamination by As discharges or emissions. A forest soil was collected from the nearby
187 countryside in Muroran (pH, 5.3; ignition loss, 13.4%) and used to make a model of
188 contaminated soil. The soil was dried at 60°C for two days, after which it was sieved through
189 a 2 mm mesh sieve. Next, 1.5 mL of 1 M As(V) solution was added to 100 g portions of the
190 soil, followed by vigorous shaking at room temperature for 12 h. After drying, the soil was
191 used as a model contaminated soil. The concentration of As in each model soil was calculated
192 at approximately 1,200 mg kg⁻¹. One gram of the model contaminated soil was added to each
193 50 mL serum bottle. The bottle was then autoclaved (1 h, 121°C), and 20 mL of
194 glucose-BSMY was added (for comparison of results, the amount of As(V) contained in the
195 5% [w/v] soil-medium mixture, if completely extracted, would equate to 0.76 mM dissolved
196 As) [38]. An anaerobically grown cell suspension was then inoculated into each bottle,
197 because As(V)-reducing activity can be readily induced under anaerobic conditions in the
198 presence of As(V).

199

200 Analytical procedures

201

202 The arsenate and selenate concentrations in filtered samples were quantified by ion
203 chromatography (IC, DX-300 system; Dionex, CA, USA) using a conductivity detector [9,

204 13]. The levels of arsenite were indirectly determined by measuring the difference in arsenate
205 concentration between oxidized samples (oxidized by 9.1 mM H₂O₂) [50] and untreated
206 samples [9, 13]. Nitrate and nitrite were determined using an ion chromatography system
207 equipped with an IonPac AS4A-SC column, an IonPac AG4S-SC guard column (Dionex) and
208 a SPD-10AV UV-VIS detector (Shimadzu, Kyoto, Japan) at 215 nm. The total arsenic in the
209 filtrates was measured using a Hitachi Z6100 polarization Zeeman atomic adsorption
210 spectrophotometer (Hitachi, Ibaraki, Japan).

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

215

216 Nucleotide sequence accession number

217

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

221

222 **Results**

223

224 Taxonomy of the isolated organism

225

226 The isolated organism was named strain NC-1. The arsenate-reducing organism is an
227 anaerobic, gram-negative, rod-shaped bacterium. NC-1 colonies were white when cultured on
228 glucose-BSMY agar with arsenate (2 mM). Strain NC-1 is able to produce β-galactosidase,

229 but not indole, arginine dihydrolase, lysine decarboxylase, or ornithine decarboxylases (data
230 not shown). The strains were positive for H₂S production and citrate utilization, but did not
231 produce urease. As shown in the phylogenetic tree (Supplementary Fig. 1), strain NC-1 was
232 identified as a *Citrobacter* sp. Strain NC-1 is a member of the γ -Proteobacteria family and is
233 most closely related to *Citrobacter freundii* AB210978 (99.9% sequence identity), but also
234 shares significant identity (99.7%) with *Citrobacter braakii* NR02868.

235

236 Growth characteristics

237

238 When NC-1 was grown in minimal medium with arsenate (5 mM) as the terminal electron
239 acceptor, the following electron donors and carbon sources supported its growth: glucose,
240 fructose, sucrose, ribose, xylose, acetate, pyruvate, lactate, formate, citrate, hydrogen,
241 fumarate, glycerol, and malate (data not shown). No growth occurred on phenol, ethanol,
242 methanol, benzoate, hydrogen, or fumarate when arsenate was absent. Phenol, ethanol,
243 methanol, and benzoate also did not support growth in the presence of arsenate, but slight
244 growth (from 5×10^6 to 9×10^6 cells mL⁻¹) was observed when hydrogen was added. When
245 NC-1 was grown with glucose (2.0 g/L) as the electron donor and carbon source, only nitrate
246 (5 mM) was able to replace arsenate as the terminal electron acceptor (data not shown). The
247 electron acceptors sulfate, thiosulfate, Fe(III), selenate, and oxygen did not support its growth.

248

249 Arsenate reduction by strain NC-1

250

251 Figure 1 shows the timing of the growth of strain NC-1 during arsenate reduction. In cultures
252 containing 5 mM, 10 mM, and 20 mM arsenate, strain NC-1 began to reduce arsenate within
253 12 h, and the arsenate was completely reduced within 20, 24 h, and 48 h, respectively (Fig. 1).

254 Cell growth occurred concurrently with arsenate reduction. However, in cultures containing
255 60 mM arsenate, the growth of strain NC-1 was significantly inhibited and the cell density
256 decreased after about 15 mM of arsenate was reduced, although the arsenate reduction
257 proceeded further (Fig. 1). During cell growth, lactic acid and pyruvic acid accumulation was
258 observed as a result of glucose consumption (data not shown). However, yeast extract (0.1
259 g/L) did not serve as a carbon and energy source, as no growth or arsenate reduction occurred
260 in the absence of glucose (data not shown).

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

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

270

271 Effect of other electron acceptors on arsenate reduction

272

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

278

279 Effect of pH and electron donors on arsenate reduction

280

281 The effect of pH on arsenate reduction by strain NC-1 was studied using washed cells grown
282 on arsenate. The NC-1 cell suspension showed arsenate reducing activity across a pH range of
283 7.2–9.0, with an optimal pH of approximately 8.5 (data not shown). In a previous study, the
284 optimal growth of strain NC-1 occurred at pH 8.0 [6].

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

295

296 Oxygen sensitivity

297

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

302

303 Arsenate and nitrate reduction by washed cell suspensions

304

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

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

313

314 Reductase activities in crude cell extracts

315

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

323

324 Inhibition of arsenate and nitrate reduction by tungstate

325

326 In the absence of tungstate, strain NC-1 actively reduced 1 mM arsenate and nitrate, with the
327 arsenate and nitrate being completely reduced within 12 h and 8 h, respectively (data not
328 shown). However, the addition of tungstate (1 mM) lowered the arsenate and nitrate reduction

329 activities. The inhibition ratios for arsenate and nitrate reduction were 55.7% and 47.3%,
330 respectively, indicating that tungstate inhibited both reduction activities.

331

332 Extraction of As from contaminated forest soil

333

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

342

343 **Discussion**

344

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

352 Strain NC-1 could grow on glucose as an electron donor and arsenate as an electron acceptor.

353 Arsenate reduction by strain NC-1 was significantly inhibited by aerobic conditions. Although

354 arsenate reduction can also be catalyzed by arsenic-resistant microbes, this can occur in the
355 presence of oxygen [19]. Thus, this inhibition by oxygen is evidence that strain NC-1 is a
356 DARB. The toxic effect of arsenite may explain the growth inhibition of strain NC-1 at high
357 concentrations (60 mM) of arsenate (Fig. 1). These results suggest that arsenate reduction by
358 strain NC-1 does not occur via the arsenic resistance system, which does not appear to be
359 involved in energy conservation [5, 15], but via dissimilatory reduction.

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

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

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

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

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

395

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397

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403

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503 Figure legends

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

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512 Fig. 2. Extraction of As from forest soil artificially contaminated with As(V). Cultures were
513 incubated for 100 h with 2.0 g/L glucose. The pre-cultivated cultures of strain NC-1 (about
514 $1.2 \times 10^8 \text{ mL}^{-1}$) were added to the soil-medium mixture. The concentration of As in each model
515 soil was calculated to be approximately 1,200 mg kg⁻¹. Data represent the averages of two
516 separate experiments (the difference between the data obtained in the two experiments was
517 within 5%).

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529 Supplementary Figure Legends

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531 Fig. 1. Phylogenetic tree based on comparison of the 16S rRNA gene sequence. The phylogenetic
532 tree was generated using the neighbor-joining method. Bootstrap values shown are based on
533 100 replications. Scale bar represents 0.005% sequence difference.

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Fig. 1

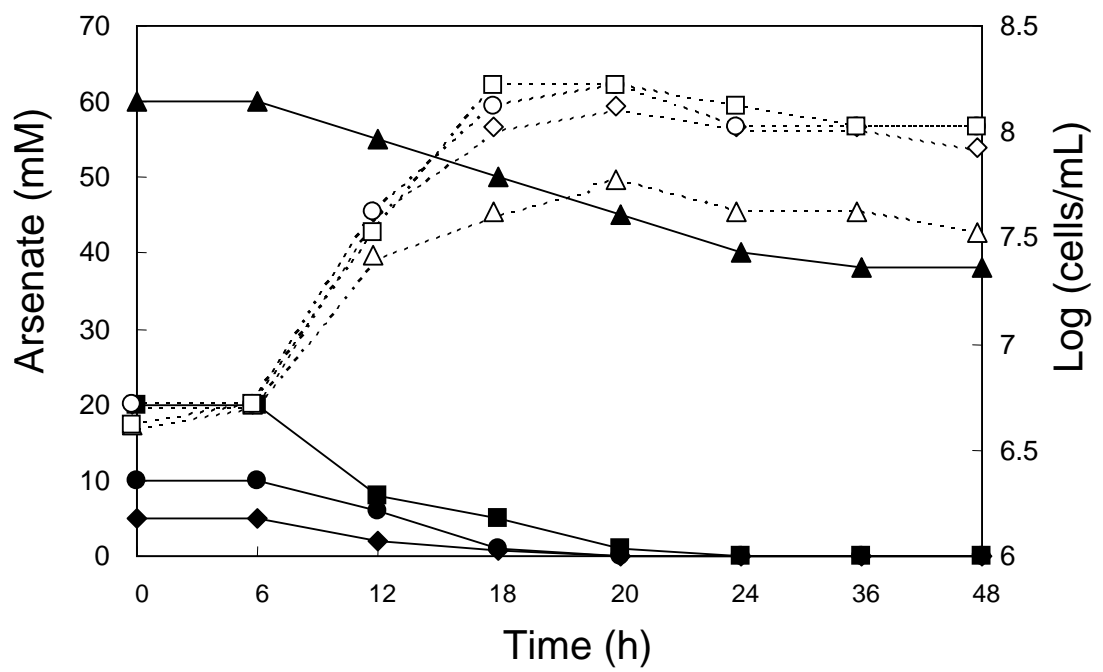


Fig. 2

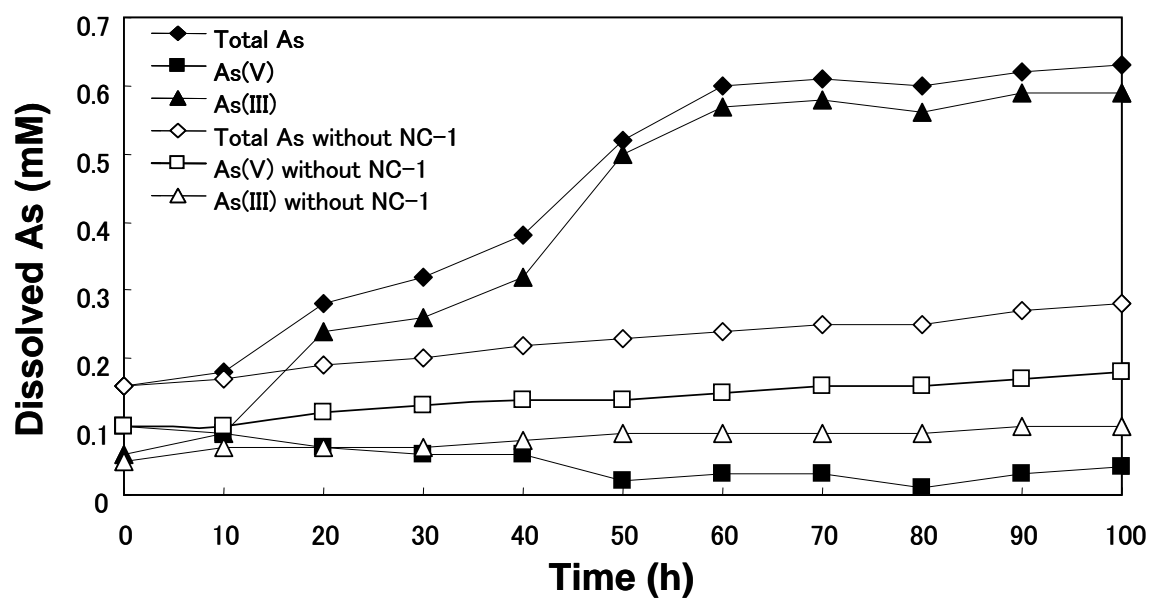


Table 1 Arsenate and nitrate-reducing activity in washed cell suspensions of strain NC-1 grown in arsenate or nitrate with glucose as the electron donor^a

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

^aThe initial glucose concentration was 2.0 g/l, the arsenate and nitrate concentration was 1.0 mM.

^bResults 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%.

^cCell 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 1 mM dithiothreitol (DTT) (Buffer A). The cells were then resuspended in 10 mL ice-cold Buffer A containing 1 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 1 mM Na₂WO₄ in anaerobic (N₂ 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
<i>Thermus</i> sp. HR13	Thermus	Arsenate, O ₂	Gihring et al. 2001
<i>Deferribacter desulfuricans</i> SSM1	Deferribacter	Arsenate, Nitrate, S(0)	Takai et al. 2003
<i>Chrysiogenes arsenatis</i> BAL-1 ^T	Chrysiogenes	Arsenate, Nitrate, Nitrate	Macy et al. 1996; Krafft and Macy 1998
<i>Bacillus arsenicoselenatis</i> E1H ^T	Low G+C Gram-positive	Arsenate, Nitrate, Selenate	Blum et al. 1998
<i>Bacillus selenitireducens</i> MLS10 ^T	Low G+C Gram-positive	Arsenate, Nitrate, Nitrate, Selenate, Trimethylamine oxide, low- O ₂	Blum et al. 1998; Afkar et al. 2003
<i>Bacillus</i> sp. JMM-4	Low G+C Gram-positive	Arsenate, Nitrate	Santini et al. 2002
<i>Bacillus</i> sp. HT-1	Low G+C Gram-positive	Arsenate	Herbel et al. 2002
<i>Bacillus</i> sp. SF-1	Low G+C Gram-positive	Arsenate, Selenate, Nitrate	Fujita et al. 1997; Yamamura et al. 2003
<i>Desulfitobacterium</i> sp. GBFH	Low G+C Gram-positive	Arsenate, Selenate, Thiosulfate, Sulfite, S(0), Fe(III), Mu(IV), Fumarate	Niggemyer et al. 2001
<i>Desulfitobacterium frappieri</i> 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
<i>Desulfitobacterium hafniense</i> 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]
<i>Desulfosporosinus auripigmenti</i> 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
<i>Citrobacter</i> sp. TSA-1	Gamma Proteobacteria	Arsenate	Herbel et al. 2002
Strain GFAJ-1	Gamma Proteobacteria	Arsenate	Felisa et al. 2010
<i>Shewanella</i> 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	Hoefl et al. 2004
<i>Desulfomicrobium</i> sp. BEN-RB	Delta Proteobacteria	Arsenate, Sulfite	Macy et al. 2000
<i>Wolinella succinogenes</i> BSA-1	Epsilon Proteobacteria	Arsenate	Herbel et al. 2002
<i>Sulfospirillum arsenophilum</i> MIT-13 ^T	Epsilon Proteobacteria	Arsenate, Nitrate, Fumarate	Ahmann et al. 1994, 1997; Stolz et al. 1999
<i>Sulfospirillum barnesii</i> 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

