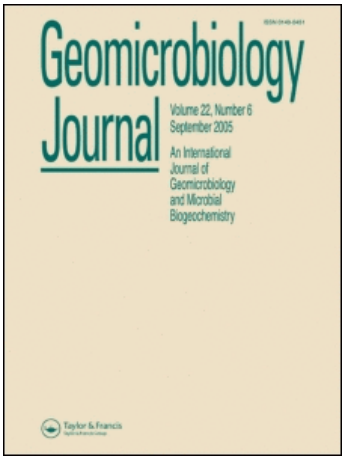

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Isolation of a New Arsenate-Respiring Bacterium—Physiological and Phylogenetic Studies

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A new strictly anaerobic arsenate-respiring bacterium has been isolated from arsenic-contaminated mud obtained from a gold mine in Bendigo, Australia. This organism, designated JMM-4, was found to be a Gram-positive, spore-forming rod, 0.6 × 2.5–3 μm, motile by means of flagella that are subpolar or along one side of the cell. JMM-4 grows using arsenate as the terminal electron acceptor and lactate as the electron donor. Arsenate is reduced to arsenite and the lactate is oxidized to CO_2 via the intermediate, acetate. The doubling time for exponential growth with arsenate (5 mM) and lactate (5 mM) was 4.3 ± 0.2 h. 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 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 $g \cdot l^{-1}$. Based upon 16S rRNA gene sequence analysis, 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. *The physiological differences between JMM-4 and* B. arsenicoselenatis *however suggest that JMM-4 is a new species of* Bacillus.

Keywords arsenic, arsenate respiration, phylogenetic, *Bacillus*

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This manuscript is dedicated to the late Professor Joan M. Macy, who made an enormous contribution to the area of microbial physiology and environmental microbiology. This work was supported by an Australian Research Council Grant (A09925054) to JMM. We would like to thank D. Flood for technical assistance. We would also like to thank R. S. Oremland for helpful suggestions.

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Introduction

The reduction of arsenate [As(V)] to arsenite [As(III)] is known to occur in anoxic environments (McGeehan and Naylor 1994; Dowdle et al. 1996; Oremland and Stolz 2000). A number of both physiologically and phylogenetically unique bacteria have been isolated that are able to use arsenate as a terminal electron acceptor, reducing it to arsenite.

A total of nine arsenate-respiring prokaryotes have been described, six of which use the nonrespiratory substrate lactate as the electron donor. These organisms group together phylogenetically as follows: (1) *Sulfurospirillum arsenophilum* str. MIT-13 (Ahmann et al. 1994; Stolz et al. 1999) and *S. barnesii* str. SES-3 (Oremland et al. 1994; Stolz et al. 1999) are both members of the ε division of the *Proteobacteria*, (2) *Bacillus arsenicoselenatis* str. E1H, *B. selenitireducens* str. MLS10 (Switzer Blum et al. 1998) and *Desulfotomaculum auripigmentum* str. OREX-4 (Newman et al. 1997a, b) are members of the low G+C group of the Gram-positive bacteria, and (3) *Desulfomicrobium* sp. str. Ben-RB is a member of the δ division of the *Proteobacteria* (Macy et al. 2000). The two *Bacillus* species mentioned previously, however, are both phylogenetically and physiologically unrelated to *D. auripigmentum*.

To date, only one arsenate-respiring bacterium has been isolated that is able to use acetate as the electron donor. This organism, *Chrysiogenes arsenatis*, is the first representative of a new phylum of the *Bacteria* (Macy et al. 1996, 2001). Recently, two hyperthermophilic members of the *Archaea, Pyrobaculum arsenaticum* and *P. aerophilum*, have been demonstrated to respire with arsenate as the terminal electron acceptor and hydrogen as the electron donor (Huber et al. 2000).

This report describes a new strictly anaerobic arsenate-respiring bacterium isolated from a gold mine in Bendigo, Australia. The physiological and phylogenetic characteristics of this organism are described.

Materials and Methods

Growth and Media Conditions

The anoxic minimal medium contained 20 mM NaCl, 4 mM KCl, 2.8 mM NH_4Cl, 1.5 mM KH_2PO_4, 0.2 mM Na_2SO_4, 2 mM $MgCl_2 \cdot 6H_2O$, 1 mM $CaCl_2 \cdot 2H_2O$, 0.05% $NaHCO_3$, 1 ml/l SL10 trace elements, and vitamins (Macy et al. 1989). At no time was a reducing agent added to the medium. The initial pH of the medium was 7.8. The standard anaerobic culture technique of Hungate was employed (Macy et al. 1989). All incubations were carried out at 28°C.

Enrichments and Isolation

The sample used for the enrichment was mud from an arsenic-contaminated gold mine in Bendigo, Victoria, Australia (pH 7.6, 2.5 mg l^{-1} arsenic). The mud was placed in anoxic minimal medium containing arsenate (5 mM) and acetate (10 mM) and the enrichment incubated for 5 days. The enrichment was subcultured twice and the third transfer culture was serially diluted and inoculated into minimal medium containing 1.5% (w/v) Oxoid agar (Oxoid, Hants, England), arsenate (5 mM) and acetate (10 mM) in Hungate roll tubes (Hungate 1969) or onto agar plates in an anaerobic chamber. Several colonies were selected, purified, and tested for their ability to respire with arsenate (5 mM) using acetate (10 mM) as the electron donor. A motile, rod-shaped bacterium was isolated and designated JMM-4.

Growth of JMM-4 on Arsenate and Lactate

JMM-4 was grown in 400 ml of anoxic minimal medium containing arsenate (5 mM or 10 mM) and lactate (5 mM or 10 mM). The initial pH of the medium was 7.8 and was

maintained between 7.8 and 8.2 by the addition of 1 M HCl. The inoculum (40 ml) was a 22-h culture grown in the same medium (without pH control). The concentration of total arsenic [As(V) and As(III)] remained constant throughout the experiment. Growth experiments were performed in duplicate on three separate occasions. Experiments were initially performed without pH control, however, when the pH rose to 8.7 growth ceased.

Electron Acceptors and Donors Used for Growth

The electron acceptors (5 mM) tested for their ability to support growth, when lactate (5 mM) was present as the electron donor, included arsenate, arsenite, nitrate, nitrite, sulfate, thiosulfate, selenate, Fe(III), and oxygen (added as 5 or 10 ml of air to a closed Balch tube containing 10 ml of medium). The initial inoculum used for these experiments had been grown in minimal medium containing arsenate (5 mM) and lactate (5 mM). Growth with a given electron acceptor was considered positive only if, after two successive subcultures, at least 90% of the electron acceptor had been reduced and the cell number had increased by at least 10-fold.

The electron donors (5 mM) tested for their ability to support growth when arsenate (5 mM) was present as the electron acceptor included acetate, lactate, pyruvate, succinate, malate, formate, citrate, glutamate, starch, dextrose, fructose, galactose, xylose, glycine, and molecular hydrogen (10 ml) (with acetate as carbon source). A 10% inoculum of an overnight culture of JMM-4 grown in minimal medium containing arsenate (5 mM) as the terminal electron acceptor and lactate (5 mM) as the electron donor was used for the initial transfer. In the cases where growth was detected the culture was subcultured twice. Growth with a given electron donor with arsenate as the terminal electron acceptor was considered positive if the number of cells had increased by at least 10-fold and if at least 90% of the arsenate had been reduced to arsenite (the amount of total arsenic remained constant).

Salinity and pH Optimum for Growth

For determining the optimum pH for growth, the medium used was identical to that described in *Growth and Media Conditions*. Arsenate (5 mM) was provided as the electron acceptor and lactate (5 mM) as the electron donor. The pHs tested were pH 7, 7.4, 7.8, 8.1, and 8.4.

For determining the optimal sodium chloride (NaCl) concentration for growth, the medium was identical to that described in *Growth and Media Conditions*. Arsenate (5 mM) was provided as the electron acceptor and lactate (5 mM) as the electron donor. The NaCl concentrations tested were ($g \cdot l^{-1}$) 1.2, 1, 10, 30, 50, 70, and 90. These experiments were performed in duplicate and the initial inoculum used for these experiments was grown in minimal medium containing arsenate (5 mM) and lactate (5 mM) (pH 7.8, NaCl 1.2 $g \cdot l^{-1}$).

Analytical Methods

Total arsenic [As(III) and As(V)] was determined by inductively coupled plasma (ICP) (Jobin Yvon 24; France) and arsenite by using a Varian Spectra AA20 atomic absorption spectrophotometer, with a VGA76 hydride generator (Varian, Melbourne, Victoria, Australia) as described previously (Macy et al. 1996). Nitrate was determined by HPLC as described previously (Macy et al. 1989). Lactate and acetate were determined using enzymatic bioanalysis/food analysis D-L/lactic acid and acetate kits (Roche).

Electron Microscopy

JMM-4 was prepared for transmission electron microscopy using the methods in Stolz (1991). Negative-stained preparations and ultrathin sections were observed on a Philips 201 transmission electron microscope at 60 kV. Transmission electron microscope negative images were captured using a Kodak DC120 digital camera, processed with Adobe PhotoShop, and printed with a Codonics dye sublimation printer. All images were digitally enhanced for improved contrast and sharpness.

16S rRNA Gene Sequence Determination and Analysis

PCR-mediated amplification of the 16S rRNA gene and purification of the PCR products were carried out as described previously (Rainey et al. 1996). Purified PCR products were sequenced using the ABI PRISM^{TM} Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Germany) as specified by the manufacturer's instructions. The reactions mixtures were sequenced using an Applied Biosystems model 373A DNA sequencer.

The near full-length 16S rRNA gene sequence (1512 bp) for strain JMM-4 was determined by the DSMZ, Germany. Additional sequences were obtained from GenBank and from the Ribosomal Database Project (RDP) (Maidak et al. 1999). Alignments were done using Clustal X (Higgins and Sharp 1989). Maximum parsimony and neighbor-joining trees were generated and sequence similarities were determined using a version of PHYLIP (Felsenstein 1988) and PAUP (Swofford 1998). The sequence determined in this study of JMM-4 has been deposited in GenBank under accession number AY032601.

Results

Isolation of an Arsenate-Respiring Bacterium

Arsenic-contaminated mud from a gold mine in Bendigo, Australia, was used for the enrichments and placed into anoxic minimal medium containing arsenate and acetate. The enrichment was subcultured twice upon which a strictly anaerobic, arsenate-respiring organism, designated JMM-4, was isolated from agar. JMM-4 is a Gram-positive, spore-forming, motile, rod-shaped bacterium that grows singly. The cells are 0.6 μm in diameter and 2.5 to 3.0 μm in length with cells near division slightly longer (Figure 1). Spores are formed in a polar position. The flagella are subpolar or arranged on one side of the cell (Figure 1A). The cell envelope is comprised of a cytoplasmic membrane and thick peptidoglycan layer, and there appears to be a distinctive wall structure reminiscent of polar organelles (Figure 1B; Tauschel 1985).

Growth of JMM-4 on Arsenate and Lactate

Growth of JMM-4 in minimal medium with arsenate (5 mM) as the terminal electron acceptor and lactate (5 mM) as the electron donor is shown in Figure 2. JMM-4 reduced arsenate to arsenite while oxidizing lactate to acetate and CO_2. As essentially no acetate (i.e., $<$0.1 mM) was detected one can assume that the acetate was further oxidized to CO_2. The total arsenic level (i.e., arsenate and arsenite) remained constant throughout the experiment. The total amount of arsenite made, therefore arsenate reduced, was 4.8 mM. A total of 1 mM lactate was oxidized to acetate and CO_2 and the acetate subsequently oxidized to CO_2. The utilization of 1 mM lactate and acetate should yield 2 and 4 mM reducing equivalents, respectively. The estimated number of reducing equivalents available from the oxidation of lactate and acetate is close to the number required to reduce the

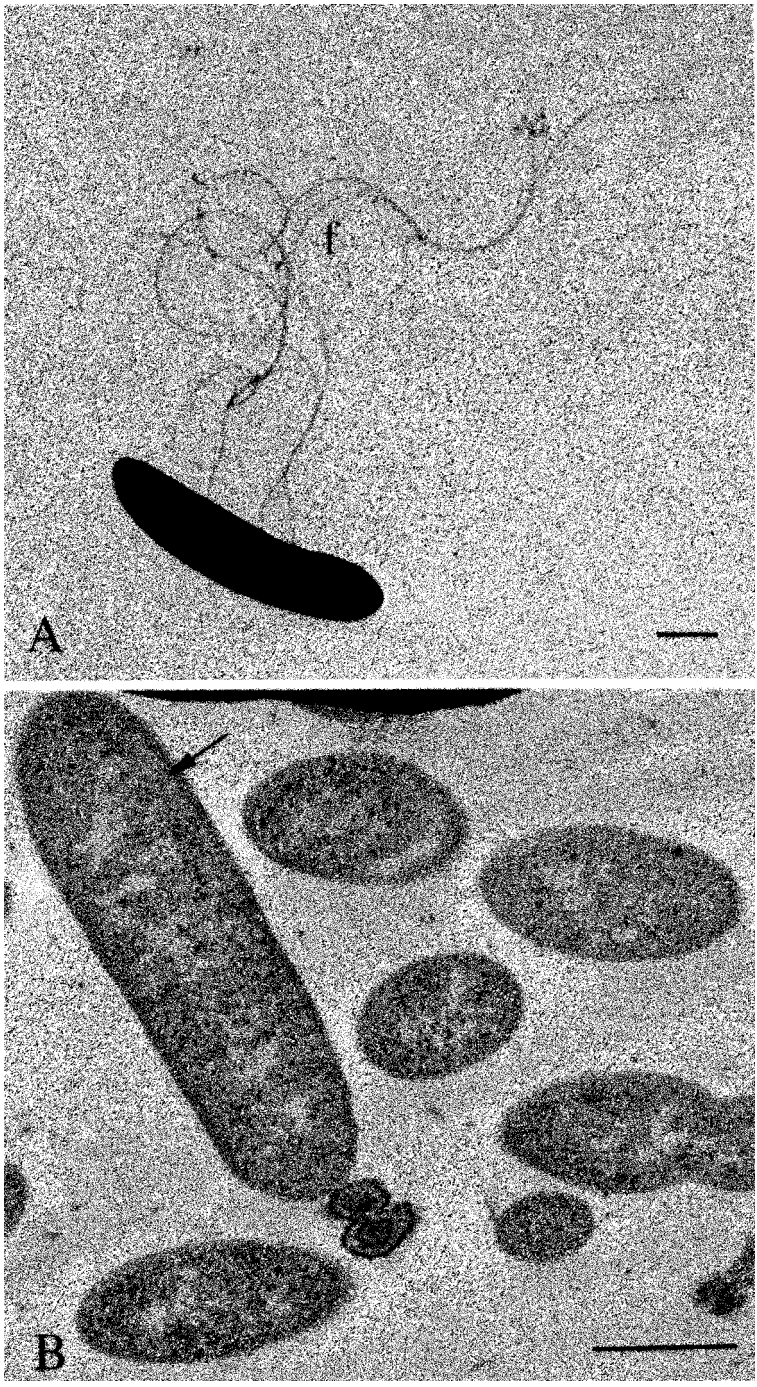

FIGURE 1 Transmission electron micrographs of JMM-4. A) Negative stain showing the overall shape of the cell and location of the flagella (f). B) Thin section showing longitudinal and cross-sections of the cells. Flagella can be seen in cross-section and longitudinal section between the cells. The polar organelle is designated by the arrow. All bars are 0.5 μm.

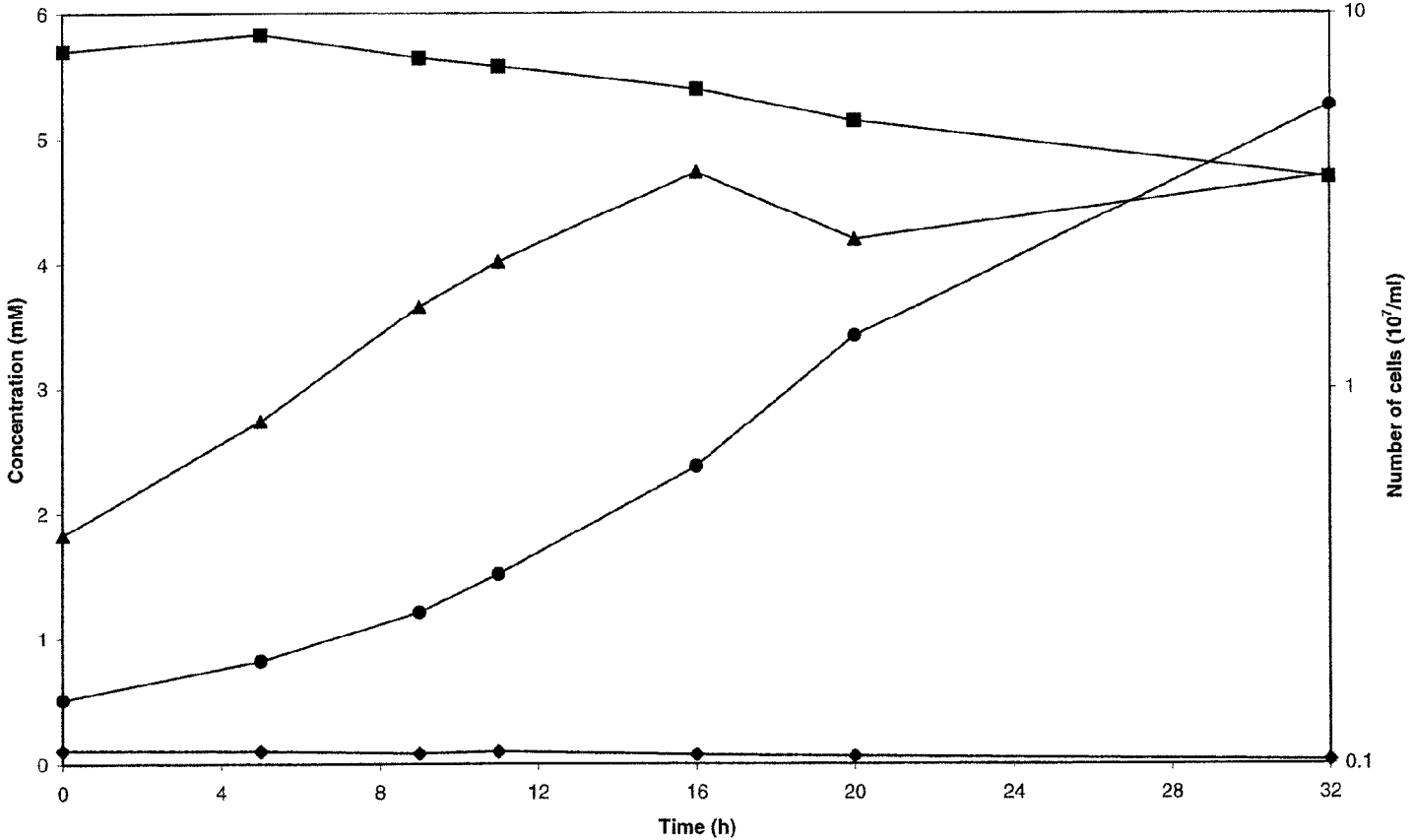

FIGURE 2 Growth of strain JMM-4 in minimal medium with arsenate (5 mM) and lactate (5 mM). An overnight culture of strain JMM-4, grown in the same medium however without pH control, was inoculated (10%) into the experimental flask at time = 0. The pH was maintained between 7.8 and 8.2. ▲, number of cells · ml^{-1}; ●, arsenite; ■, lactate; ◆, acetate.

arsenate by this organism. The generation time during exponential growth was 4.3 ± 0.2 h (average of four experiments). A similar generation time of 4.45 ± 0.15 h (average of two experiments) was observed when JMM-4 was grown with arsenate (10 mM) and lactate (10 mM). Growth did not occur when either arsenate or lactate (data not shown) was absent from the medium. Optimal growth of this organism occurs in a minimal medium at pH 7.8 with a NaCl concentration of 1.2 $g \cdot l^{-1}$ (see Table 1).

TABLE 1 Comparisons of strain JMM-4 with *Bacillus arsenicoselenatis* strain E1H

	JMM-4	*B. arsenicoselenatis* strain E1H[a]
Cell dimensions	0.6 × 2.5–3 μm	1 × 3 μm
Motility	+	−
Spores	+	+
pH range	7–8.4	7–10.2
pH optimum	7.8	9.8
NaCl range	1.2–30	20–120
NaCl optimum	1.2	60
Electron acceptors		
Arsenate	+	+
Arsenite	−	ND
Nitrate	+	+
Nitrite	−	−
Selenate	−	+
Selenite	−	−
Oxygen	−	−
Iron(III)	−	+
Sulfate	−	−
Thiosulfate	−	−
Electron donors		
Acetate	+	−
Lactate	+	+
Pyruvate	+	−
Succinate	+	−
Malate	+	+
Citrate	−	+
Formate	−	−
Glutamate	+	−
Hydrogen + acetate	+	−
Starch	−	+
Dextrose	−	−
Fructose	−	+[b]
Galactose	−	−
Xylose	−	ND
Glycine	−	−

[a] The information for *B. arsenicoselenatis* strain E1H was obtained from Switzer Blum et al. (1998).

[b] Growth occurs in the absence of an electron acceptor.

ND = not determined.

Growth of JMM-4 on Other Electron Acceptors and Electron Donors

When JMM-4 was grown in minimal medium with lactate as the electron donor, only nitrate could replace arsenate as the terminal electron acceptor (Table 1). The electron acceptors, oxygen, arsenite, Fe(III), nitrite, selenate, sulfate and thiosulfate, did not support growth (Table 1).

When JMM-4 was grown with arsenate as the terminal electron acceptor, the following electron donors supported growth: acetate, pyruvate, succinate, malate, glutamate, and hydrogen (acetate was used as the carbon source) (Table 1). No growth occurred on any of these substrates in the absence of arsenate.

Phylogenetic Characterization

The phylogenetic analysis of the 16S rRNA gene sequence (1512 bp) of JMM-4 revealed that it fell within the low G+C, Gram-positive, aerobic, spore-forming bacilli cluster (Figure 3). The nearest known phylogenetic relatives of strain JMM-4 are alkalophilic *Bacillus* species such as *B. pseudofirmus* (95.1% sequence similarity), *B. pseudoalcaliphilus* (94.4% sequence similarity), and *B. alcalophilus* (93.9% sequence similarity). Interestingly, strain JMM-4 showed the highest 16S rRNA gene sequence similarity (97.3%) to the Gram-positive haloalkalophilic arsenate/selenate-respiring bacterium, *Bacillus arsenicoselenatis* (Switzer Blum et al. 1998). JMM-4 however differs substantially from two other arsenate-respiring organisms also present in this group of the *Bacteria*, *B. selenitireducens* (92.3% sequence similarity) and *D. auripigmentum* (83.6% sequence similarity) (Figure 3). Based on the 16S rRNA gene sequence similarities, strain JMM-4 appears to represent a new species within the genus *Bacillus*.

Discussion

A number of phylogenetically and physiologically diverse bacteria have been isolated that are able to grow by respiring with arsenate as the terminal electron acceptor using various organic compounds (e.g., lactate) or hydrogen as electron donors (see Introduction).

This study reports the characterization of a new arsenate-respiring bacterium, JMM-4, isolated from arsenic-contaminated mud from a gold mine in Bendigo, Victoria, Australia. The arsenic levels in this location and other mining areas in Victoria, Australia, can be as high 12 mg l^{-1}. Two other arsenate-respiring bacteria have been isolated from mines in this area, namely *C. arsenatis* from Ballarat (Macy et al. 1996), approximately 100 km from Bendigo, and the arsenate/sulfate-reducing bacterium *Desulfomicrobium* sp. str. Ben-RB isolated from Bendigo (Macy et al. 2000). Moreover, arsenite-oxidizing bacteria have also been isolated from Bendigo (Santini et al. in press). These organisms have adapted mechanisms to live in these environments by either respiring with arsenate or oxidizing arsenite.

JMM-4 is a Gram-positive, spore-forming, rod-shaped, motile bacterium that phylogenetically groups with the low G+C Gram-positive bacilli. In fact, JMM-4 was found to be, based on 16S rRNA gene sequence analysis, most closely related to known alkalophilic *Bacillus* species [e.g., *B. pseudofirmus* (95.1% sequence similarity)]. The highest sequence similarity (97.3%) however was to the haloalkalophilic arsenate/selenate-respiring bacterium, *B. arsenicoselenatis* (Switzer Blum et al. 1998). JMM-4 however differs physiologically (see below) from both *B. arsenicoselenatis* and *B. pseudofirmus*. The latter organisms are alkalophiles, the pH optima of *B. arsenicoselenatis* and *B. pseudofirmus* are 9.8 and 9.5, respectively (Guffanti et al. 1986; Switzer Blum et al. 1998), whereas that of JMM-4 is 7.8, which is consistent with the environment where the organism was isolated (pH 7.6) (see Table 1). Moreover, JMM-4 differs from *B. arsenicoselenatis* in that the

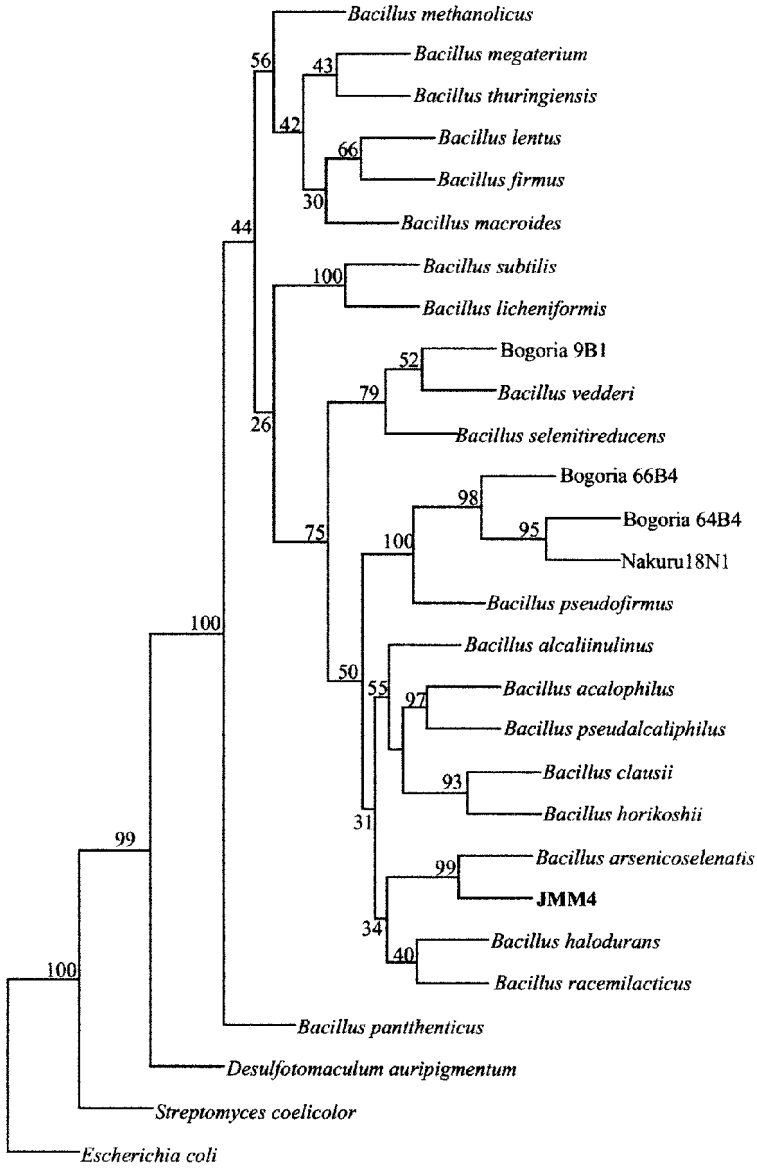

FIGURE 3 Maximum parsimony tree (PHYLYP) showing the phylogenetic relationship of strain JMM-4 with representatives of the low G+C, Gram-positive, spore-forming bacilli cluster. The analysis included data from 1,200 unambiguous nucleotide positions and *Escherichia coli* was used as the outgroup. The bootstrap values are shown at the nodes. The accession numbers for the additional species were *Bacillus alcaliinulinus* AB018595, *B. alcalophilus* X60603, *B. arsenicoselenatis* AF064704, *B. clausii* AJ297498, *B. firmus* X60616, *B. halodurans* AB043973, *B. horikoshii* X76443, *B. lentus* X60601, *B. licheniformis* X60623, *B. macroides* X70312, *B. megaterium* X60629, *B. methanolicus* X64465, *B. pantthenticus* X60627, *B. pseudoalcaliphilus* X76449, *B. pseudofirmus* X76439, *B. racemilacticus* D16278, *B. selenitireducens* AF064705, *B. subtilis* D88802, *B. thuringiensis* X55062, *B. vedderi* Z48306, *Desulfotomaculum auripigmentum* U85624, *Escherichia coli* J01695, *Streptomyces coelicolor* U00411, Lake Bogoria 64B4 X92160, Lake Bogoria 66B4 X92158, Lake Bogoria 9B1 X92167, and Lake Nakuru 18N1 X92159.

latter organism was isolated from a hypersaline environment and thus requires high sodium chloride concentrations for growth (see Table 1).

JMM-4 grows by coupling the oxidation of lactate to CO_2 via the intermediate acetate with concomitant reduction of arsenate to arsenite. Growth of JMM-4 on arsenate as the terminal electron acceptor and lactate as the electron donor occurs with a generation time of 4.3 ± 0.2 h, which is similar to the growth rate of *B. arsenicoselenatis* grown with arsenate and lactate (Switzer Blum et al. 1998). *B. arsenicoselenatis*, however, couples the oxidation of lactate to acetate plus CO_2 with the reduction of arsenate to arsenite. Growth of JMM-4 and *B. arsenicoselenatis* (Switzer Blum et al. 1998) does not occur in the absence of arsenate, thereby suggesting that growth of both organisms is dependent on arsenate reduction.

The electron acceptors and electron donors used by JMM-4 are compared with those used by *B. arsenicoselenatis* in Table 1. As can be seen for JMM-4, arsenate can only be replaced with nitrate as terminal electron acceptor. Alternatively, *B. arsenicoselenatis* can use arsenate, nitrate, selenate, and Fe(III) as terminal electron acceptors. Both JMM-4 and *B. arsenicoselenatis* (Switzer Blum et al. 1998) are strict anaerobes differing from *B. pseudofirmus*, which is a strict aerobe (Guffanti et al. 1986). Electron donors used by these organisms also vary (see Table 1). The electron donors tested for *B. pseudofirmus* are limited, however, growth occurs on acetate, malate but not lactate and also occurs on various sugars (i.e., glucose and fructose) (Guffanti et al. 1986). Apart from *C. arsenatis*, JMM-4 represents the second example of an organism that can couple arsenate reduction with the oxidation of acetate to CO_2.

From both the phylogenetic and the physiological characteristics JMM-4 appears to represent a new species within the genus *Bacillus*.

The mechanism used by these organisms to reduce arsenate to arsenite has only been studied in detail for *C. arsenatis*. The *C. arsenatis* enzyme responsible for catalyzing the reduction of arsenate to arsenite, arsenate reductase, has been purified and characterized and is located in the periplasm of the cell (Krafft and Macy 1998). It consists of two subunits [ArrA (87 kDa) and ArrB (29 kDa)], is a heterodimer $\alpha_1\beta_1$ with a native molecular weight of 123 kDa, and contains molybdenum, iron, and acid-labile sulfur as cofactor constituents. Preliminary biochemical studies of the equivalent enzyme in *S. barnesii* show that it is an integral membrane protein with a calculated mass of 100 kDa, consisting of three different subunits of 65, 31, and 22 kDa (Newman et al. 1998; Stolz and Oremland 1999). The *Desulfomicrobium* sp. str. Ben-RB arsenate reductase, like that of *S. barnesii*, is also membrane-bound (Macy et al. 2000). This enzyme is either a *c*-type cytochrome or associated in the membrane with such a cytochrome (Macy et al. 2000).

Interestingly, all of the organisms mentioned above are unrelated phylogenetically, and the enzymes involved in arsenate reduction also appear to be different. Whether the enzymes have common features remains to be determined. As JMM-4 and *B. arsenicoselenatis* are more related to each other than the organisms outlined above, it would be interesting to determine whether the mechanisms used by these organisms to reduce arsenate to arsenite are similar.

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