Bacillus macyae sp. nov., an arsenate-respiring bacterium isolated from an Australian gold mine

Joanne M. Santini, Illo C. A. Streimann and Rachel N. vanden Hoven

Department of Microbiology, La Trobe University, 3086, Victoria, Australia

Correspondence
Joanne M. Santini
j.santini@latrobe.edu.au

A strictly anaerobic arsenate-respiring bacterium isolated from a gold mine in Bendigo, Victoria, Australia, belonging to the genus *Bacillus* is described. Cells are Gram-positive, motile rods capable of respiring with arsenate and nitrate as terminal electron acceptors using a variety of substrates, including acetate as the electron donor. Reduction of arsenate to arsenite is catalysed by a membrane-bound arsenate reductase that displays activity over a broad pH range. Synthesis of the enzyme is regulated; maximal activity is obtained when the organism is grown with arsenate as the terminal electron acceptor and no activity is detectable when it is grown with nitrate. Mass of the catalytic subunit was determined to be approximately 87 kDa based on ingel activity stains. The closest phylogenetic relative, based on 16S rRNA gene sequence analysis, is *Bacillus arseniciselenatis*, but DNA–DNA hybridization experiments clearly show that strain JMM-4^T represents a novel *Bacillus* species, for which the name *Bacillus macyae* sp. nov. is proposed. The type strain is JMM-4^T (=DSM 16346^T=JCM 12340^T).

The arsenic oxyanion arsenate [As(V)] can be used by prokaryotes in anaerobic respiration as a terminal electron acceptor. The arsenate is reduced to arsenite coupled with the oxidation of several electron donors, thereby generating energy for growth. Eighteen species of arsenate-respiring prokaryotes have been isolated from diverse environments that are physiologically and phylogenetically unique (Oremland & Stolz, 2003). These organisms range from mesophiles to extremophiles, and can be adapted to extremes of temperature, pH and salinity.

The mechanism of arsenate respiration has only been studied in three of these organisms, namely *Chrysiogenes arsenatis* (Krafft & Macy, 1998), *Bacillus selenitireducens* (Afkar *et al.*, 2003) and *Shewanella* sp. strain ANA-3 (Saltikov & Newman, 2003); the arsenate reductases (Arr) of *C. arsenatis* and *B. selenitireducens* have been purified and characterized and the genes encoding the Arr of *Shewanella* sp. strain ANA-3 have been cloned and sequenced. The three enzymes share similarities, suggesting that the differences between them have occurred by divergent evolution.

A Gram-positive, strictly anaerobic, arsenate-respiring bacterium was isolated from an arsenic-contaminated environment in Bendigo, Victoria, Australia (Santini *et al.*, 2002). This strain (JMM-4^T) can couple the reduction of arsenate to arsenite with the oxidation of lactate to CO_2 via the intermediate acetate. Based on 16S rRNA gene sequence analysis, JMM-4^T falls within the low-G+C Gram-positive, aerobic, spore-forming bacilli cluster and is most closely related to the haloalkalophilic arsenate/selenate-respiring bacterium *Bacillus arseniciselenatis* (Switzer Blum *et al.*, 1998). Here we determine, based on physiological, phylogenetic and molecular analyses, that JMM-4^T represents a novel *Bacillus* species, for which the name *Bacillus macyae* sp. nov. is proposed. We also provide preliminary data on the arsenate reductase of this organism.

JMM-4^T was grown as described by Santini *et al.* (2002) except that yeast extract (0·08 %) was used instead of vitamins for all experiments. Sodium lactate (10 mM) was used as the electron donor and sodium arsenate (5 mM) or sodium nitrate (5 mM) as the terminal electron acceptors. For enzyme studies, JMM-4^T was grown in 2 litre batch cultures until late-exponential phase. *B. arseniciselenatis* (ATCC 700614^T) was grown as described by Switzer Blum *et al.* (1998) but in the absence of a reducing agent and with NaCl (40 g l^{-1}) and 0·1 % yeast extract. Sodium lactate (10 mM) was used as the electron donor and sodium nitrate (10 mM) as the terminal electron acceptor.

For genomic DNA isolations, JMM-4^T and *B. arseniciselenatis* were grown in 2 litre batches containing 5 mM arsenate, 10 mM lactate and 0·1 % yeast extract, and 10 mM nitrate, 10 mM lactate and 0·1 % yeast extract, respectively, until mid- to late-exponential phase. Cells were harvested by centrifugation for 20 min at 21 000 ***g*** (4 °C). JMM-4^T genomic DNA was isolated using the Wizard SV Genomic

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Abbreviation: Arr, arsenate reductase.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of JMM-4^T is AY032601.

DNA Purification System (Promega). *B. arseniciselenatis* genomic DNA was isolated by the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). *B. arseniciselenatis* cells were disrupted by French press and the DNA purified by hydroxyapatite chromatography as described by Cashion *et al.* (1977). DNA base composition of JMM-4^T was determined by HPLC again at the DSMZ. DNA–DNA hybridization of strain JMM-4^T and *B. arseniciselenatis* was carried out as described by De Ley *et al.* (1970) with the modifications described by Huss *et al.* (1983) and Escara & Hutton (1980) in 2× SSC at 62 °C by the DSMZ.

For biochemical studies, JMM-4^T cells were harvested by centrifugation for 20 min at 21 000 ***g*** (4 °C), suspended in 50 mM Tris/HCl (pH 8·5) and the cells disrupted by two passages through a French press ($1{\cdot}4 \times 10^9$ Pa). Unbroken cells were removed by centrifugation at 17 000 ***g*** (4 °C). The soluble and membrane fractions were separated by ultracentrifugation for 1 h at 100 000 ***g*** (4 °C). The pellet (i.e. membranes) was suspended in 50 mM Tris/HCl (pH 8·5) (0 °C); the volume used was identical to that of the supernatant (i.e. the soluble fraction).

Arsenate and nitrate reductase activity was determined using anaerobic assays (25 °C) in cuvettes (1 cm) closed with butyl-rubber stoppers. Enzyme activity was determined using a Cary 1E spectrophotometer (Varian) by monitoring oxidation of the artificial electron donors, reduced benzyl (1 mM) or methyl viologen (1 mM) at 546 nm or 540 nm, respectively, using arsenate (10 mM) or nitrate (10 mM) as the electron acceptors (ε of reduced benzyl and methyl viologen is 19·5 and 4·95 cm^{-1} mM^{-1}, respectively). The artificial electron donors were reduced chemically with sodium hydrosulfite (0·15 mg ml^{-1}). The reaction was started by addition of the electron acceptor. One unit (U) of activity corresponded to the reduction of 1 μmol arsenate or nitrate per minute. To determine the optimum buffer and pH for the enzyme, Arr activity was tested in 50 mM MES (pH 5·5, 6 and 6·5), MOPS (pH 6·5, 7 and 7·5), Tris/HCl (pH 7·5, 8 and 8·5) and CHES (pH 9). All subsequent Arr assays were done in 50 mM Tris/HCl (pH 8·5). All enzyme assays were performed at least in duplicate and on two separate occasions. To identify the Arr, ingel activity stains were performed as described by Bluemle & Zumft (1991) except that the nitrate was replaced with arsenate.

Colonies observed in Hungate roll tubes were circular and white. JMM-4^T is a Gram-positive, motile (four to six peritrichous flagella), spore-forming, rod-shaped bacterium that grows singly. Spores are terminal and ellipsoidal and swelling of the sporangia is not observed. JMM-4^T was found to be catalase-positive and oxidase-negative. JMM-4^T can grow with either arsenate or nitrate as terminal electron acceptors; the arsenate is reduced to arsenite and nitrate to nitrite (Santini *et al.*, 2002) (Table 1). The electron acceptors oxygen, arsenite, Fe(III), nitrite, selenate, sulfate, thiosulfate (Santini *et al.*, 2002) and fumarate did not support growth (Table 1). Comparisons with its closest phylogenetic relative, based on 16S rRNA gene sequence analysis, the haloalkalophile *B. arseniciselenatis* strain E1H^T (97·3 % similarity), are given in Table 1. Electron donors used by JMM-4^T are also given in Table 1. JMM-4^T grew just as well at 37 °C as it did at 28 °C but it did not grow at 4 or 42 °C.

Table 1. Comparison of strain JMM-4^T with *B. arseniciselenatis* strain E1H^T

Both JMM-4^T and *B. arseniciselenatis* E1H^T produce spores. Both use arsenate and nitrate as terminal electron acceptors, and use lactate and malate as electron donors. Neither uses nitrite, selenite, oxygen, sulfate and thiosulfate as electron acceptors, nor formate, dextrose, galactose and glycine as electron donors. JMM-4^T was grown under the same conditions for all results presented in this table. ND, Not determined.

Characteristic	Strain JMM-4^T*	Strain E1H^T†
Cell dimensions (μm)	0·6 × 2·5–3	1 × 3
Motility	+	+‡
Temperature range for growth (°C)	28–37‡	ND
pH for growth:		
Range	7–8·4	7–10·2
Optimum	7·8	9·8
NaCl for growth:		
Range	1·2–30	20–120
Optimum	1·2	60
Electron acceptors:		
Arsenite	−	ND
Selenate	−	+
Fumarate	−‡	+
Iron(III)	−	+
Electron donors:		
Acetate	+	−
Pyruvate	+	−
Succinate	+	−
Citrate	−	+
Glutamate	+	−
Hydrogen + acetate	+	−
Starch	−	+
Fructose	−	+§
Xylose	−	ND

*Results obtained from Santini *et al.* (2002) unless specified otherwise.

†Results obtained from Switzer Blum *et al.* (1998) unless specified otherwise.

‡Results obtained in this study.

§Growth was also obtained in the absence of an electron acceptor.

Phylogenetic analysis of the 16S rRNA gene sequence (1512 bp) of JMM-4^T showed that it fell within the low-G + C, Gram-positive, aerobic, spore-forming bacilli cluster (Santini *et al.*, 2002). The nearest phylogenetic relatives of JMM-4^T were the alkalophilic *Bacillus* species, including

B. arseniciselenatis (97·3 %), *Bacillus pseudofirmus* (95·1 %) (GenBank/EMBL/DDBJ accession no. X76439), *Bacillus pseudalcaliphilus* (94·4 %) (X76449), *Bacillus alcalophilus* (93·9 %) (X60603) and *B. selenitireducens* (92·3 %) (AF064704) (Santini *et al.*, 2002). Based on the fact that the 16S RNA gene sequence of JMM-4^T was more than 97 % similar to that of *B. arseniciselenatis*, both DNA–DNA hybridization values and G+C content were determined (Stackebrandt & Goebel, 1994; Stackebrandt *et al.*, 2002).

DNA–DNA hybridization between JMM-4^T and *B. arseniciselenatis* was found to be 30·4 %, below the recommended value delimiting different species (Wayne *et al.*, 1987; Stackebrandt *et al.*, 2002). The DNA G+C content of strain JMM-4^T was 37 mol% compared with 40 mol% for *B. arseniciselenatis* (Switzer Blum *et al.*, 1998).

Preliminary experiments of the Arr of strain JMM-4^T showed activity in total cell extracts to be stable over a broad pH range. Optimal activity occurred with methyl viologen as the artificial electron donor in Tris/HCl (pH 8) ($2{\cdot}3 \pm 0{\cdot}15$ U mg^{-1}), Tris/HCl (pH 8·5) ($2{\cdot}15 \pm 0{\cdot}35$ U mg^{-1}) and CHES (pH 9) ($2{\cdot}3 \pm 0{\cdot}2$ U mg^{-1}). Arr activity was still detected in MES (pH 5·5) buffer, where the enzyme displayed 33 % maximal activity. Arr activity with benzyl viologen as the artificial electron donor was found to be 3·5-fold less than [$0{\cdot}595 \pm 0{\cdot}005$ U mg^{-1} in Tris/HCl (pH 8·5)] with methyl viologen. Tris/HCl (pH 8·5) and methyl viologen were used for all further experiments. The Arr of *B. selenitireducens* also displays a broad pH range (pH 6–11), with optimum activity at pH 9·5, which is similar to the optimum pH for growth of the organism (Afkar *et al.*, 2003). The purified *B. selenitireducens* Arr displays a maximum activity of 2·5 U mg^{-1}, which is similar to the specific activity of the Arr of strain JMM-4^T in total cell extracts. These results suggest either (1) that JMM-4^T expresses more Arr than *B. selenitireducens* or (2) that the Arr of JMM-4^T displays a higher enzyme turnover.

The effect of different growth conditions on Arr activity was determined. These experiments were performed to determine whether the enzyme is constitutively expressed or whether its synthesis is regulated. When JMM-4^T was grown with only nitrate as the terminal electron acceptor no Arr activity was detected ($<0{\cdot}01$ U mg^{-1}), whereas nitrate reductase activity was detected ($7{\cdot}7 \pm 0{\cdot}3$ U mg^{-1}). This suggests that arsenate is required for induction of enzyme synthesis. This was also found to be the case for the Arr of *C. arsenatis* (Krafft & Macy, 1998). When JMM-4^T was grown with both nitrate and arsenate, Arr activity decreased by about 10-fold as compared with activity in cell extracts from cells grown with only arsenate as the electron acceptor ($0{\cdot}25 \pm 0{\cdot}0$ versus $2{\cdot}15 \pm 0{\cdot}35$ U mg^{-1}). Nitrate reductase activity was slightly higher ($8{\cdot}5 \pm 0{\cdot}0$ U mg^{-1}) in cell extracts grown with nitrate/arsenate. No nitrate reductase activity was detected in cell extracts grown with arsenate ($<0{\cdot}01$ U mg^{-1}). These results contrast with those for the *C. arsenatis* Arr, in which the activity remains the same when the organism is grown with either arsenate or arsenate/nitrate (Krafft & Macy, 1998). These preliminary results suggest that arsenate and nitrate respiration may be co-regulated.

Strain JMM-4^T Arr was found to be membrane-bound; 81·6 % of the activity was associated with the membranes and 18·4 % in the soluble fraction. These results suggest that the Arr is not an integral membrane protein as a significant amount of activity was found in the soluble fraction. The Arr of *C. arsenatis* is periplasmic (Krafft & Macy, 1998), whereas the Arr of *B. selenitireducens* is membrane-bound (Afkar *et al.*, 2003).

Arr activity was monitored using SDS-polyacrylamide gels (Fig. 1). Reductase activity was detected in total cell and membrane fractions. The estimated size of the band displaying activity was 87 kDa. This result corresponds well with the molecular masses of the catalytic subunits (ArrA) of the arsenate reductases of *C. arsenatis* (87 kDa) and *B. selenitireducens* (110 kDa).

Description of *Bacillus macyae* sp. nov.

Bacillus macyae (ma.cy′ae. N.L. fem. n. *macyae* of Macy, named after the late Professor Joan M. Macy, Chair of

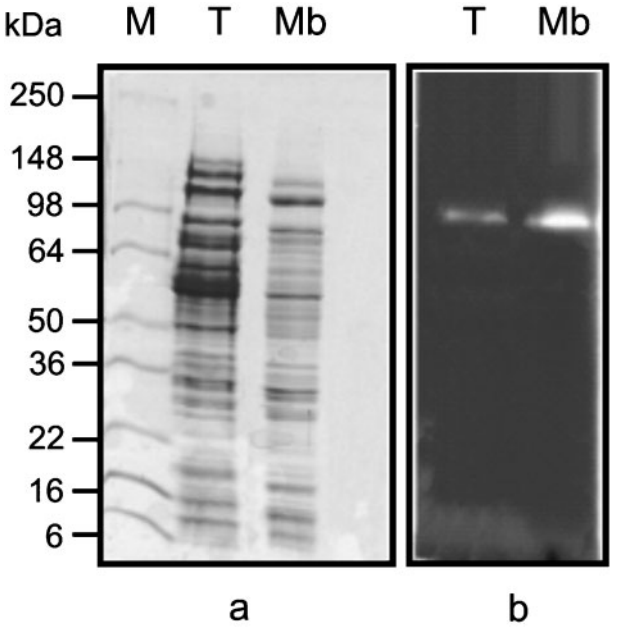

Fig. 1. SDS-polyacrylamide gradient (6–13·5 %) gel and Arr ingel activity stain of the arsenate reductase (Arr) of JMM-4^T. (a) Coomassie blue-stained gel. Molecular mass markers (M): myosin (250 kDa), phosphorylase B (148 kDa), bovine serum albumin (98 kDa), glutamic dehydrogenase (64 kDa), alcohol dehydrogenase (50 kDa), carbonic anhydrase (36 kDa), myoglobin red (22 kDa), lysozyme (16 kDa), aprotinin (6 kDa); JMM-4^T total cell extract (T); and membrane (Mb) fractions. (b) Ingel activity stain of Arr of JMM-4^T.

Microbiology, La Trobe University, in tribute to her research in the area of environmental microbiology).

Cells are Gram-positive, motile rods (2·5–3 μm long and 0·6 μm wide) and produce subterminally located ellipsoidal spores. Spores do not cause swelling of sporangia. Colonies are round and white. Catalase reaction is positive and oxidase is negative. Strict anaerobe that respires with arsenate and nitrate as terminal electron acceptors. Arsenate is reduced to arsenite and nitrate to nitrite. The electron donors used for anaerobic respiration are acetate, lactate, pyruvate, succinate, malate, glutamate and hydrogen (with acetate as carbon source). Growth occurs at 28–37 °C, pH 7–8·4 and 0·12–3 % NaCl. The DNA G+C content is 37 mol%.

The type strain, JMM-4^T (=DSM 16346^T=JCM 12340^T), was isolated from arsenic-contaminated mud from a gold mine in Bendigo, Victoria, Australia.

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