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총설

이화형비산염환원균의 특성

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Characteristics of Dissimilatory Arsenate-reducing Bacteria

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Abstract: Although, microbial arsenic mobilization by dissimilatory arsenate-reducing bacteria (DARB) and the practical use to the removal technology of arsenic from contaminated soil are expected, most previous research mainly has been focused on the geochemical circulation of arsenic. Therefore, in this review we summarized the previously reported DARB to grasp the characteristic for bioremediation of arsenic. Evidence of microbial growth on arsenate is presented based on isolate analyses, after which a summary of the physiology of the following arsenate-respiring bacteria is provided: *Chrysiogenes arsenatis* strain BAL-1^T, *Sulfurospirillum barnesii*, *Desulfotomaculum* strain Ben-RB, *Desulfotomaculum auripigmentum* strains OREX-4, GFAJ-1, *Bacillus* sp., *Desulfitobacterium hafniense* DCB-2^T, strain SES-3, *Citrobacter* sp. (TSA-1 and NC-1), *Sulfurospirillum arsenophilum* sp. nov., *Shewanella* sp., *Chrysiogenes arsenatis* BAL-l^T, *Deferribacter desulfuricans*. Among the DARB, *Citrobacter* sp. NC-1 is superior to other dissimilatory arsenate-reducing bacteria with respect to arsenate reduction, particularly at high concentrations as high as 60 mM. A gram-negative anaerobic bacterium, *Citrobacter* sp. NC-1, which was isolated from arsenic contaminated soil, can grow on glucose as an electron donor and arsenate as an electron acceptor. Strain NC-1 rapidly reduced arsenate at 5 mM to arsenite with concomitant cell growth, indicating that arsenate can act as the terminal electron acceptor for anaerobic respiration (dissimilatory arsenate reduction). To characterize the reductase systems in strain NC-1, arsenate and nitrate reduction activities were investigated with 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. Tungstate, which is a typical inhibitory antagonist of molybdenum containing dissimilatory reductases, strongly inhibited the reduction of arsenate and nitrate in anaerobic growth cultures. These results suggest that strain NC-1 catalyzes the reduction of arsenate and nitrate by distinct terminal reductases containing a molybdenum cofactor. This may be advantageous during bioremediation processes where both contaminants are present. Moreover, a brief explanation of arsenic extraction from a model soil artificially contaminated with As (V) using a novel DARB (*Citrobacter* sp. NC-1) is given in this article. We conclude with a discussion of the importance of microbial arsenate reduction in the environment. The successful application and use of DARB should facilitate the effective bioremediation of arsenic contaminated sites.

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

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1. Introduction

Contamination of drinking water supplies with inorganic soluble forms of arsenic (a combination of arsenate and arsenite) has commonly been reported, and arsenic has been identified as a major risk factor for human health [1]. Arsenic is toxic to bacteria, as well as to most other forms of life. Indeed, arsenic has been found to pose a major risk to human health in northeast India, Bangladesh, the northwest United States, and other parts of the world [2,3]. 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 [4].

In Japan, soil contamination by arsenic from anthropogenic sources has become a serious problem in urban areas. To address this soil contamination, the Japanese Ministry of Environment enacted the Soil Contamination Countermeasure Law in 2003 [5]. This law sets a soil concentration standard for arsenic of 150 mg/kg. Remediation methods for arsenic contamination include containment, solidification, and stabilization however, these all require appropriate controls and long-term monitoring because they result in the arsenic being retained in the treated soil and continuing 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 [6,7]. Consequently, a cost-effective method of remediation that readily reduces the environmental risk posed by arsenic with less damage to the soil must be developed.

Arsenic primarily exists in inorganic forms as oxyanions of As (III) (arsenite) or As (V) (arsenate) in the subsurface environment. Under oxidizing conditions in the surface soil, the predominant form of arsenic is As (V). However, 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) [8-10]. Once the As (III) is present in the liquid phase, it can be easily removed through precipitation or complexation with sulfide or sulfide-containing materials or via adsorption to Fe (II)-based solids [11-13]. The concept of microbial arsenic removal from contaminated soil is shown in Fig. 1.

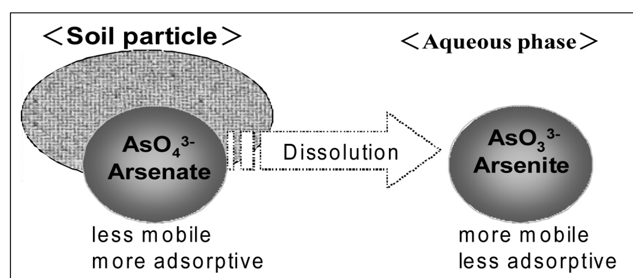

Fig. 1. Concept of microbial arsenic removal from contaminated soil.

Microorganisms can remove a number of metals and metalloids from the environment or waste streams by reducing them to a lower oxidation state [14]. Microbial arsenic mobilization has the potential for use in the removal of arsenic from contaminated soils [15,16] 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 [17]. Accordingly, DARB could be useful in arsenic contaminated sites as an arsenic extraction agent. However, there is currently little information available regarding the reducing reactions of DARB at arsenic contaminated sites.

In this review, we summarize DARB and introduce the characteristics of *Citrobacter*sp. NC-1 during the reduction of arsenate. Arsenic extraction was also described experimentally to evaluate whether strain NC-1 could efficiently remove arsenate from soils containing As (V).

2. DARB

DARB are able to reduce As (V) to As (III) and utilize this toxic metalloid as a terminal electron acceptor during anaerobic respiration [6]. Since the first report of an anaerobic bacterium capable of using the toxic oxyanion As (V) as an electron acceptor for growth, at least 11 other phylogenetically diverse prokaryotes that can achieve growth via dissimilatory arsenate reduction to As (III) have been identified [18]. DARBs are considered to be attractive agents with the potential for cost-effective bioremediation of arsenic [19]. Representative DARBs are summarized in Table 1.

2.1. *Deferribacter desulfuricans* SSM1 [21]

A novel anaerobic, heterotrophic thermophile was isolated from a deep-sea hydrothermal vent chimney at the Suiyo Seamount in the Izu-Bonin Arc, Japan. The cells are bent, flexible rods, with a single polar flagellum. Growth occurs between 40℃ and 70℃ (optimum temperature: 60℃-65℃; doubling time, 40 min) and between pH 5.0 and 7.5 (optimum pH: 6.5). The isolate is a strictly anaerobic heterotroph capable of using complex organic compounds (yeast extract, tryptone, peptone, casein and Casamino acids), ethanol and various organic acids as energy and carbon sources. Hydrogen can serve as a supplementary energy source. Elemental sulfur (S^0), nitrate or arsenate are required for growth as an electron acceptor. The G+C content of the genomic DNA is 38.6 mol%. Phylogenetic analysis based on 16S rDNA sequences indicated that isolate $SSM1^T$ is closely related to *Deferribacter thermophilus* BMA^T (98.1%). However, the novel isolate can be clearly differentiated from *D.*

Table 1. Dissimilatory arsenate-reducing bacteria (DARB)

Species	Phylogeny	Electron acceptors	References
Thermus sp. HR13	Thermus	Arsenate, O_2	Gihring et al. 2001 [20]
Deferribacter desulfuricans SSM1	Deferribacter	Arsenate, Nitrate, S (0)	Takai et. 2003 [21]
Chrysiogenes arsenatis BAL-1^T	Chrysiogenes	Arsenate, Nitrate, Nitrate	Macy et al. 1996 [3]; Krafft and Macy 1998 [14]
Bacillus arsenicoselenatis E1H^T	Low G+C Gram-positive	Arsenate, Nitrate, Selenate	Blum et al. 1998 [22]
Bacillus selenitireducens MLS10^T	Low G+C Gram-positive	Arsenate, Nitrate, Nitrate, Selenate, Trimethylamine oxide, low- O_2	Blum et al. 1998 [22]; Afkar et al. 2003 [23]
Bacillus sp. JMM-4	Low G+C Gram-positive	Arsenate, Nitrate	Santini et al. 2002 [24]
Bacillus sp. HT-1	Low G+C Gram-positive	Arsenate	Herbel et al. 2002 [18]
Bacillus sp. SF-1	Low G+C Gram-positive	Arsenate, Selenate, Nitrate	Fujita et al. 1997 [25]; Yamamura et al. 2003 [26]
Desulfitobacterium sp. GBFH	Low G+C Gram-positive	Arsenate, Selenate, Thiosulfate, Sulfite, S (0), Fe (III), Mu (IV), Fumarate	Niggemyer et al. 2001 [27]
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 [28]; Niggemyer et al. 2001 [27]
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 [29]; Niggemyer et al. 2001 [27]
Desulfotomaculum auripigmenti OREX-4^T	Low G+C Gram-positive	Arsenate, Sulfite, Thiosulfate, Sulfite, Fumarate	Newman et al. 1997 [13]; Stackebrandt et al. 2003 [30]
Strain Y5	Low G+C Gram-positive	Arsenate, Nitrate, Sulfite, Thiosulfate, Fe (III)	Liu et al. 2004 [11]
Citrobacter sp. TSA-1	Grmma Proteobacteria	Arsenate	Herbel et al. 2002 [18]
Citrobacter sp. NC-1	Grmma Proteobacteria	Arsenate	Chang et al. 2012 [31]
Shewanella sp. ANA-3	Gamma Proteobacteria	Arsenate, Nitrate, Thiosulfate, Fumarate, O_2, Mn O_2 Fe $(OH)_{,3}$, AQDS	Saltikov et al. 2003 [32]
Strain GFAJ-1	Gamma Proteobacteria	Arsenate	Felisa et al. 2011 [33]
Strain MLMS-1	Delta Proteobacteria	Arsenate	Hoeft et al. 2004 [34]
Desulfotomaculum sp. Ben-RB	Delta Proteobacteria	Arsenate, Sulfite	Macy et al. 2000 [35]
Wolinella succinogenes BSA-1	Epsilon Proteobacteria	Arsenate	Herbel et al. 2002 [18]
Sulfurospirillum arsenophilum MIT-13^T	Epsilon Proteobacteria	Arsenate, Nitrate, Fumarate	Ahmann et al. 1994, 1997 [6,17]; Stolz et al. 1999 [36]
Sulfurospirillum 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 [10,37]; Laverman et al. 1995 [8]; Stolz et al. 1997, 1999 [36,37]; Zobrist et al. 2000 [38]

thermophilus BMA^T based on its physiological and genetic properties. The name *Deferribacter desulfuricans* sp. nov. (type strain SSM1^T = JCM 11476^T = DSM 14783^T) is proposed for this organism.

2.2. *Chrysiogenes arsenatis* BAL-1T [3,14]

A strictly anaerobic bacterium (strain BAL-1^T) was isolated from a reed bed at Ballarat Goldfields in Australia. This organism grows by reducing arsenate while using acetate as the electron donor and carbon source; however, acetate alone does not support growth. When grown with arsenate as the terminal electron acceptor, acetate can be replaced by pyruvate, *l*- and *d*-lactate, succinate, malate, and fumarate, but not by H_2, formate, citrate, glutamate, other amino acids, sugars, or benzoate. When acetate is the electron donor, arsenate can be replaced by nitrate or nitrite, but not by sulphate, thiosulphate, or iron oxide. BAL-1^T reduces nitrate to ammonia via nitrite. The doubling time for growth on acetate (5 mM) plus arsenate (5 mM) or nitrate (5 mM) is 4 h. The organism has a DNA G+C content of 49 mol%. The 16S rRNA sequence data for the organism support the hypothesis that this species is phylogenetically unique, and it is currently

the first representative of a new deeply branching lineage of the Bacteria. This organism is described as *Chrysiogenes arsenatis* gen. nov., sp. nov.

2.3. Strains E1H and MLS10 [22,23]

Two gram-positive anaerobic bacteria (strains E1H and MLS10) were isolated from anoxic muds of Mono Lake, California, an alkaline, hypersaline, arsenic rich water body. Both bacteria grow via dissimilatory reduction of As (V) to As (III) with the concomitant oxidation of lactate to acetate plus CO_2. *Bacillus arsenicoselenatis* (strain E1H) is a spore-forming rod that also grows via dissimilatory reduction of Se (VI) to Se (IV). *Bacillus selenitireducens* (strain MLS10) is a short, non-spore-forming rod that grows by dissimilatory reduction of Se (IV) to Se^0. When the two isolates are cocultured, complete reduction of Se (VI) to Se^0 is achieved. Both isolates are alkaliphiles and have optimal specific growth rates in the pH range of 8.5-10. Strain E1H has an optimum salinity of 60 g/L NaCl, while that of strain MLS10 is 24-60 g/L NaCl. Strain MLS10 demonstrates weak growth as a microaerophile and is capable of fermentative growth on glucose, while strain E1H is a strict anaerobe.

2.4. Strain JMM-4 [24]

A strictly anaerobic arsenate-respiring bacterium was isolated from arsenic contaminated mud obtained from a gold mine in Bendigo, Australia. This organism, designated JMM-4, isa Gram-positive, spore-forming rod, motile by means of flagella that are subpolar or located along one side of the cell. JMM-4 grows using arsenate as the terminal electron acceptor and lactate as the electron donor. During the growth of JMM-4, arsenate is reduced to arsenite and lactate is oxidized to CO_2 via the intermediate, acetate. The doubling time for exponential growth with arsenate (5 mM) and lactate (5 mM) is 4.3-0.2 h. The alternative electron donors used by JMM-4 when grown with arsenate as the terminal electron acceptor are acetate, pyruvate, succinate, malate, glutamate, and hydrogen (with acetate as the carbon source). Apart from arsenate, nitrate can serve as an alternative electron acceptor. Optimal growth occurs at pH 7.8 with a sodium chloride concentration of 1.2 g/L. Sequence analysis of the 16S rRNA gene revealed that JMM-4 falls within the low G+C, Gram-positive, aerobic, spore-forming bacilli cluster and is most closely related to the previously described haloalkalophilic arsenate/selenate respiring bacterium, *Bacillus arsenicoselenatis*. However, the physiological differences between JMM-4 and *B. arsenicoselenatis* suggest that JMM-4 is a new species of *Bacillus*.

2.5. *Bacillus* sp. SF-1 [25,26]

A gram-positive and facultative anaerobic bacterium, *Bacillus* sp. SF-1, was isolated from selenium-polluted sediment. This organism can utilise lactate as an electron donor and selenate, arsenate or nitrate as an alternate electron acceptor in the absence of oxygen. This strain appears to be a promising agent for bioremediation of selenium and arsenic. In an effort to characterize the reductase systems of strain SF-1, its arsenate, selenite and nitrate reduction activities were investigated using washed-cell suspensions and crude cell extracts from cells grown on arsenate, selenite and nitrate. These reductase activities were induced individually by their respective electron acceptors. Tungstate, which is a typical inhibitory antagonist of molybdenum-containing dissimilatory reductases, strongly inhibited reduction of arsenate, selenate and nitrate in the anaerobic growth cultures. Strain SF-1 effectively extracted arsenic from various arsenic-contaminated solids via the reduction of solid phase arsenate to arsenite [19].

2.6. *Desulfitobacterium* sp. GBFH [27]

GBFH is the first As (V)-reducing organism capable of conserving energy for growth by coupling the reduction of As (V) to the oxidation of formate when formate is supplied as the sole electron donor and carbon source. However, formate alone cannot support the growth of GBFH in the absence of an exogenous tetraethylammonium such as As (V). Yeast extract (0.1 g/L) cannot serve as a carbon and energy source as no growth or As (V) reduction occurs in the absence of formate.

2.7. *Desulfitobacterium hafniense* DCB-2^T [27, 29]

Strain DCB-2^T (T = type strain) is an anaerobic, spore-forming bacterium capable of reductive dechlorination of chlorophenols. The cells of this strain are rod shaped, 3.3 to 6 μm long by 0.6 to 0.7 μm wide, occurring singly, in pairs or short chains are formed. Spores are terminal. This bacterium is motile, and each cell has one or two terminal flagella. Cells in the exponential and stationary phases are gram negative. This organism does not hydrolyze gelatin,is indole positive and catalase negative and has a cellular DNA guanine-plus-cytosine content of47 mol%. The optimum temperature for growth of strain DCB-2^T is 37℃. The organism can only use pyruvate and tryptophan as substrates. Pyruvate and 2,4,6-trichloropheno1 are converted to acetate, CO, and 4-chlorophenol by strain DCB-2^T. When grown on pyruvate, this bacterium produces sulphide if thiosulphate or sulphite is added as an electron acceptor. Fe (III) is reduced to Fe (II), but Mn (IV) is not reduced. Sulphate is not reduced to sulphidein the presence of pyruvate or other carbon sources typically used by sulphate-reducing bacteria. Cytochrome c is present, but desulfoviridin is not. DCB-2^T reductively dechlorinates 3-chloro-4-hydroxyphenylacetatteo

4-hydroxyphenylacetate and conserves energy from the reaction.

2.8. *Desulfotomaculum auripigmentum* OREX-4 [13,30]

The arsenate-reducing bacterium, strain OREX-4, differs significantly from the previously described arsenate-reducing isolates, MIT-13 and SES-3, which grow on nitrate but not sulphate. In contrast, strain OREX-4 does not respire nitrate, but does grow on lactate. During growth, either arsenate or sulphate can be used as the electron acceptor, although arsenate is preferred. The reduction of both arsenate and sulphate by OREX-4 are inhibited by molybdate. Strain OREX-4 is a gram-positive bacterium with a hexagonal S-layer on its cell wall that metabolizes compounds commonly used by sulphate reducers. Scorodite ($FeAsO_4 \cdot 2H_2O$), which is an arsenate-containing mineral, was found to provide micromolar concentrations of arsenate that supported cell growth. Strain OREX-4 is physiologically and phylogenetically distant from strains MIT-13 and SES-3. Specifically, strain OREX-4 grows on different electron donors and electron acceptors than MIT-13 and SES-3, and falls within the gram-positive group of the Bacteria, whereas MIT-13 and SES-3 fall together within the ε-subdivision of the Proteobacteria. Together, these results suggest that diverse bacterial phyla can use arsenate as a terminal electron acceptor, and that dissimilatory arsenate reduction might occur in the sulfidogenic zone at arsenate concentrations of environmental interest.

2.9. *Citrobacter* sp. TSA-1 [18]

An isolate from the termite was shown to be capable of growth using H_2 as the electron donor and As (V) as their electron acceptor with the caveat that acetate was provided as a building block for cell carbon. Strain TSA-1 is clearly a species of *Citrobacter*.

2.10. *Shewanella* sp. ANA-3 [32]

The gram-negative strain stoichiometrically couples the oxidation of lactate to acetate with the reduction of As (V) to arsenite [As (III); $HAsO_2$]. The generation time and lactate molar growth yield ($Y_{lactate}$) are 2.8 h and 10.0 g of cells per mol of lactate^{-1}, respectively, when grown anaerobically on lactate and As (V). ANA-3 uses a wide variety of terminal electron acceptors, including oxygen, soluble ferric iron, oxides of iron and manganese, nitrate, fumarate, the humic acid functional analog 2,6-anthraquinone disulphonate, and thiosulfate. ANA-3 also reduces As (V) to As (III) in the presence of oxygen and resists high concentrations of As (III) (up to 10 mM) when grown under either aerobic or anaerobic conditions. ANA-3 possesses an ars operon (arsDABC) that allows it to resist high levels of As (III), which is the same operon that confers resistance to the As-sensitive strains *Shewanella oneidensis* MR-1 and *Escherichia coli* AW3110. When the gene encoding the As (III) efflux pump, arsB, is inactivated in ANA-3 by a polar mutation that also eliminates the expression of arsC, the resulting As (III)-sensitive strain still respires As (V); however, the generation time and $Y_{lactate}$ value are two- and threefold lower, respectively, than those of the wild type.

2.11. Strain GFAJ-1 [33]

Strain GFAJ-1 of Halomonadaceae, which was isolated from Mono Lake, California, is able to substitute arsenic for phosphorus to sustain its growth. Mono Lake is hypersaline and alkaline, with high dissolved arsenic concentrations [200 μM on average [9]]. The lake sediments were used as inocula into an aerobic defined artificial medium with a pH of 9.8 [10,11] that contained 10 mM glucose, vitamins, and trace metals, but no added PO_4^{3-} orcomplex organic supplements (such as yeast extract or peptone), with a regimen of increasing AsO_4^{3-} levels ranging from 100 μM to 5 mM. These enrichments were made through many decimal-dilution transfers, greatly reducing any potential carryover of autochthonous phosphorus. The average background PO_4^{3-} in the medium was 3.1 μM, regardless of whether AsO_4^{3-} was added, and this PO_4^{3-} originated from trace impurities in the major salts. Strain GFAJ-1 has been identified as a member of the Halomonadaceae family of Gammaproteobacteria based on its 16S ribosomal RNA sequence phylogeny. This strain is currently maintained aerobically with 40 mM AsO_4^{3-}, 10 mM glucose, and no added PO_4^{3-} (+As/-P condition). Members of this family have previously been shown to accumulate intracellular As. GFAJ-1 grows at an average mmax of 0.53 day^{-1} under +As/-P, and its concentration has been shown to increase by over 20-fold after six days. This organism also grows faster and more extensively with the addition of 1.5 mM PO_4^{3-} (-As/+P, μmax of 0.86/day). However, no growth is observed when neither AsO_4^{3-} nor PO_4^{3-} are added.

2.12. Strain MLMS-1 [34]

Anoxic bottom water from Mono Lake, California, can biologically reduce added arsenate without the addition of electron donors. Of the possible in situ inorganic electron donors present, only sulphide is present in levels sufficient to drive this reaction. Research group tested the ability of sulphideto serve as an electron donor for arsenate reduction in experiments with lake water. Reduction of arsenate to arsenite occurred simultaneously with the removal of sulphide. However, no loss of sulphideoccurred in controls without arsenate or in sterilized samples containing both arsenate and sulphide. The rate of arsenate reduction in lake water was dependent on the amount of available arsenate. Research

group isolated a bacterium that could achieve growth with sulphide and arsenate by enrichment of a mineral medium and subsequent purification by serial dilution. The isolate, strain MLMS-1, is a gram-negative, motile, curved rod that grows by oxidizing sulphide to sulphatewhile reducing arsenate to arsenite. Chemoautotrophy was confirmed by the incorporation of $H^{14}CO_3^-$ into dark-incubated cells, but preliminary gene probing tests with primers for ribulose-1,5-biphosphate carboxylase/oxygenase did not yield PCR-amplified products. Alignment of 16S rRNA sequences indicated that strain MLMS-1 is a δ-Proteobacteria related to sulphate reducers such as *Desulfobulbus* sp. (88 to 90% similarity), but more closely related (97%) to unidentified sequences previously amplified from Mono Lake. However, strain MLMS-1 does not grow using sulphate as its electron acceptor.

2.13. *Desulfotomaculum* sp. Ben-RB [35]

Desulfotomaculum strain Ben-RB is able to grow using lactate as a substrate and arsenate as the sole electron acceptor. When using lactate as the electron donor, strain Ben-RB rapidly reduces (doubling time = 8 h) 5.1 mM arsenate concurrently with the reduction of sulfate (9.6 mM). Sulfate reduction is not inhibited by the presence of arsenate. Arsenate can act as the terminal electron acceptor in minimal medium (doubling time = 9 h) in the absence of sulphate. Arsenate is reduced by a membrane bound enzyme that is either a c-type cytochrome or is associated with such a cytochrome. Benzyl-viologen-dependent arsenate reductase activity is greater in cells grown with arsenate/sulfate than those grown with sulfate only. It has been proposed that arsenate reductase is associated with the respiratory chain of this organism because >98% of the arsenate reductase is bound to the plasma membrane.

2.14. *Sulfurospirillum barnesii* SES-3^T [8,10,36-38]

Sulfurospirillum barnesii SES-3^T is a gram-negative organism characterised by vibrioid to spirillum-shaped cells that are 0.3 μm wide and 1-2 μm in length. This organism is motile by polar flagellum. The optimum growth conditions are 33℃, pH 7.5 and 0.05% NaCl. Selenate, arsenate, thiosulphate, elemental sulphur, trimethylamine oxide, Fe (III), nitrate, fumarate, aspartate and manganese dioxide can all be used as a terminal electron acceptor by this bacterium, which is also capable of microaerobic growth. The organism reduces selenate to elemental selenium through selenite and nitrate to ammonium. Lactate and H_2 can both serve as electron donors. When grown with hydrogen, acetate is required as the carbon source. Strain SES-3^T (= ATCC 700032^T) is the type strain. A membrane-bound arsenate reductase was isolated from strain SES-3 and determined to be an a1b1g1-heterotrimic enzyme complex. The enzyme has a composite molecular mass of 100 kDa, and a-, b-, and g-subunits with masses of 65, 31, and 22, respectively. This enzyme couples the reduction of As (V) to As (III) via oxidation of methyl viologen, with an apparent Km of 0.2 mM. Preliminary compositional analysis suggests that iron-sulfur and molybdenum prosthetic groups are present. A b-type cytochrome is associated with the membrane of *S. barnesii*, and arsenate reductase is believed to be linked to the electron-transport system of the plasma membrane.

3. Characterization of *Citrobacter* sp. NC-1 in dissimilatory arsenate-reduction

3.1. Arsenate reduction by strain NC-1

Strain NC-1 was isolated from an old industrial site located in Hyogo Prefecture, Japan. The representative soil sample contained 5,000 mg As/kg soil. 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 $MnCl_2 \cdot 4H_2O$, 0.22 mg of $ZnCl_2$, and 0.1 g (0.01%) of yeast extract (BSMY) in 1000 mL of Tris-HCl buffer (pH 8.0). In addition, *L*-cysteine (1.5 g/L) and either 10 mM or 100 mM of $Na_2HAsO_4 \cdot 7H_2O$ 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. 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℃, 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.

The results showed that 5 mM arsenate was completely reduced within 20 h, although there was no decrease in the concentration of arsenite generated, and the total arsenic concentration remained constant even after prolonged incubation (Fig. 2). Approximate arsenic mass balances were determined based on the amounts of arsenate reduced and arsenite formed.

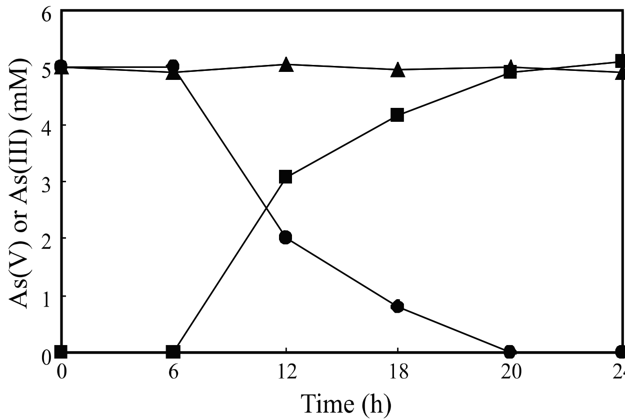

Fig. 2. Time course of arsenate reduction and arsenite accumulation by strain NC-1. Cultures were incubated with glucose (2.0 g/L) and 5 mM arsenate. Triangles represent total arsenic; solid circles represent arsenate; squares represent arsenite.

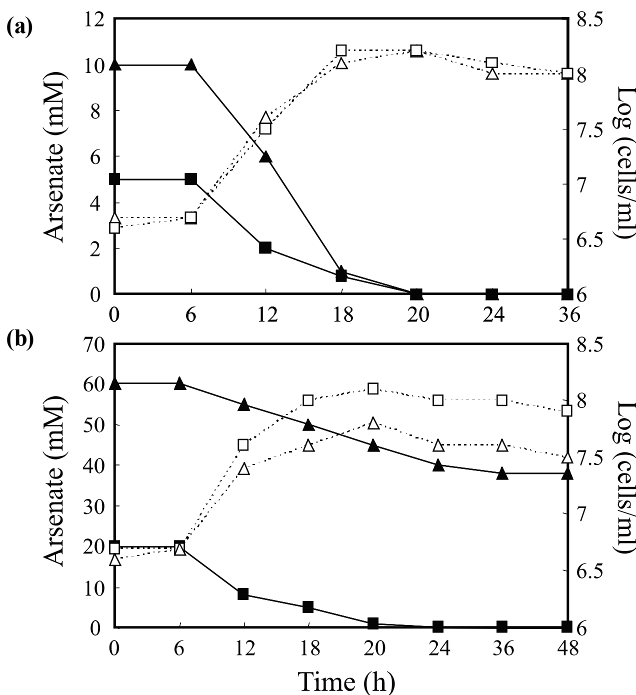

Fig. 3. Arsenate reduction by strain NC-1 and cell growth. Cultures were incubated with glucose (2.0 g/L) and 5, 10, 20, 60 mM arsenate. Solid symbols represent arsenate concentrations (A: squares = 10 mM; triangles = 5 mM; B: squares = 20 mM; triangles = 60 mM); open symbols represent the number of cells.

Fig. 3 shows the timing of the growth of strain NC-1 during the 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. 3(a)). 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 20 mM of arsenate was reduced, although the arsenate reduction proceeded further (Fig. 3(b)). During cell growth, lactic acid and pyruvic acid accumulation were observed in response to glucose consumption (data not shown).

Fig. 4 compares the growth of strain NC-1 in the presence and absence of arsenate under anaerobic conditions. 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). However, when glucose in the medium was replaced with acetate (20 mM), strain NC-1 showed visible growth in the presence of arsenate, but not in the absence of arsenate (data not shown).

When 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. The toxic effect of arsenite may explain the growth inhibition of strain NC-1 at high concentrations (60 mM) of arsenate. This result suggests 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 [5,19], but rather through dissimilatory reduction. 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).

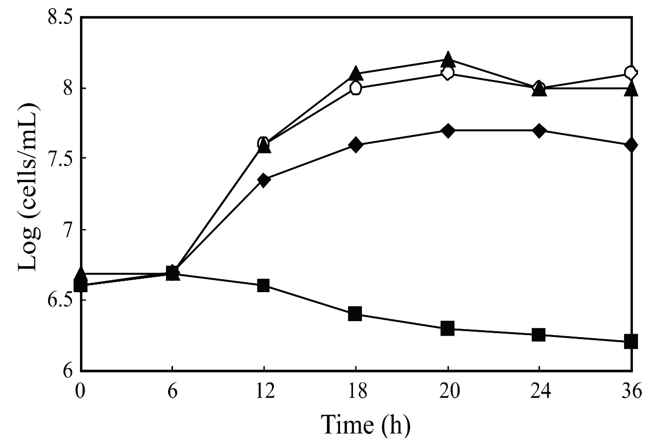

Fig. 4. Cell growth of strain NC-1 in the absence and presence of arsenic. Cultures were grown on 2.0 g/L glucose and 0, 5, or 10 mM arsenate or 10 mM arsenite. Symbols: triangles represent 10 mM arsenate, open circles represent 5 mM arsenate; diamonds represent 0 mM arsenate; squares represent 10 mM arsenate plus 10 mM arsenite.

3.2. 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). Since this oxyanion may coexist with arsenate in a contaminated soil environment, its effect on arsenate reduction was investigated in growth experiments using glucose-BSMY (Fig. 5). When nitrate was present with arsenate,

arsenate reduction proceeded concomitantly with nitrate reduction, although a slight inhibitory effect was observed (Fig. 5). These findings indicate thatnitrate did not significantly inhibit the arsenate-reducing activity of strain NC-1.

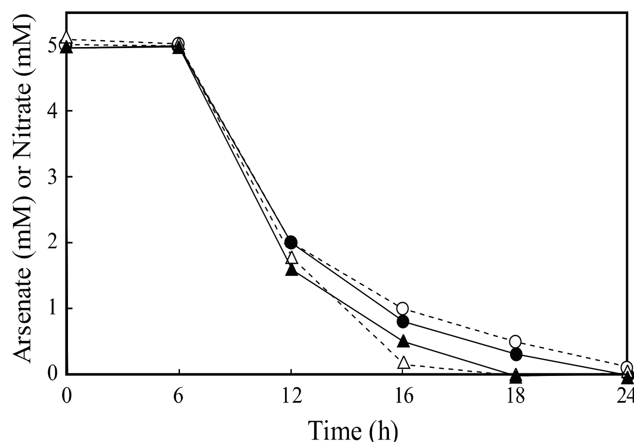

Fig. 5. Effect of nitrate on arsenate reduction. Cultures were incubated with 2.0 g/Lglucose and 5 mM arsenate and/or nitrate. Symbols: open circles represent arsenate reduction in the presence of nitrate; solid circles represent arsenate reduction in the absence of nitrate; open triangles represent nitrate reduction in the presence of arsenate; solid triangles represent nitrate reduction in the absence of arsenate.

3.3. Effects of electron donors on arsenate reduction

The effects of electron donors on arsenate reduction were also studied using washed cells that were grown on arsenate (Table 2). Although no arsenate reduction was observed in the control experiment (lacking an electron donor), 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 to the other carbon sources. 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 with lactate or glucose, possibly owing to poor growth of NC-1. 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.

Table 2. Arsenate reduction by arsenate-grown washed cell suspensions of strain NC-1 provided with various electron donors*

Electron donor	Arsenate reduced (%) after	
	6 h	10 h
Control	0	2.9
Acetate	37	58.3
Lactate	42	70.5
Pyruvate	32.4	47.1
Formate	32.4	52.9
Glucose	58.8	87.4
Fumarate	18	37.8
Hydrogen**	17.6	26.5
Phenol	0	0
Ethanol	0	0
Methanol	0	0
Benzoate	0	0

*Arsenate concentration was 1 mM, electron donors were added to suspensions at 5 mM. **Hydrogen (0.9 μM) was tested at a partial pressure of 0.5×10^5 Pa.

3.4. 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 the 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 did not significantly reduce arsenate (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, while those grown on nitrate did.

Arsenate and selenate reducing activities were only observed in washed cells grown on arsenate. These findings indicate that arsenate induced the synthesis of corresponding reductases in strain NC-1, suggesting that this strain synthesizes separate inducible enzymes, dissimilatory arsenate reductase and nitrate reductase, which are specifically induced by the corresponding oxyanion to use either arsenate or nitrate, respectively, as the terminal electron acceptor. Interestingly, there appears 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 [8,37].

3.5. Reductase activities in crude cell extracts

To determine the dissimilatory arsenate and nitrate reductase

Table 3. Comparison of the reducing activity of different crude cell extracts with each terminal electron acceptor[a]

Crude extracts from cells grown on:	Enzyme activity (%)[b] for each of electron acceptor	
	Arsenate	Nitrate
Arsenate	100[c]	4
Nitrate	2	100[d]

[a]The enzyme activity was measured as benzyl viologen (artificial electron donor) oxidized per min.
[b]The percent enzyme activity was calculated by assigning a value of 100% to the enzyme activity of each crude cell extract on its specific substrate. Each value represents an average of two analyses (the difference of the data obtained in the two analyses was within 2.5%).
[c]The activity of arsenate-grown cell extracts with arsenate was 61.2 U.
[d]The activity of nitrate-grown cell extracts with arsenate was 57.8 U.

activities in strain NC-1, crude cell 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. The reductase activities of each cell extract are shown in Table 3. Crude extracts from cells grown on arsenate exhibited the greatest 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 (Table 3).

3.6. Inhibition of arsenate and nitrate reduction by tungstate

The effects of tungstate on arsenate and nitrate reduction were investigated in anaerobic growth experiments (Fig. 6). In the absence of tungstate, strain NC-1 actively reduced l mM arsenate and nitrate, and they were completely reduced within 12 h and 8 h, respectively. However, the addition of tungstate (1 mM) reduced the arsenate and nitrate reduction activities by 55.7% and 47.3%, respectively, indicating that tungstate inhibited both reduction activities.

Tungstate, which is known to block a number of molybdoenzymes (including nitrate reductase) by substituting tungsten for molybdenum at the active site, [4,15,41] had strong inhibitory effects against arsenate, selenate and nitrate reduction under anaerobic conditions. These findings indicate that 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* [32] and *B. selenitireducens* [5].

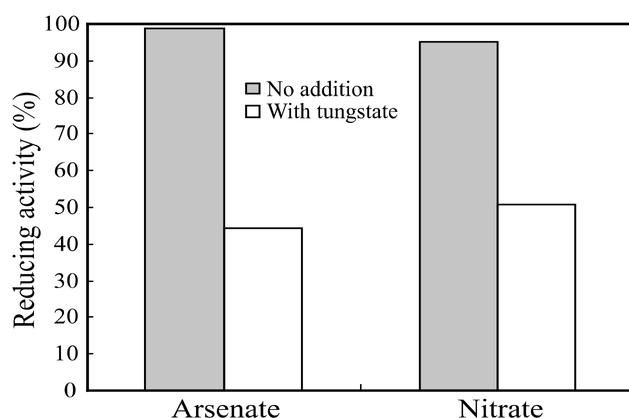

Fig. 6. Effect of tungstate on arsenate and nitrate reduction in growth experiments. Cultures were incubated with glucose (2.0 g/L) and 1 mM arsenate or 1 mM nitrate in the presence or absence of 1 mM tungstate. Reducing activity was expressed as the percentage of initial arsenate or nitrate reduced after 8 h (nitrate) or 12 h (arsenate) of incubation.

3.7. Extraction of As from contaminated forest soil

A forest soil was collected from the countryside outside of Muroran (pH, 5.3; ignition loss, 13.4%) and used to prepare a model As contaminated soil. The soil was dried at 60℃ for two days and then passed 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 and the samples were vigorously shaken 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 to be approximately 1200 mg kg^{-1}. One gram aliquots of the model contaminated soil were placed in 50 mL serum bottles, which were then autoclaved (1 h, 121℃), after which 20 mL of glucose-BSMY were added to the samples [19]. An anaerobically grown cell suspension was then inoculated into each bottle because the As (V)-reducing activity can be readily induced under anaerobic conditions in the presence of As (V).

In the experiment using the model contaminated forest soil, after 60 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 dissolved As was present as As (III) (Fig. 7). In the control (no NC-1) experiment, a slight increase in the dissolved As concentration was followed by a plateau however, 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 indicate that the dissolution of As observed in the control experiment was caused by the desorption of excess As (V) from soil.

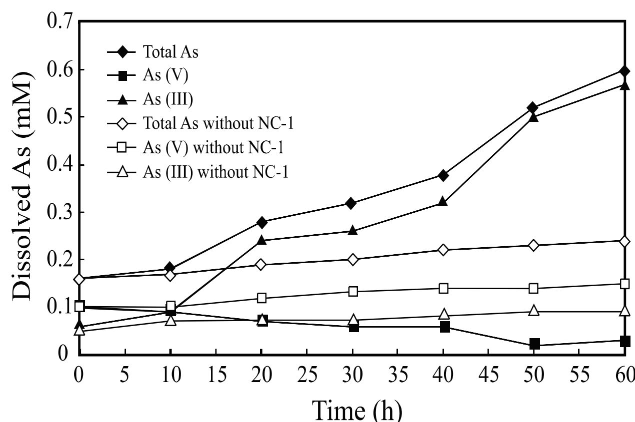

Fig. 7. Extraction of As from forest soil artificially contaminated with As (V). Cultures were incubated with 2.0 g/L glucose. Data represent the averages of two separate experiments.

4. Perspectives

Reduction is the dominant mechanism controlling As mobilization under reducing conditions. Although additional work is necessary to assess the environmental importance of arsenate respiration, the successful extraction of As from

contaminated forest soil by strain NC-1 suggests that arsenate-reducing organisms may be active in nature.

To date, more than 18 DARB have been isolated in pure culture. These organisms have been isolated from diverse environments, including freshwater marshes, acidic hot springs, and alkaline, hypersaline sediments. DARB are considered to be attractive agents for the bioremediation of arsenic contaminated soils and sediments [42,43] because they can mobilize arsenic from the solid phase into the liquid phase [17,38]. The experimental results reported here indicate that strain NC-1 has several properties that make it advantageous for bioremediation. The arsenate reducing activity of strain NC-1 is comparable or superior to that of previously reported DARB, and occurred even at an extremely high concentration of arsenate (~60 mM). This report presents data that reveal, for the first time, that bacterial reduction of high concentrations (~60 mM) of arsenate may be possible. The presence of other electron acceptors such as nitrate did not inhibit the reduction of arsenate, and various electron donors supported the arsenate reduction. Strain NC-1 has separate pathways for the dissimilatory reduction of arsenate and nitrate. Thus, controlling the expression of the reductases may lead to effective removal of target contaminants, even in the presence of alternative electron acceptors.

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. These findings 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. However, further experiments are still necessary to complete the investigation of transformation mechanisms. The molecular properties of respiratory enzymes are currently being studied and may provide important information for sites polluted with As.

Conversely, dissimilatory arsenate-reducing bacteria could also be applied for microbial recovery of rare metals such as Se, Mo or Pd via reduction of the metals, which is emerging as a clean alternative to traditional reclamation treatments, and the potential advantages of this technique are numerous. Collaboration between environmental engineers, microbiologists and geochemists is required to bring this promising technology to fruition.

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