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ORIGINAL PAPER

Dianne K. Newman · Erin K. Kennedy · John D. Coates · Dianne Ahmann · Debra J. Ellis · Derek R. Lovley · François M. M. Morel

Dissimilatory arsenate and sulfate reduction in *Desulfotomaculum auripigmentum* sp. nov.

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Abstract A newly discovered arsenate-reducing bacterium, strain OREX-4, differed significantly from strains MIT-13 and SES-3, the previously described arsenate-reducing isolates, which grew on nitrate but not on sulfate. In contrast, strain OREX-4 did not respire nitrate but grew on lactate, with either arsenate or sulfate serving as the electron acceptor, and even preferred arsenate. Both arsenate and sulfate reduction were inhibited by molybdate. Strain OREX-4, a gram-positive bacterium with a hexagonal S-layer on its cell wall, metabolized compounds commonly used by sulfate reducers. Scorodite ($FeAsO_4 2 \cdot H_2O$) an arsenate-containing mineral, provided micromolar concentrations of arsenate that supported cell growth. Physiologically and phylogenetically, strain OREX-4 was far-removed from strains MIT-13 and SES-3: strain OREX-4 grew on different electron donors and electron acceptors, and fell within the gram-positive group of the Bacteria, whereas MIT-13 and SES-3 fell together in the ε-subdivision of the Proteobacteria. Together, these results suggest that organisms spread among 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. 16S rRNA sequence analysis indicated that strain OREX-4 is a new species of the genus *Desulfotomaculum*, and accordingly, the name *Desulfotomaculum auripigmentum* is proposed.

Key words *Desulfotomaculum* · Arsenate reduction · Sulfate reduction

D. K. Newman
Department of Civil and Environmental Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

D. K. Newman (✉) · E. K. Kennedy · F. M. M. Morel
Department of Geosciences, Guyot Hall, Princeton University, Princeton, NJ 08544, USA
Tel. +1-609-258-1052; Fax +1-609-258-5242
e-mail: dianne@phoenix.princeton.edu

J. D. Coates
Department of Microbiology, Southern Illinois University, Carbondale, IL 62901, USA

D. Ahmann
School of the Environment, Duke University, Durham, NC 27704, USA

D. J. Ellis
American Type Culture Collection, 12031 Parklawn Drive, Rockville, MD 20852, USA

D. R. Lovley
Department of Microbiology, University of Massachusetts, Amherst, MA 01003, USA

Introduction

Arsenic is relatively abundant in the environment owing to contamination from a variety of anthropogenic sources in addition to its natural occurrence in minerals (Bumbla and Keefer 1994). Over the past three decades, evidence has been growing that suggests microorganisms play an important role in arsenic's geochemical cycle despite its toxicity. Bacteria and phytoplankton that oxidize (Osborn and Ehrlich 1976), methylate (Anderson and Bruland 1991), and reductively detoxify arsenic (Cervantes et al. 1994) have been described, and organisms that grow by reducing arsenate [As(V)] to arsenite [As(III)] during anaerobic respiration have recently been found (Ahmann et al. 1994; Laverman et al. 1995). Indeed, evidence is mounting that dissimilatory arsenate reduction may be an important process in environments where arsenic concentrations are high (Dowdle et al. 1996). For example, arsenic in the contaminated Aberjona Watershed in eastern Massachusetts (from which the isolate described in this study originates) has been measured at concentrations as high as 1 mM (Aurilio et al. 1994).

Previous discussion of arsenate-reducing microorganisms has suggested that arsenic may inhibit a variety of ecologically important anaerobic respiratory processes (Dowdle et al. 1996). This conclusion was based upon a study of subsurface aquifer sediments where arsenic was

shown to inhibit denitrification (Bradley and Chappelle 1991) and upon a study of various anoxic sediments that showed that arsenate and/or arsenite dramatically inhibited sulfate reduction and methanogenesis, presumably through toxic effects (Dowdle et al. 1996). Furthermore, in the latter study, molybdate was found to have no effect on arsenate reduction; thus, it was argued that sulfate-respiring bacteria were not involved in dissimilatory arsenate reduction in these sediments. This finding fit the physiological characterization of the previously described arsenate-reducing isolates, MIT-13 (Ahmann 1996) and SES-3 (Laverman et al. 1995) – which are unable to reduce sulfate - and allowed the inference to be made that the inhibition of sulfate reduction by arsenic in these sediments was noncompetitive (Dowdle et al. 1996). We now report that a novel arsenate-reducer, strain OREX-4, possesses metabolic abilities that challenge the conclusions drawn regarding the compatibility of dissimilatory arsenate reduction with sulfate reduction. Because strain OREX-4 is physiologically and phylogenetically distinct from the two other known arsenate-reducers, it now seems possible that microorganisms that respire arsenate may occupy wider niches than previously believed.

Materials and methods

Isolation and cultivation

Surface sediments were taken from Upper Mystic Lake, located in Woburn, Mass., USA (Aurilio et al. 1994; Spliethoff et al. 1995). Initial enrichments were made in freshwater minimal medium amended after autoclaving with sterile 14 mM sodium sulfate (Na_2SO_4), 20 mM sodium lactate, and 1 mM cysteine under an N_2:CO_2 atmosphere (80:20, v/v). After several transfers, the enrichment was inoculated into medium in which 10 mM dibasic sodium arsenate ($Na_2HAsO_4 \cdot 7\ H_2O$) was substituted for sulfate. A yellow precipitate formed and was determined to be As_2S_3 (Newman et al. 1997) cysteine was the source of reduced sulfur in this medium. The bacterium responsible for this precipitate, strain OREX-4, was isolated by successive colony transfers in agar shake tubes (Pfennig et al. 1981) and was maintained on 5–20 mM lactate, 1–10 mM arsenate, and 1–14 mM sulfate. In each shake-tube series, single yellow colonies were picked for further purification. The medium was buffered at a pH of 6.8 with $NaHCO_3$ (1.9 g/l) and was reduced with $PdCl_2$ + H_2 (300 μg/ml) (Aranki and Freter 1972). Salts supplied per liter of medium were: 0.14 g KH_2PO_4, 0.25 g NH_4Cl, 0.50 g KCl, 0.15 g $CaCl_2 \cdot 2\ H_2O$, 1.0 g NaCl, and 0.62 g $MgCl_2 \cdot 6\ H_2O$. The medium contained per liter: 0.05 mg *p*-aminobenzoic acid, 0.02 mg biotin, 0.05 mg nicotinic acid, 0.05 mg calcium pantothenate, 0.05 mg thiamine HCl, 0.1 mg pyridoxin HCl (B_6), and 0.001 mg cyanocobalamin (B_{12}); trace metals, added from a concentrated mixture, were (l^{-1}) 0.001 ml concentrated HCl, 0.1 mg $MnCl_2 \cdot 4\ H_2O$, 0.12 mg $CoCl_2 \cdot 6\ H_2O$, 0.07 mg $ZnCl_2$, 0.06 mg H_3BO_3, 0.025 mg $NiCl_2 \cdot 6\ H_2O$, 0.015 mg $CuCl_2 \cdot 2\ H_2$, 0.025 mg $Na_2MoO_4 \cdot 2\ H_2O$, and 1.5 mg $FeCl_2 \cdot 4\ H_2O$. All preparations and manipulations were performed under strict anoxic conditions using the Hungate technique (Miller 1974). Cultures were incubated in the dark at 25° C unless otherwise noted.

Analyses

Total arsenic (oxidized sample), arsenate (untreated sample), and background phosphate (reduced sample) were measured directly by the molybdenum blue spectrophotometric assay (Johnson and Pilson 1972); arsenite was indirectly determined by measuring the difference between the oxidized and untreated samples. Sulfate was measured by a slight modification of the barium-sulfate technique; the $BaCl_2$ gel was prepared by microwaving for convenience (Tabatabai 1974). Sulfide was measured by the methylene blue method (Franson 1981). Samples were taken directly for arsenic and sulfide measurements, but were filtered through a 0.2-μm cellulose filter (Nalgene) before sulfate measurements were taken. Cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) and counted. Lactate and acetate concentrations were determined by HPLC on a polysulfonate ion-exclusion column (Hamilton PRP X 300) with UV detection at 210 nm.

Growth experiments

The optimal pH and temperature ranges for growth of strain OREX-4 were determined by growing the organism on sodium lactate (10 mM) and sodium sulfate (10 mM) and by performing direct cell counts. The pH range tested was 6.0–8.0 as set by sterile additions of 0.1 M HCl or 0.1 N NaOH to TES (*N*-tris[Hydroxymethyl]methyl-2-aminoethane-sulfonic acid) buffered medium. For the pH experiments, the medium was the same as given above, except that TES (4.6 g/l) and $NaHCO_3$ (1 g/l) were used in place of $NaHCO_3$ (1.9 g/l) alone. The pH of the media was measured after autoclaving. The temperature range studied at pH 6.8 was 17–50° C, incrementing by roughly 5° C. Alternative electron donors and acceptors were added to the medium from anoxic sterile stocks to give the concentrations listed in Table 1. Colloidal elemental sulfur (S^0), synthetic MnO_2, and Fe(III) gel were added to the medium as previously described (Lovley and Phillips 1988; Blumentals et al. 1990). Hydrophobic hydrocarbon electron donors (toluene, benzene, and cyclohexane) were prepared in degassed mineral oil at 2% (v/v) (Rabus et al. 1993). In all of the growth experiments, cysteine-HCl (0.025%, w/v) was used as the reductant instead of $PdCl_2$.

Growth on an arsenate mineral

An arsenate-containing mineral, scorodite ($FeAsO_4 \cdot 2\ H_2O$), was obtained from the Princeton University mineral collection and ground through a mesh screen to 40–70 μm. A Scintag PAD-V Automated Powder X-ray Diffractometer (XRD) was used to confirm its identity and purity. One gram of the ground sample was added to each culture bottle containing 20 ml medium and autoclaved prior to inoculation. XRD analysis confirmed the mineral's identity after autoclaving. Dissolved arsenate and arsenite (samples passed through a 0.2-μm Nalgene cellulose filter) and cell growth were measured on day 0 and day 14.

Sequencing of the 16S-rRNA-encoding DNA and phylogenetic analysis

Nucleic acids were isolated from a cell pellet of strain OREX-4, and the partial 16S rRNA gene was amplified using eubacterial primers 50F and 1492R and sequenced as described previously (Caccavo 1994). Sequencing was performed at the Michigan State University Sequencing Facility. Preliminary identification of strain OREX-4 was obtained using the BLAST program (Altschul 1990). Sequence alignments were performed manually or were obtained from the Ribosomal Database Project (Maidak 1996). The phylogenetic tree was inferred using the distance matrix method with the least-squares algorithm (De Soete 1983). The method of Jukes and Cantor (1969) was used to compute the evolutionary distances.

Nucleotide sequence accession numbers

The 16S rRNA sequences of *Campylobacter helveticus* (Cam.helvet), *Campylobacter jejuni* (Cam.jejun5), *Clostridium quercicolum* (C.quercico), *Desulfitobacterium dehalogenans* (Dfi.dehalo), *Desulfobulbus propionicus* (Dbb.propio), *Desulfotomacu-*

lum nigrificans (Dfm.nigrif), *Desulfotomaculum orientis* (Dfm.orient), *Desulfotomaculum ruminis* (Dfm.rumin2), *Desulfovibrio desulfuricans* (Dsv.desulf), *Desulfovibrio vulgaris* (Dsv.vulgar), *Escherichia coli* (E.coli), *Selenomonas lacticifex* (Sln.lactic), and *Staphylococcus aureus* (Stp.aureus) were obtained from the Ribosomal Database Project (Maidak 1996). The 16S rRNA sequences of strain MIT-13, strain SES-3, and *Desulfitobacterium hafniense* were obtained from Genbank (accession nos. U85964, U41564, and X94975, respectively). The partial 16S rDNA sequence of strain OREX-4 has been submitted to Genbank (accession no. U85624).

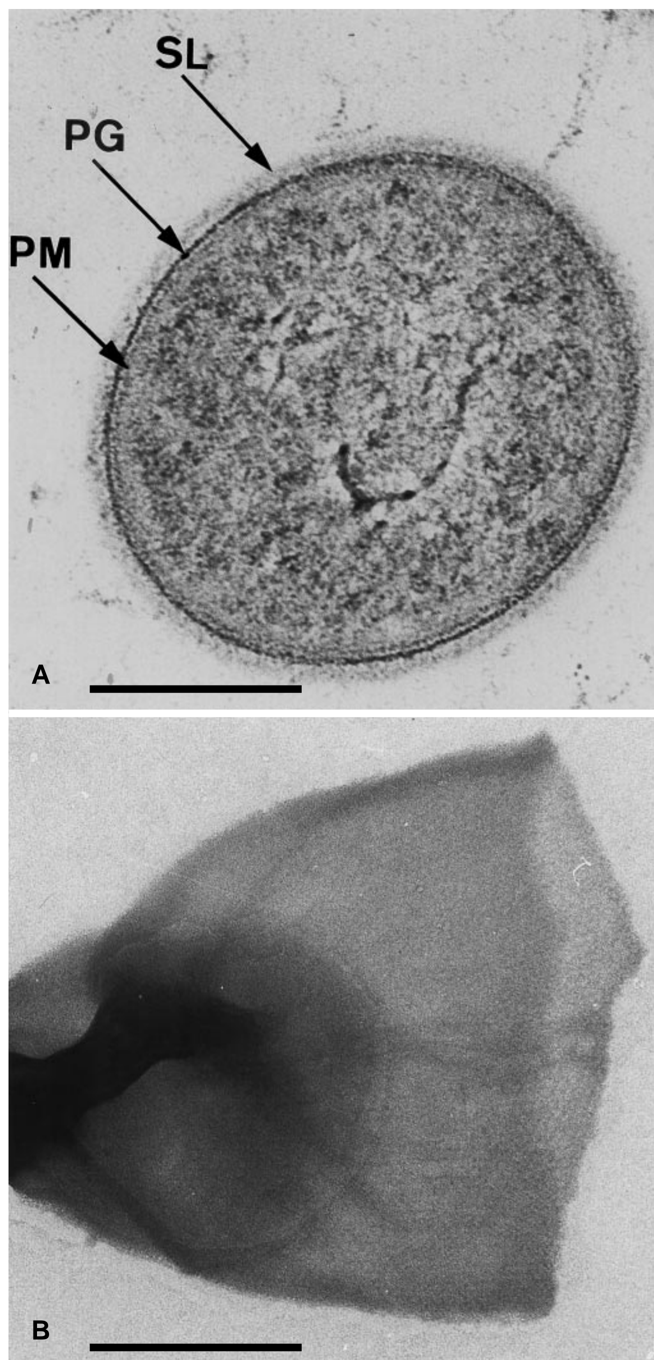


Fig. 1A, B Transmission electron micrographs of strain OREX-4. **A** Thin-section profile showing the gram-positive cell wall. *PG* points to the peptidoglycan layer, *PM* points to the plasma membrane, and *SL* points to the S-layer (*bar* 200 nm). **B** A fragment of the hexagonal surface array (S-layer) of strain OREX-4 negatively stained with 2% uranyl acetate (*bar* 200 nm)

Transmission electron micrographs

Thin-sections of strain OREX-4 grown on lactate and sulfate were cut from pellets embedded in L. R. White resin as previously described (Newman et al. 1997). Cells were negatively stained with 2% uranyl acetate to visualize the S-layer.

Results

Cell and colony morphology

Strain OREX-4 is a gram-positive, slightly curved, nonmotile rod that does not appear to contain endospores. Cells are approximately 2.5 μm in length and 0.4 μm in diameter, and electron micrographs show the murein sacculus (peptidoglycan) of strain OREX-4 to be thinner than usual for a gram-positive bacterium (Fig. 1A). A hexagonal surface array (S-layer) is attached to its cell wall (Fig. 1B). On solid medium amended with sulfate but no arsenate, the cells are light gray. On solid medium amended with both arsenate and sulfate, the cells are bright yellow due to precipitation of As_2S_3 (Newman et al. 1997).

Respiration of arsenate and sulfate

Strain OREX-4 grew exponentially while oxidizing lactate to acetate when either arsenate or sulfate was provided as the electron acceptor. For cells grown on lactate

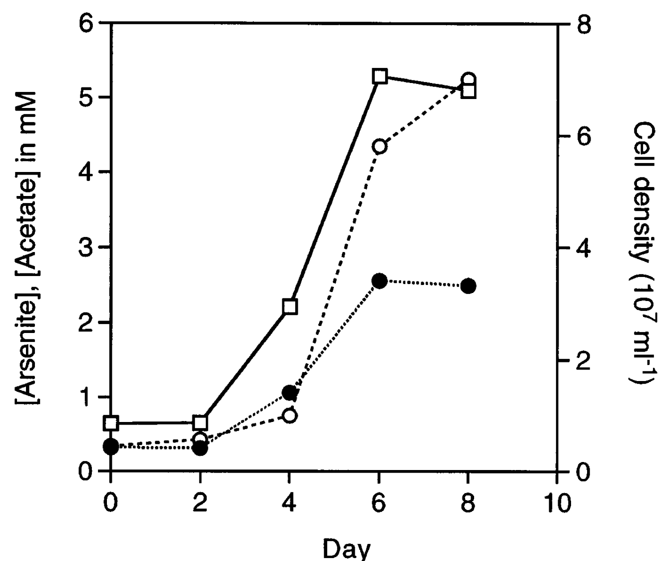
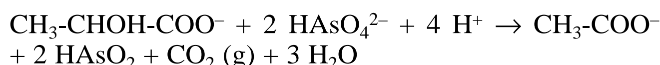
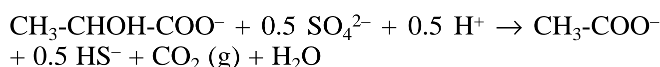


Fig. 2 Growth of strain OREX-4 on 10 mM lactate with 5 mM arsenate as the electron acceptor. Growth coincides with the stoichiometric evolution of acetate and the production of arsenite. □ Arsenite production, ○ cell density, and ● acetate production. *Symbols* represent single data points that are representative of duplicate cultures

and arsenate, the average molar ratio (of duplicate cultures) of acetate produced to arsenite produced was 0.47 (0.49–0.46; Fig. 2); for cells grown on lactate and sulfate, the average molar ratio of acetate produced to sulfide produced was 1.65 (1.73–1.56; data not shown). When it is considered that some carbon was probably incorporated into cell biomass, these results are in fairly good agreement with the theoretical values of 0.5 and 2 as predicted by the following reactions:



$$CH_3\text{-}CHOH\text{-}COO^- + 2\ HAsO_4^{2-} + 4\ H^+ \rightarrow CH_3\text{-}COO^- + 2\ HAsO_2 + CO_2\ (g) + 3\ H_2O$$



$$CH_3\text{-}CHOH\text{-}COO^- + 0.5\ SO_4^{2-} + 0.5\ H^+ \rightarrow CH_3\text{-}COO^- + 0.5\ HS^- + CO_2\ (g) + H_2O$$

Final cell numbers of strain OREX-4 were proportional to the arsenate concentration in the presence of 5 mM lactate, further demonstrating its use of arsenate as an electron acceptor (Fig. 3). It appears that higher arsenate concentrations eventually inhibit growth, perhaps because of the build-up of high arsenite concentrations. Growth slowed and the rate of arsenate reduction decreased after 5 mM arsenate had been reduced in cultures supplied with 10 mM arsenate. Growth on nonfermentable substrates (H_2 + acetate and arsenate; H_2 + acetate and sulfate) further established that arsenate and sulfate are used as electron acceptors (data not shown). Arsenate was never observed to be reduced further than to arsenite.

In all cultures where arsenate and sulfate were added together, we observed that arsenate reduction precedes that of sulfate. This was examined by growing strain OREX-4 in medium amended with 1 mM arsenate, 10 mM sulfate, and 20 mM lactate. Arsenate reduction was nearly complete by the time sulfate reduction began (days 2–4; Fig. 4A). On day 4, As_2S_3 precipitated in the medium, causing small amounts of sulfide to be removed from the solution (Fig. 4B) (the arsenite and sulfide present in As_2S_3 are not measured by either the molybdenum blue or methylene blue assay). As time progressed, sulfide levels began to rise again as more and more sulfate was reduced, yet the rate of sulfate reduction was poor, with most of the sulfide going to As_2S_3 (Fig. 4B). Preferential reduction of arsenate to sulfate was also observed when the medium was amended with a fivefold concentration of arsenate (data not shown). A complementary experiment was performed in medium amended with 2.5 mM arsenate, 1 mM sulfate, and 10 mM lactate with samples previously grown on arsenate alone, on sulfate alone, and on both arsenate and

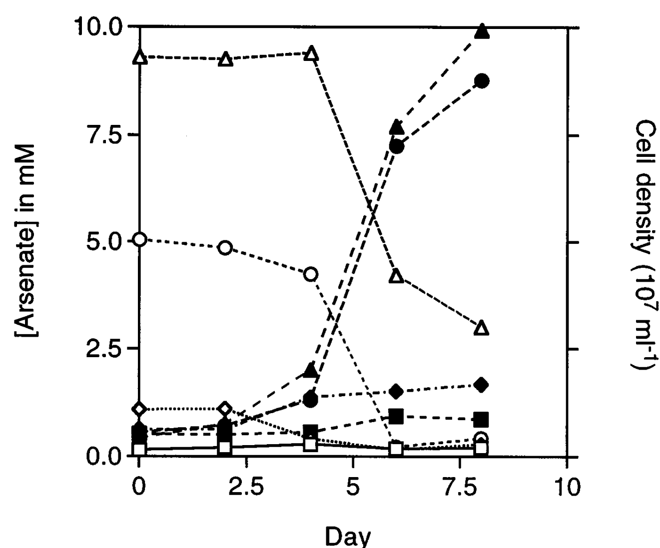


Fig. 3 Cell growth in response to increasing concentrations of arsenate. Cultures were grown on 5 mM lactate and on 0, 1, 5, and 10 mM arsenate. *Open symbols* represent values for arsenate concentrations; *filled symbols* represent cell numbers. □, ■ 0 mM arsenate; ◇, ◆ 1 mM arsenate; ○, ● 5 mM arsenate; and △, ▲ 10 mM arsenate. *Symbols* represent single data points that are representative of duplicate cultures

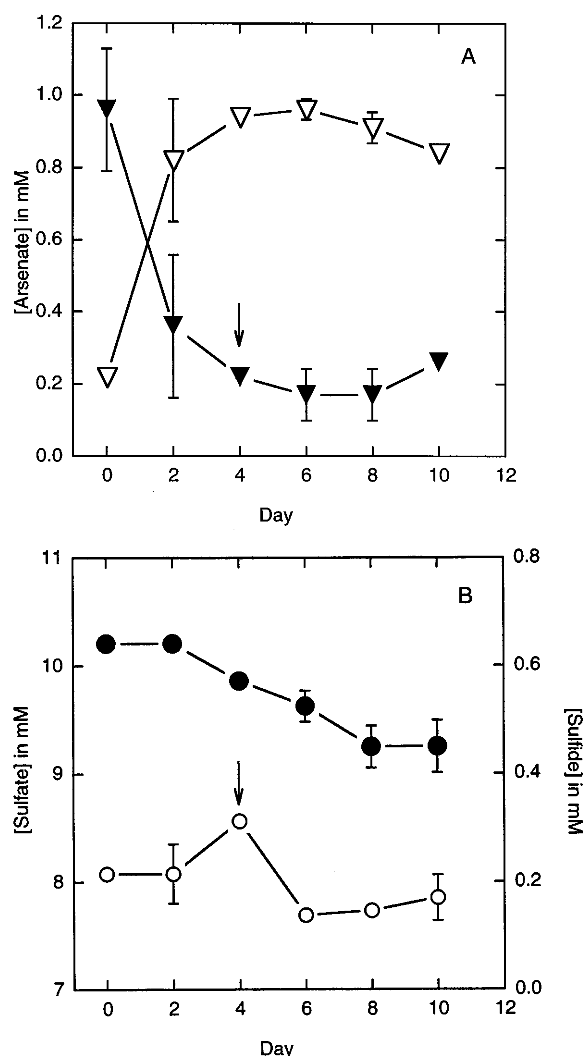


Fig. 4A, B Preferential reduction of arsenate in comparison to sulfate. Cultures were amended with 20 mM lacate, 1 mM arsenate, and 10 mM sulfate. **A** Arsenate reduction to arsenite: ▲ arsenate, △ arsenite. **B** Sulfate reduction to sulfide: ● sulfate, ○ sulfide. *Arrows* indicate precipitation of As_2S_3. *Symbols* represent the means of duplicate cultures, and *bars* indicate the data range

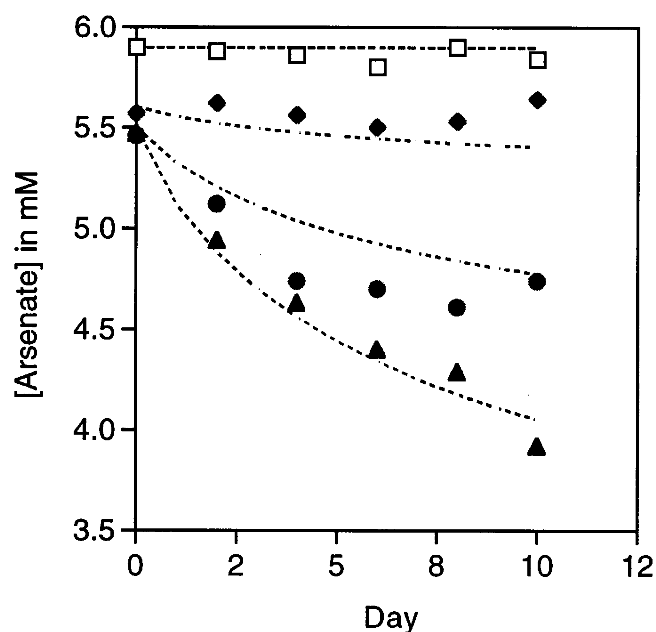


Fig. 5 Kinetics of arsenate reduction by various concentrations of sulfide (pH 6.8, ionic strength 0.038 M). Data points represent single measurements. □ 0 mM sulfide, ◆ 0.3 mM sulfide, ● 1 mM sulfide, and ▲ 3 mM sulfide. The lines were fit to the data by modeling the disappearance of arsenate over time using the measured initial concentrations of arsenate and sulfide (1:1 stoichiometry) and k = 25 $M^{-1}day^{-1}$ as a second-order rate constant

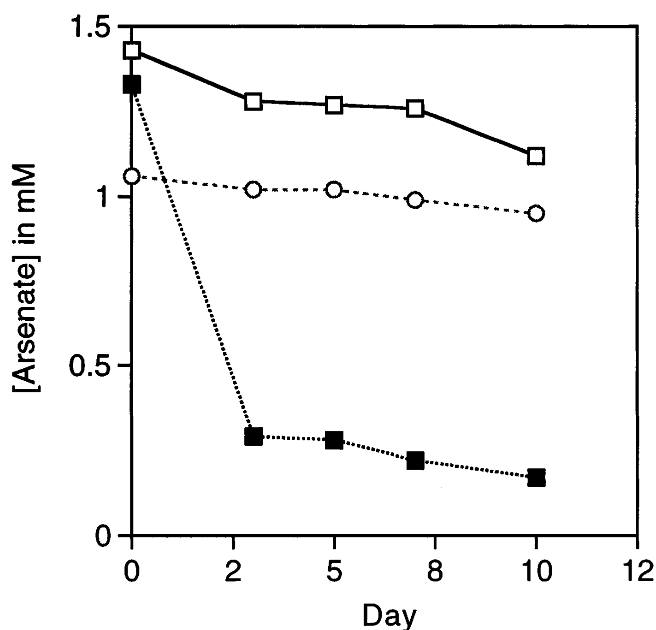


Fig. 6 Molybdate inhibition of arsenate reduction. Cultures were grown on 10 mM pyruvate and 1 mM arsenate, 1 mM sulfate, and 1 mM molybdate as indicated. □ Arsenate + molybdate, ○ arsenate + sulfate + molybdate, and ■ arsenate – molybdate. The data are representative of duplicate cultures

sulfate. Arsenate reduction rapidly commenced in the bottles that had been inoculated from an arsenate culture, whereas bottles inoculated from sulfate or dual-substrate cultures took slightly longer to reduce the same amount of arsenate (data not shown). More than 1 mM arsenate was reduced before a significant fraction of the sulfate had been depleted, and by the end of the experiment, only 0.5 mM sulfate had been reduced; however, all of the 2.5 mM arsenate had been reduced to arsenite (data not shown).

To verify that arsenate reduction in the presence of sulfate did not result from bacterial sulfide production and subsequent abiotic reduction of arsenate, we studied the kinetics of arsenate reduction by sulfide over the concentration range of interest. Bottles of 1 and 5 mM arsenate were amended with several concentrations of sulfide (0, 0.3, 1.0, and 3.0 mM), and arsenate reduction was followed over a 2-week period. Thiosulfate (1 mM) and sulfite (1 mM) were also tested for their ability to reduce arsenate. Reduction of arsenate by sulfide was kinetically slow, and thiosulfate and sulfite showed negligible reduction of arsenate. Assuming a 1:1 stoichiometry of arsenate reduced by sulfide, an apparent second-order rate constant of 20–25 $M^{-1}day^{-1}$ for the reduction of arsenate by sulfide enabled us to fit all experimental data. This is shown for the 5 mM arsenate data set in Fig. 5. Assuming k = 25 $M^{-1}day^{-1}$, an initial arsenate concentration of 2.5 mM, and an initial sulfide concentration of 0.5 mM, we predict a maximum of 0.2 mM arsenate to be reduced over 10 days. This is well below the concentration of arsenate reduced in our microbial experiments (2.5 mM arsenate reduced in 2 days) given these conditions.

When cultures growing either on 1 mM arsenate and 1 mM sulfate or on 1 mM arsenate alone were amended with 1 mM sodium molybdate, near total inhibition (95%) of arsenate reduction was achieved (Fig. 6). As expected, complete inhibition of sulfate reduction was also observed for cultures growing on 10 mM sulfate alone (data not shown).

Growth conditions and substrates

Strain OREX-4 has a temperature optimum between 25 and 30°C and a pH optimum between 6.4 and 7.0. The ability of strain OREX-4 to utilize a wide variety of electron donors and acceptors was tested and compared to that of strains MIT-13 and SES-3 (Table 1). Strain OREX-4 can obtain energy for growth by coupling the reduction of sulfate to the oxidation of pyruvate, ethanol, glycerol, butyrate, formate, and malate, in addition to lactate. Strain OREX-4 can also ferment pyruvate and appears to be capable of autotrophic growth on H_2. For incubation times of approximately 1 week, strain OREX-4 grows best on pyruvate (10^8 cells/ml) and glycerol (10^8 cells/ml), well on lactate (10^7 cells/ml), but only poorly on butyrate (10^6 cells/ml) when sulfate serves as the electron acceptor and both the electron acceptor and the electron donor are provided at concentrations of 10 mM. In addition to arsenate and sulfate, electron acceptors that support growth include thiosulfate, sulfite, and fumarate.

Table 1 Compounds tested as electron donors and electron acceptors for strain OREX-4, as compared to strains SES-3 and MIT-13. Electron donors were tested using sulfate as the electron acceptor (applies only to strain OREX-4). Electron acceptors were tested using H_2 + acetate as the electron donor (applies only to strain OREX-4). Data for strains SES-3 and MIT-13 were taken from the literature (Laverman et al. 1995; Ahmann 1996) (*0* not reported)

Electron donor (mM)	OREX-4	SES-3	MIT-13
H_2 (1 atm)	+	–	–
H_2 + acetate (1 atm + 10)	+	+	+
Methane + acetate (1 atm + 0.1)	–	0	0
Formate (10)	–	+[a]	0
Acetate (10)	–	–	–
Propionate (5)	–	0	–
Butyrate (5)	+	0	+[a]
Pyruvate (10)	+	0	+
Lactate (10)	+	+	+
Fumarate (10)	–	0	+
Succinate (1)	–	+	0
Citrate (10)	–	+	0
Malate (1)	+	0	0
Palmatate (1)	–	0	0
Benzoate (1)	–	0	–
Glucose (10)	–	0	0
Glycerol (5)	+	0	0
Ethanol (10)	+	0	0
Methanol (10)	–	0	0
Phenol (0.5)	–	0	0
Casamino acids (1g/l)	–	0	0
Yeast axtract (1g/l)	–	0	0
Octane (1)	–	0	0
Cyclohexane (1)	–	0	0
Benzene (1)	–	0	0
Toluene (1)	–	0	0
Electron acceptor (mM)	**OREX-4**	**SES-3**	**MIT-13**
Nitrate (5)	–	+	+
Mn(IV) (20)	–	+	–
Fe(III) (50)	–	+	0
Sulfate (10)	+	–	–
Sulfite (5)	+	0	0
Thiosulfate (5)	+	+	0
S_0 (10)	0	+	–
Se(IV) (5)	–	+	–
As(V) (5)	+	+	+
Fumarate (20)	+	+	+
Malate (10)	–	0	0
DMSO (10)	–	0	0

[a] Growth occurs only in the presence of acetate

Growth on scorodite

Strain OREX-4 was able to utilize scorodite ($FeAsO_4 \cdot 2H_2O$) as a source of arsenate for respiratory growth. Scorodite is one of the most abundant arsenate-containing minerals (Azcue and Nriagu 1994) and, thus, is representative of what bacteria might encounter in the natural environment. Uninoculated controls show the dissolution of scorodite to be slow: less than 1 mM of arsenate dissolved abiotically over the course of the experiment (14 days). No growth was observed in inoculated controls lacking scorodite (cell numbers remained constant at 2×10^6 cells/ml). In cultures amended with 10 mM acetate, 1 atm H_2, and 1 g scorodite, however, cell numbers increased significantly (from 2×10^6 cells/ml initially to 2×10^7 cells/ml at the end of the incubation) as dissolved arsenate decreased by approximately 300 µM and dissolved arsenite increased by approximately 400 µM. Since growth of strain OREX-4 by a factor of ten was greater than expected given these measured concentrations of arsenate and arsenite (compare Fig. 3, where 1 mM arsenate produces an equivalent increase in cell numbers), it is probable that more arsenate was reduced than could be measured due to adsorption of arsenite onto scorodite.

Phylogenetic analysis

Phylogenetic analysis of the nearly complete 16S rRNA sequence of strain OREX-4 placed it within the gram-positive phylum of the Bacteria (Maidak 1996; Fig. 7). In contrast, 16S rRNA-based phylogenetic analysis of the other known arsenate reducers, strain MIT-13 and strain SES-3, has placed these two microorganisms within the -subdivision of the Proteobacteria (Ahmann 1996, Lonergan et al. 1996). Thus, strain OREX-4 represents the first known example of a gram-positive microorganism able to obtain energy for growth from the reduction of arsenate.

The closest known relative of strain OREX-4 is *Desulfotomaculum orientis* (96.2% sequence identity, 1,020 nucleotides considered). Based on the consistent phylogenetic placement of strain OREX-4 within the *Desulfotomaculum* group of the gram-positive phylum regardless of the algorithm used to infer the tree [maximum-likelihood (Felsenstein 1981) or distance-matrix (De Soete 1983)] and its phylogenetic relationship and physiological similarity to *D. orientis* as compared to *D. dehalogenans*, we propose that it is a new species within the genus *Desulfotomaculum*: *Desulfotomaculum auripigmentum* (Fig. 7).

Discussion

D. auripigmentum is the first example of a sulfate-reducing bacterium that can also grow with arsenate as a terminal electron acceptor. No other dissimilatory arsenate-reducing bacterium has been found that can also reduce sulfate, although strain SES-3 is capable of reducing other sulfur compounds such as thiosulfate and elemental sulfur (Laverman et al. 1995). *D. auripigmentum* achieves a higher molar growth yield when grown on arsenate (5.6 g cells/mol lactate) than when grown on sulfate (2.3 g cells/mol lactate), assuming a cell dry wt. of 2×10^{-10} mg/cell and the measured values of lactate consumed to achieve the observed cell densities for growth on 5 mM arsenate and 10 mM sulfate (Fig. 2). This is not surprising given that arsenate is a better oxidant than sulfate and would correspondingly yield a higher free energy when coupled to the oxidation of lactate to acetate at pH 7 (Peters 1974; Morel and Hering 1993):

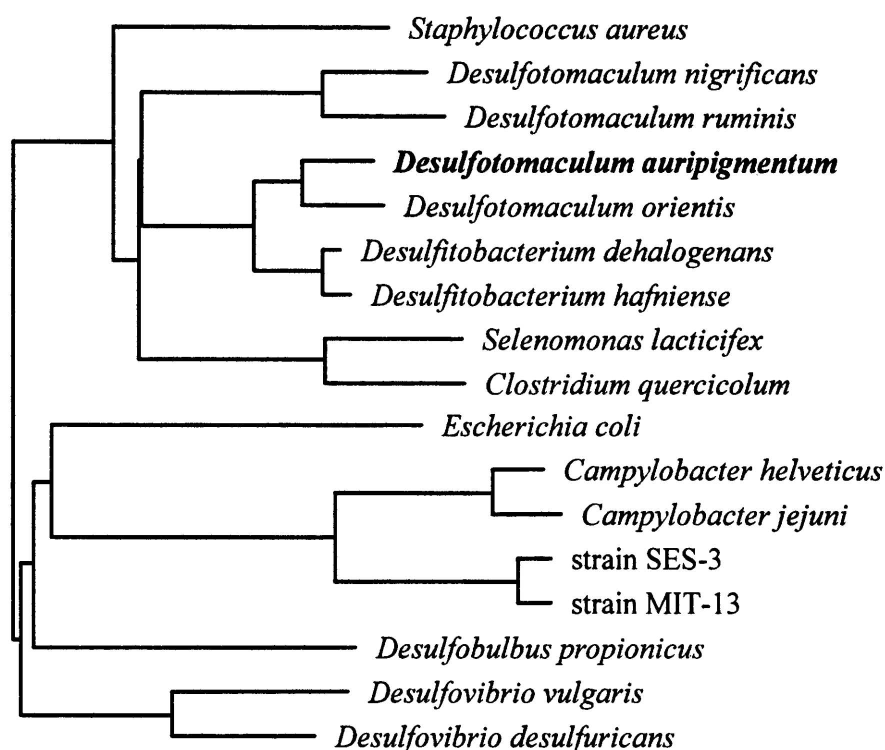
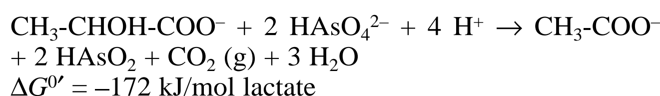
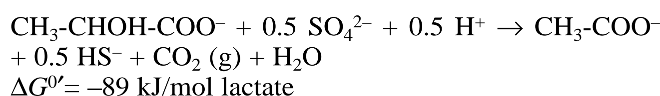


Fig. 7 Phylogenetic tree inferred from partial 16S rDNA sequences showing the placement of *Desulfotomaculum auripigmentum* (strain OREX-4) within the *Desulfotomaculum* group of the gram-positive bacteria (Maidak 1996). The phylogenetic tree was inferred from 913 positions using the least-squares algorithm (De Soete 1983) with evolutionary distances computed by the method of Jukes and Cantor (1969). *Bar length* represents one evolutionary distance unit



$$CH_3\text{-}CHOH\text{-}COO^- + 2\ HAsO_4^{2-} + 4\ H^+ \rightarrow CH_3\text{-}COO^- + 2\ HAsO_2 + CO_2\ (g) + 3\ H_2O$$
$$\Delta G^{0\prime} = -172 \text{ kJ/mol lactate}$$



$$CH_3\text{-}CHOH\text{-}COO^- + 0.5\ SO_4^{2-} + 0.5\ H^+ \rightarrow CH_3\text{-}COO^- + 0.5\ HS^- + CO_2\ (g) + H_2O$$
$$\Delta G^{0\prime} = -89 \text{ kJ/mol lactate}$$

The molar growth yield for arsenate is in good agreement with the value found for growth of strain SES-3 on 5 mM arsenate and lactate (5.3 g/mol lactate; Laverman et al. 1995). When supplied with both electron acceptors, for a time, *D. auripigmentum* reduces arsenate and sulfate concomitantly, but shows a preference for arsenate. Stoichiometrically, the reduction of arsenate in dual arsenate/sulfate cultures cannot be explained by the abiotic reduction of arsenate by sulfide. For example, in the experiments where 2.5 mM arsenate and 1 mM sulfate were supplied, only 0.5 mM sulfate was consumed over the course of the experiment; even if we assume that all of this was converted to sulfide, stoichiometrically there would still be too little to account for the full reduction of 2.5 mM arsenate (assuming a two-electron transfer from sulfide to arsenate). Furthermore, our kinetic data show that the reduction of arsenate by sulfide is too slow to account for our results, regardless of the stoichiometry of the redox reaction.

Preference for arsenate raises the question whether sulfate reduction is inhibited by arsenate - an issue of potential importance in contaminated environments. Our data suggest that the pathways for the dissimilatory reduction of arsenate and sulfate are linked in *D. auripigmentum.* Significantly, the rate of arsenate reduction in cultures amended with both arsenate and sulfate is always faster than that of sulfate reduction, independent of the relative concentrations of arsenate and sulfate or the substrate history of the inoculum. This may reflect the energetic advantage of arsenate over sulfate reduction. Alternatively, the reduction of arsenate to arsenite may be necessary before sulfate is reduced either because arsenate is toxic to the bacterium (and reduction to arsenite, although itself toxic, allows As to be exported) or because it inhibits sulfate reduction biochemically. This last possibility is reinforced by the near total inhibition of arsenate reduction by molybdate. Since molybdate is a known inhibitor of sulfate reduction (Oremland and Capone 1988), the enzyme ATP sulfurylase (which activates the sulfate ion) may also be involved in the reduction of arsenate to arsenite. Of course, it is also possible that by causing ATP sulfurylase to form a futile cycle, molybdate simply forces the cell to run out of energy, and this indirectly affects arsenate metabolism.

In a recent study, the lack of inhibition of arsenate reduction by molybdate was used to argue against the involvement of sulfate-respiring bacteria in dissimilatory arsenate reduction in certain anoxic sediments (Dowdle et al. 1996). The discovery of *D. auripigmentum*, a sulfate reducer that also reduces arsenate, suggests that this might not be true for all environments. In the experiments of Dowdle et al. (1996), 8–10 mM arsenic (arsenate or arsenite) completely inhibited sulfate reduction; however,

sulfate reduction has been observed in other sedimentary enrichments in the presence of 1.3 mM arsenite (Rittle et al. 1995). In an attempt to reconcile these results, it has been suggested that due to its toxicity, the concentration of arsenic determines the degree to which sulfate reduction is inhibited (Dowdle et al. 1996). Our data showing consistent preferential reduction of arsenate to sulfate in *D. auripigmentum*, however, suggest another possible explanation: toxicity aside, arsenate may competitively inhibit sulfate reduction in some highly contaminated environments because its reduction is more energetically favorable. Indeed, it is possible that dissimilatory arsenate reduction in contaminated sediments differs from community to community, and while some may contain bacteria such as strains MIT-13 and SES-3 (which are unaffected by molybdate and cannot respire sulfate), others may harbor organisms more similar to *D. auripigmentum*. As shown in Table 1, strains MIT-13 and SES-3 differ fundamentally from *D. auripigmentum* in important metabolic capabilities [such as in their ability to use nitrate, Fe(III), or sulfate as electron acceptors]; thus, it would not be surprising if the pathway responsible for the dissimilatory reduction of arsenate in *D. auripigmentum* differed from the one responsible for this reduction in strains MIT-13 and SES-3. *D. auripigmentum* is a gram-positive bacterium, whereas strains SES-3 and MIT-13 are gram-negative (Laverman et al. 1995; Ahmann 1996). Precedent for biochemical differences in arsenate reduction between gram-positive and gram-negative bacteria can be found in comparisons between *Escherichia coli* and *Staphylococcus aureus* (Gladysheva et al. 1994; Ji et al. 1994), which couple nonrespiratory arsenate reduction to the oxidation of glutaredoxin and thioredoxin, respectively.

The concentration of arsenic in most environments is very low (nM). Thus, we would normally expect only a negligible fraction of the organic carbon pool to be mineralized through dissimilatory arsenate reduction. The capacity to grow by dissimilatory arsenate reduction, however, may provide a selective advantage to microorganisms in some particular environments. In the anoxic hypolimnion of Upper Mystic Lake (from which *D. auripigmentum* was isolated), significant amounts of both arsenite and arsenate (< 200 nM) were found to persist in sulfidic waters (25 µM; Spliethoff et al. 1995). Interestingly, the sampling date when *D. auripigmentum* was retrieved from the surface sediments was the only time during the year when sulfide appeared in the lake, and one of only two times when the hypolimnetic concentrations of arsenite exceeded those of arsenate (Spliethoff et al. 1995). While this chemical profile fits with what we might expect if arsenate-reducing bacteria were active in this environment, clearly more work would be necessary to demonstrate in situ microbial arsenate reduction: since *D. auripigmentum* was isolated from an enrichment culture, it is difficult to draw conclusions about its prevalence and/or arsenate-reducing activity in Upper Mystic Lake without further study. Specific molecular probes (both organismal and functional) present one attractive way to build on culture studies such as this one to assess the importance of microbial arsenate reduction in the environment. The ability of *D. auripigmentum* to grow on scorodite (which essentially supplies low concentrations of arsenate) also suggests that microbial arsenate reduction might occur in uncontaminated environments in the presence of natural arsenate-containing minerals. Nonetheless, it is only in highly contaminated environments such as certain locations in the Abjerjona Watershed, where arsenic concentrations have been measured at mM levels (Aurilio et al. 1994), that we would expect dissimilatory arsenate reduction to significantly contribute to the mineralization of organic matter.

Description of *Desulfotomaculum auripigmentum* sp. nov.

Desulfotomaculum auripigmentum (sp. nov.) au.ri.pig.men'tum. N.L.chem.n., golden pigment, because it reduces arsenate and sulfate, causing As_2S_3 to precipitate (Newman et al. 1997). *D. auripigmentum* is a freshwater, gram-positive, non-motile, strictly anaerobic chemoorganotroph with sausage-shaped cells 2.5 µm in length × 0.4 µm in diameter. The murein sacculus of *D. auripigmentum* is thinner than usual for gram-positive bacteria, and a hexagonal S-layer is attached to its cell wall. *D. auripigmentum* grows by oxidizing H_2, lactate, pyruvate, butyrate, ethanol, glycerol, and malate with the concomitant reduction of sulfate. *D. auripigmentum* is an incomplete oxidizer, producing acetate from its organic substrates. *D. auripigmentum* can also obtain energy for growth by the reduction of thiosulfate, sulfite, and fumarate. *D. auripigmentum* has a temperature optimum of 25–30° C and a pH optimum of 6.4–7.0 for growth.

The type strain of *D. auripigmentum*, strain OREX-4, was enriched from freshwater sediment samples taken from Upper Mystic Lake in Woburn, Mass., USA, with lactate as the electron donor and arsenate and sulfate as the electron acceptors. The strain has been deposited in the American Type Culture Collection (ATCC 700205). The 16S rRNA sequence has been deposited in GenBank (accession no. U85624).

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References

Ahmann D (1996) Dissimilatory arsenate-reduction in a freshwater sedimentary microorganism. PhD Thesis, Massachusetts Institute of Technology, Cambridge, MA, USA

Ahmann D, Roberts AL, Krumholz LR, Morel FMM (1994) Microbe grows by reducing arsenic. Nature 371:750
Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410
Anderson LCD, Bruland KW (1991) Biogeochemistry of arsenic in natural waters: importance of methylated species. Environ Sci Technol 25:420–427
Aranki A, Freter R (1972) Use of anaerobic glove boxes for the cultivation of strictly anaerobic bacteria. Am J Clin Nutr 25: 1329–1334
Aurilio AC, Mason RP, Hemond HF (1994) Speciation and fate of arsenic in three lakes of the Aberjona Watershed. Environ Sci Technol 28:577–585
Azcue JM, Nriagu JO (1994) Arsenic: historical perspectives. In: Nriagu JO (ed) Arsenic in the environment. 1. Cycling and characterization. Wiley, New York, pp 1–15
Blumentals II, Itoh M, Olson GJ, Kelly RM (1990) Role of polysulfides in reduction of elemental sulfur by the hyperthermophilic archaebacterium *Pyrococcus furiosus*. Appl Environ Microbiol 56:1255–1262
Bradley PM, Chapelle FH (1991) Arsenate inhibition of denitrification in nitrate contaminated sediments. Soil Biol Biochem 25:1459–1462
Bumbla DK, Keefer RF (1994) Arsenic mobilization and bioavailability in soils. In: Nriagu JO (ed) Arsenic in the environment. 1. Cycling and characterization. Wiley, New York, pp 51–82
Caccavo FJ, Lonergan DJ, Lovley DR, Davis M, Stolz JF, McInerney MJ (1994) *Geobacter sulfurreducens* sp. nov., a hydrogen- and acetate-oxidizing dissimilatory metal-reducing microorganism. Appl Environ Microbiol 60:3752–3759
Cervantes C, Ji G, Ramirez JL, Silver S (1994) Resistance to arsenic compounds in microorganisms. FEMS Microbiol Rev 15:355–367
De Soete G (1983) A least-squares algorithm for fitting additive trees to proximity data. Psychometrika 48:621–626
Dowdle PR, Laverman AM, Oremland RS (1996) Bacterial dissimilatory reduction of arsenic(V) to arsenic(III) in anoxic sediments. Appl Environ Microbiol 62:1664–1669
Felsenstein J (1981) Evolutionary trees from DNA sequences: a maximum-likelihood approach. J Mol Evol 17:368–376
Franson MAH (ed) (1981) Standard methods for the examination of water and wastewater. American Public Health Association, Washington, DC, pp 447–448
Gladysheva TB, Oden KL, Rosen BP (1994) Properties of the arsenate reductase of plasmid R773. Biochemistry 33:7288–7293
Ji G, Garber EAE, Armes LG, Chen CM, Fuchs JA, Silver S (1994) Arsenate reductase of *Staphylococcus aureus* plasmid pI258. Biochemistry 33:7294–7299
Johnson DL, Pilson MEQ (1972) Spectrophotometric determination of arsenite, arsenate, and phosphate in natural waters. Anal Chem Acta 58:289–299
Jukes TH, Cantor CR (1969) Evolution of protein molecules. In: Munro HN (ed) Mammalian protein metabolism, vol 3. Academic Press, New York, pp 21–132
Laverman AM, Switzer Blum J, Schaefer JK, Philips EJP, Lovley DR, Oremland RS (1995) Growth of strain SES-3 with arsenate and other diverse electron acceptors. Appl Environ Microbiol 61:3556–3561
Lonergan DJ, Jenter HL, Coates JD, Phillips EJP, Schmidt TM, Lovley DR (1996) Phylogenetic analysis of dissimilatory Fe(III)-reducing bacteria. J Bacteriol 178:2402–2408
Lovley DR, Phillips EJP (1988) Novel mode of microbial energy metabolism: organic carbon oxidation coupled to dissimilatory reduction of iron or manganese. Appl Environ Microbiol 54:1472–1480
Maidak BL, Olsen GL, Larsen N, Overbeek R, McCaughey MJ, Woese CR (1996) The ribosomal database project (RDP). Nucleic Acids Res 24:82–85
Miller TL, Wolin MJ (1974) A serum bottle modification of the Hungate technique for cultivating obligate anaerobes. Appl Microbiol 27:985–987
Morel FMM, Hering JG (1993) Principles and applications of aquatic chemistry. Wiley, New York
Newman DK, Beveridge TJ, Morel FMM (1997) Precipitation of As_2S_3 by *Desulfotomaculum auripigmentum*. Appl Environ Microbiol 63:2022–2028
Oremland RS, Capone DG (1988) Use of “specific” inhibitors in biogeochemistry and microbial ecology. Adv Microb Ecol 10:285–383
Osborn FH, Ehrlich HL (1976) Oxidation of arsenite by a soil isolate of *Alcaligenes*. J Appl Bacteriol 41:295–305
Peters DG (1974) Chemical separations and measurements: theory and practice of analytical chemistry. Saunders, Philadelphia
Pfennig N, Widdel F, Trüper HG (1981) The dissimilatory sulfate-reducing bacteria. In: Starr MP, Stolp H, Trüper HG, Balows A, Schlegel HG (eds) The prokaryotes, vol 1. Springer, Berlin Heidelberg New York, pp 926–947
Rabus R, Nordhaus R, Ludwig W, Widdel F (1993) Complete oxidation of toluene under strictly anoxic conditions by a new sulfate-reducing bacterium. Appl Environ Microbiol 59:1444–1451
Rittle KA, Drever JI, Colberg PJS (1995) Precipitation of arsenic during bacterial sulfate reduction. Geomicrobiol J 13:1–12
Spliethoff HM, Mason RP, Hemond HF (1995) Interannual variablity in the speciation and mobility of arsenic in a dimictic lake. Environ Sci Technol 29(8):2157-2161
Tabatabai MA (1974) A rapid method for determination of sulfate in water samples. Environ Lett 7:237–243