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Desulfitobacterium metallireducens sp. nov., an anaerobic bacterium that couples growth to the reduction of metals and humic acids as well as chlorinated compounds

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Strain 853-15A^T was enriched and isolated from uranium-contaminated aquifer sediment by its ability to grow under anaerobic conditions via the oxidation of lactate coupled to the reduction of anthraquinone-2,6-disulfonate (AQDS) to anthrahydroquinone-2,6-disulfonate (AHQDS). Lactate was oxidized incompletely to acetate and carbon dioxide according to the reaction $CH_3CHOHCOO^- + 2AQDS + H_2O \rightarrow CH_3COO^- + 2AHQDS + CO_2$. Additional electron donors utilized included formate, ethanol, butanol, butyrate, malate and pyruvate. Lactate also supported growth with Fe(III) citrate, Mn(IV) oxide, humic substances, elemental sulfur, 3-chloro-4-hydroxyphenylacetate, trichloroethylene or tetrachloroethylene serving as the electron acceptor. Growth was not observed with sulfate, sulfite, nitrate or fumarate as the terminal electron acceptor. The temperature optimum for growth was 30 °C, but growth was also observed at 20 and 37 °C. The pH optimum was approximately 7·0. The 16S rDNA sequence of strain 853-15A^T suggested that it was most closely related to *Desulfitobacterium dehalogenans* and closely related to *Desulfitobacterium chlororespirans* and *Desulfitobacterium frappieri*. The phylogenetic and physiological properties exhibited by strain 853-15A^T (= ATCC BAA-636^T) place it within the genus *Desulfitobacterium* as the type strain of a novel species, *Desulfitobacterium metallireducens* sp. nov.

Keywords: Fe(III) reduction, reductive dechlorination, humic compounds, Gram-positive bacteria, *Desulfitobacterium*

INTRODUCTION

Dissimilatory Fe(III) reduction can be an important process for the oxidation of organic compounds and can have a significant influence on the inorganic geochemistry of subsurface environments, aquatic sediments and soils (Lovley, 1995, 2000). Microorganisms that can oxidize organic compounds with Fe(III) serving as the electron acceptor can play an important role in the carbon cycle of aquatic sediments (Canfield *et al*., 1993; Lovley, 2000) as well as the degradation of organic contaminants (Anderson & Lovley, 1997; Finneran & Lovley, 2001; Lovley & Anderson, 2000). Microbial reduction of Fe(III) can release trace metals and phosphate bound to Fe(III) oxides, influencing water quality (Lovley, 1995). The production of Fe(II) minerals as the result of microbial Fe(III) reduction can serve as an important geological signature of microbial activity (Ehrlich, 1999). Furthermore, many dissimilatory Fe(III)-reducing microorganisms can use a variety of other metals such as Mn(IV), U(VI) and Co(III) as electron acceptors

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Abbreviations: AHQDS, anthrahydroquinone-2,6-disulfonate; AQDS, anthraquinone-2,6-disulfonate.

The GenBank accession number for the 16S rRNA sequence of strain 853-15A^T is AF297871.

(Lovley, 2000). Although these metals are much less abundant than Fe(III) in most environments, microbial reduction of these metals can greatly influence their geochemistry.

Humic substances can have a major effect on microbial reduction of Fe(III) (Lovley *et al.*, 1996). Most Fe(III)-reducing micro-organisms have the ability to transfer electrons onto the quinone moieties in humic substances (Lovley, 2000; Lovley *et al.*, 1998; Scott *et al.*, 1998). The hydroquinone moieties that are generated can react abiotically with Fe(III) oxides, reducing Fe(III) to Fe(II) and regenerating the humic substances to the oxidized form, which can then accept electrons again from Fe(III)-reducing micro-organisms. In this manner, a small amount of humic substances can transfer electrons repeatedly from Fe(III)-reducing micro-organisms to Fe(III) oxides. This electron shuttling via humic substances can greatly accelerate microbial reduction of Fe(III) oxides (Lovley *et al.*, 1996; Nevin & Lovley, 2000a), presumably because, in the presence of the humic substances, the Fe(III)-reducing micro-organisms no longer have to establish direct physical contact with the Fe(III) oxides in order to reduce them.

Despite the environmental significance of dissimilatory Fe(III) reduction and related forms of electron transfer in soils and sediments, little is known about the diversity of micro-organisms that are capable of carrying out this form of respiration. Here, we describe a Gram-positive, Fe(III)-and humic substance-reducing micro-organism recovered from a uranium-contaminated subsurface environment in New Mexico that is also capable of using chlorinated compounds as a terminal electron acceptor.

METHODS

Origin of enrichment cultures and isolates. Strain 853-15A^T was enriched from anaerobic sediment collected within the floodplain of the San Juan River at the Department of Energy (DOE) uranium mill tailings site in Shiprock, NM, USA. The closest relative site location is monitoring well number 853 and the depth of the core was 15 feet (4·6 m). All sediment was collected anaerobically and shipped to the laboratory via overnight carrier. It was placed in a N_2-filled glove bag and dispensed into the appropriate containers.

Media and growth conditions. Techniques for strict anaerobic culturing (Lovley *et al.*, 1993) were used throughout. The enrichment medium was a defined freshwater medium (Lovley *et al.*, 1993) that contained lactate (10 mM) as the electron donor and 5 mM of the humic-substance analogue anthraquinone-2,6-disulfonate (AQDS) (Sigma) as the sole electron acceptor. The medium consisted of (g l^{-1} unless specified otherwise): $NaHCO_3$ (2·5), NH_4Cl (0·25), $NaH_2PO_4 . H_2O$ (0·6), KCl (0·1), modified Wolfe's vitamin and mineral mixtures (each 10 ml l^{-1}) and 1 mM Na_2SeO_4 (1 ml l^{-1}). The medium (10 ml) was dispensed in anaerobic pressure tubes and bubbled with N_2/CO_2 (80:20) to remove dissolved oxygen. The final pH was approximately 6·7, buffered by bicarbonate. No reducing agent was used in any enrichment or pure culture medium. The enrichment culture was initiated with a 1 g sediment inoculum. The cultures were incubated at 20 °C in the dark. Positive AQDS-reducing enrichments were visually apparent from the bright-orange colour of anthahydroquinone-2,6-disulfonate (AHQDS). Positive enrichments were transferred (10% inoculum) at least five times. In order to obtain pure culture isolates, the enrichment was streaked on medium solidified with purified agar (1·5%) in wide-mouthed glass tubes (Bellco Glass) that were then sealed with a butyl rubber stopper. Distinct colonies were picked and restreaked at least three times on solid agar slants before being suspended in liquid medium. Once purified, the isolate was grown on medium that was supplemented with 0·1% yeast extract.

Characterization of anaerobic growth and electron donor and acceptor utilization. Cells were enumerated by epifluorescent microscopy (Hobbie *et al.*, 1977). Production of AHQDS was measured spectrophotometrically at 450 nm (Lovley *et al.*, 1996). Fe(II) was assayed with Ferrozine as described previously (Lovley & Phillips, 1987). Lactate and acetate were quantified by HPLC. Electron donor utilization was evaluated with AQDS (5 mM) as the electron acceptor. Lactate served as the electron donor for studies on the range of electron acceptors reduced. Reduction of purified humic acids was determined by the electron shuttle assay (Nevin & Lovley, 2000b). Briefly, filtrates (0·2 μm pore diameter) of culture media with humic acid as the electron acceptor were allowed to react with poorly crystalline Fe(III) oxide in pressure tubes under anaerobic conditions and the Fe(II) produced was quantified with ferrozine. Reduction of all other electron acceptors was determined visually by observing precipitation, colour change or turbidity.

16S rDNA and phylogenetic analysis. Cells from a 10 ml culture grown on lactate and AQDS were collected by centrifugation and genomic DNA was extracted as described previously (Rooney-Varga *et al.*, 1999). Nearly the entire 16S rDNA of strain 853-15A^T was amplified using primers 8 Forward (Eden *et al.*, 1991) and 1525 Reverse (Achenbach & Woese, 1995). PCR mixtures were as described previously (Murray *et al.*, 1996), with the following modifications: each 100 μl reaction contained 1·5 mM $MgCl_2$ and 2·5 U AmpliTaq (Perkin Elmer Cetus). Amplification was performed in a GeneAmp PCR System 2400 thermal cycler (Perkin Elmer Cetus) with an initial denaturation step at 94 °C for 1 min followed by 30 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min. There was a final extension at 72 °C for 6 min. PCR products were prepared for sequencing using a QIAquick purification kit (Qiagen). Sequencing was performed at the University of Massachusetts at Amherst Sequencing Facility. Complete bidirectional sequences were obtained from the PCR product. The sequences were compared to the GenBank and Ribosomal Database Project (RDP) databases using the BLAST (National Center for Biotechnology Information) and SIMILARITY_RANK (RDP) algorithms. The secondary structure was verified manually. The sequences were then aligned with related 16S rDNA sequences from GenBank and the RDP using the GCG sequence editor (Wisconsin Package version 10). Phylogenetic trees were inferred using the distance, maximum-likelihood and parsimony tools of PAUP (Swofford, 1998).

RESULTS AND DISCUSSION

Enrichment and isolation

As part of a study on the diversity of metal-reducing micro-organisms in a uranium-contaminated aquifer, subsurface sediments served as the inoculum for an

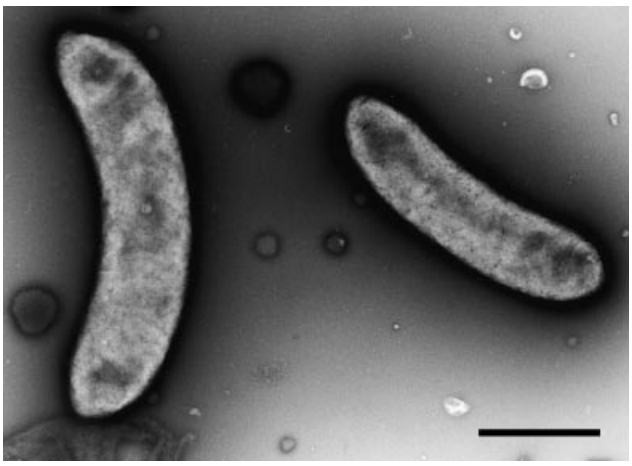

Fig. 1. Negatively stained electron micrograph of strain 853-15A^T indicating the typical curved morphology of exponentially growing cells. Bar, 1 μm.

enrichment culture in which lactate (10 mM) served as the electron donor and the humic-substances analogue AQDS (5 mM) served as the electron acceptor. The enrichment culture turned orange, indicative of the reduction of AQDS to AHQDS. After five repeated transfers (10 % inoculum), the dominant cell type in the enrichment culture was a curved rod. The organism was isolated by streaking the liquid culture onto agar slants of the same medium. Isolated colonies were picked and streaked again. After restreaking three times, the only colonies were white, round, smooth, domed and entire. The medium around the colonies turned orange, indicating the production of AHQDS. A single colony was picked and resuspended in liquid medium with lactate and AQDS. The culture continued to reduce AQDS. This culture, designated strain 853-15A^T (the strain name is derived from its isolation from sediments from near well 853 at a depth of 15 feet, A being first organism isolated), was chosen for further study.

During the exponential growth phase, cells of 853-15A^T were slightly curved rods, 2–5 μm long and about 0·5 μm in diameter and non-motile (Fig. 1). As the culture approached stationary phase, some cells began to form short and long chains and some individual cell morphologies changed, sometimes taking on a 'C' shape in late stationary phase. These late stationary phase cells also formed short and long chains. The Gram-stain reaction (Koneman *et al.*, 1992) was positive during all growth phases. A thick peptidoglycan layer characteristic of Gram-positive organisms was visible in thin-section electron micrographs (data not shown). The cells did not form visible spores in response to heat stress and pasteurized cultures could not be regrown after heat treatment. Spectral analysis of whole cells (Gorby & Lovley, 1991) did not indicate the presence of *c*-type cytochromes, which are present in several Fe(III)-reducing micro-organisms (Lovley, 2000).

Electron donors and acceptors utilized

Strain 853-15A^T grew at 20, 30 and 37 °C, with optimal growth at 30 °C. It did not grow at 4, 15 or 50 °C. Adding 0·1 % yeast extract to the medium increased the rate of AQDS reduction and all subsequent studies were therefore conducted with media supplemented with yeast extract. AQDS was fully reduced within 24 h at 30 °C with lactate as the electron donor (Fig. 2a). AQDS reduction was accompanied by a loss of lactate, an increase in cell numbers and the accumulation of acetate over time. The loss of about 3 mM lactate was accompanied by the production of about 6 mM AHQDS. This is consistent with the metabolism of lactate according to the reaction $CH_3CHOHCOO^- + H_2O + 2AQDS \rightarrow CH_3COO^- + CO_2 + 2AHQDS$. Growth and lactate consumption stopped once the AQDS was consumed, indicating that lactate was not being fermented.

Strain 853-15A^T also grew with lactate as the electron donor and Fe(III) citrate as the electron acceptor (Fig. 2b). This finding is consistent with previous findings that have suggested that micro-organisms that can grow with AQDS as the electron acceptor can also use Fe(III) as an electron acceptor (Coates *et al.*, 1998; Lovley *et al.*, 1996, 1998).

In addition to lactate, formate also supported growth with either AQDS (Fig. 2c) or Fe(III) citrate (Fig. 2d) as the sole electron acceptor. Approximately 23 mM formate was oxidized coupled to the reduction of about 45 mM Fe(III). This is consistent with the stoichiometry expected for oxidation of formate with Fe(III) serving as the sole electron acceptor according to the reaction $HCOO^- + 2Fe^{3+} \rightarrow 2Fe^{2+} + CO_2 + H^+$. The loss of about 7 mM formate was also coupled to the production of 6 mM AHQDS, which is consistent with the reaction: $HCOO^- + H^+ + AQDS \rightarrow CO_2 +$ AHQDS. To the best of our knowledge, this is the first report of a micro-organism that can oxidize formate with AQDS as the electron acceptor.

Other electron donors utilized in yeast extract-supplemented media with 5 mM AQDS serving as the sole terminal electron acceptor included lactate (20 mM), formate (20 mM), ethanol (20 mM), butanol (10 mM), malate (10 mM) and pyruvate (20 mM). Under the same conditions, the following compounds were not utilized: acetate + H_2 (10 mM + 160 kPa), benzoate (1 mM), butyrate (10 mM), isobutyrate (10 mM), valerate (10 mM), fumarate (20 mM), nicotinate (0·5 mM), caproate (10 mM), benzyl alcohol (0·5 mM), *p*-hydroxybenzaldehyde (0·5 mM), salicylic acid (0·5 mM), peptone (0·1 %), Casamino acids (0·2 %), methanol (20 mM), 2-propanol (10 mM), phenol (0·5 mM), benzene (0·5 mM), glucose (2 mM), fructose (2 mM) and glycerol (20 mM). Strain 853-15A^T did not ferment pyruvate, glucose, lactate or citrate. Yeast extract

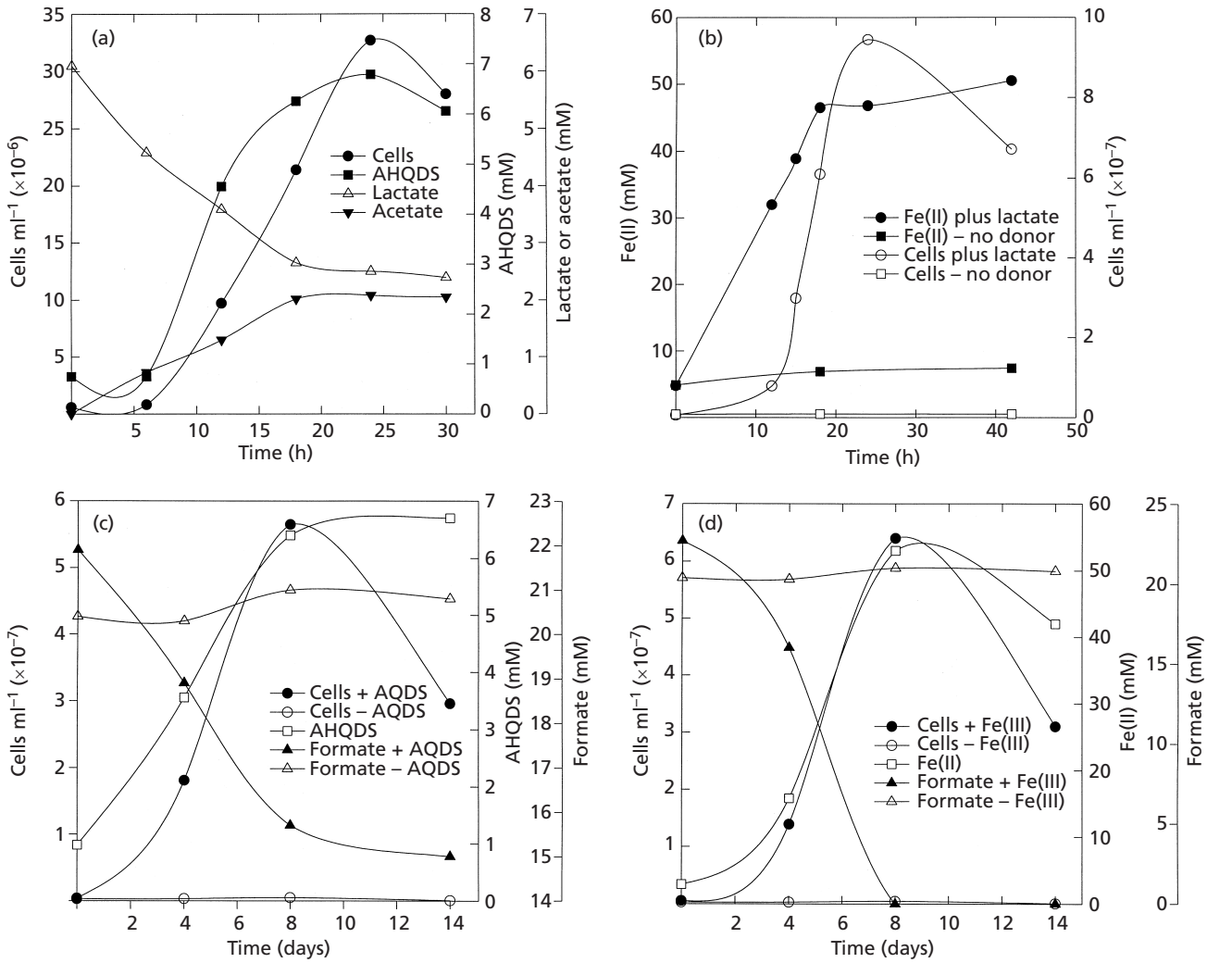

Fig. 2. Growth of strain 853-15A^T. (a) Increase in cell numbers and AHQDS concentration, loss of lactate and production of acetate. (b) Increase in cell numbers and Fe(II) concentration in the presence or absence of lactate. (c) Increase in cell numbers and loss of formate in the presence or absence of AQDS and increase in AHQDS concentration. (d) Increase in cell numbers and loss of formate in the presence or absence of Fe(III) and increase in Fe(II) concentration. Data are means of duplicate analyses.

(0·1 %) alone did not support growth. Strain 853-15A^T did not grow by converting H_2 and CO_2 to acetate.

Several chlorinated compounds were utilized as electron acceptors in yeast extract-supplemented media with either formate or lactate as the sole electron donor. Optical density continued to increase after several repeated transfers in media with 3-chloro-4-hydroxyphenylacetate (5 mM), trichloroethylene (1 mM) and tetrachloroethylene (1 mM).

Other electron acceptors utilized in yeast extract-supplemented media with 20 mM lactate serving as the electron donor included humic substances (0·15 g l^{-1}) (Sigma; and International Humic Substances Society-purified humic acid), Fe(III) nitrilotriacetate (10 mM), Mn(IV) oxide (10 mmol l^{-1}), thiosulfate (20 mM) and colloidal sulfur (1 g l^{-1}). Cr(VI) (5 mM) and selenite (10 mM) were reduced several times during repeated transfer, but there was a lag period of several days with each transfer before any activity could be identified. Toxicity could be a factor with regard to these acceptors. The following electron acceptors were not utilized: poorly crystalline Fe(III) oxide (100 mmol l^{-1}), sulfate (20 mM), sulfite (20 mM), nitrate (20 mM), nitrite (20 mM), oxygen (1 % and atmospheric), selenate (10 mM), cobalt EDTA (0·05 mM) and fumarate (20 mM).

Phylogeny

Analysis of the 16S rDNA sequence of strain 853-15A^T indicated that its closest known relatives are *Desulfitobacterium dehalogenans* (95·5 % similar, 1453 bp considered), *Desulfitobacterium chlororespirans* (95·1 % similar, 1503 bp) and *Desulfosporosinus orientis* (93·8 % similar, 1506 bp). These results suggest that

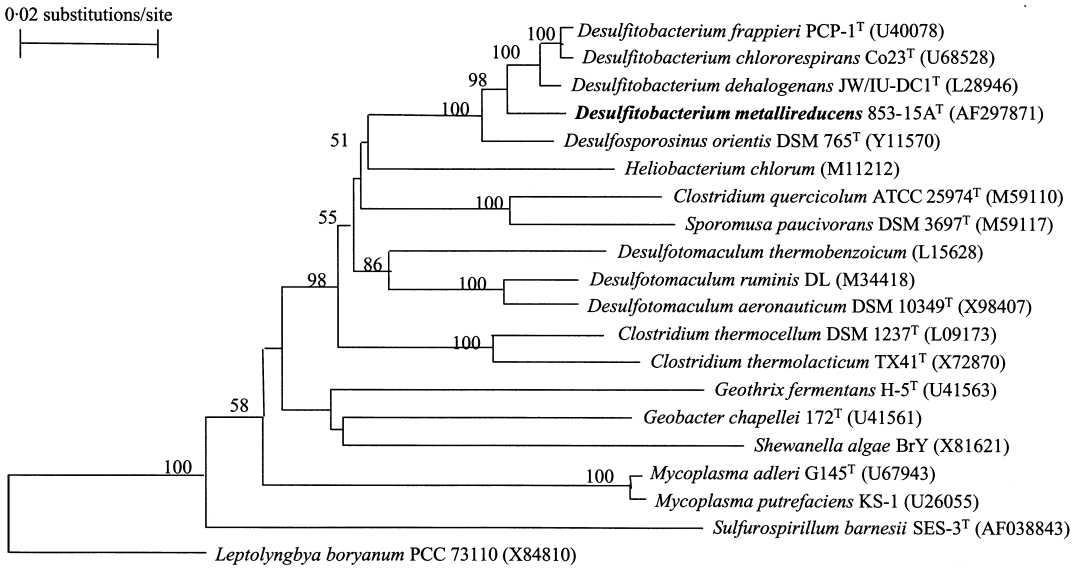

Fig. 3. Phylogenetic tree inferred from neighbour-joining of distances calculated by the Kimura two-parameter model in TREECON for Windows with 1252 bases considered. Bootstrap values at nodes were calculated with 100 replicates. Accession numbers are given in parentheses.

strain 853-15A^T should be placed within the genus *Desulfitobacterium* (Fig. 3). On the basis of the results of the 16S rDNA analyses and the ability of the organism to reduce several metals and humic compounds, as well as its inability to ferment pyruvate or reduce sulfite, we propose that strain 853-15A^T represents a novel species within the genus *Desulfitobacterium*, *Desulfitobacterium metallireducens* sp. nov.

Comparing the morphology and physiology of these and other close relatives within the genus *Desulfitobacterium* further confirms that *D. metallireducens* 853-15A^T represents a novel species within the genus (Table 1). The most significant difference is the lack of pyruvate fermentation and sulfite reduction for *D. metallireducens*. All other species within the genus can ferment pyruvate and all couple growth to sulfite reduction. Several other species reduce nitrate or 3-chloro-4-hydroxyphenylacetate. All of the *Desulfitobacterium* species can reduce thiosulfate. *D. metallireducens* has a lower optimal growth temperature than any of the other species. The morphology of *D. metallireducens* is consistent with that of the other members of the genus.

Potential ecological significance

To our knowledge, this is the first report of a member of the genus *Desulfitobacterium* that can conserve energy to support growth from the reduction of Fe(III) or extracellular quinones. *Desulfitobacterium hafniense* was previously reported to reduce Fe(III) with pyruvate as the electron donor (Christiansen & Ahring, 1996), but this organism also grows fermentatively with pyruvate and it was therefore unclear whether energy was conserved from electron transport to Fe(III). Furthermore, *Desulfitobacterium frappieri* did not reduce 25 mM Fe(III) EDTA (Gerritse *et al.*, 1999), but it should be kept in mind that such high concentrations of Fe(III) EDTA are poor electron acceptors for most known dissimilatory Fe(III)-reducing micro-organisms. Washed cell suspensions of *D. dehalogenans* were previously reported to reduce AQDS with lactate as the electron donor, but it was never shown to conserve energy for growth via AQDS reduction (Lovley *et al.*, 1999). Preliminary studies have demonstrated that the type species of *Desulfitobacterium*, *D. dehalogenans*, can grow with Fe(III) citrate or Fe(III) nitrilotriacetate as the sole electron acceptor (unpublished data). *Desulfitobacterium* species that can reduce Fe(III) may have a competitive advantage in anaerobic subsurface environments in which Fe(III) is often abundant (Lovley, 2000), but which often lack significant quantities of the other electron acceptors such as nitrate, thiosulfate and sulfite, known to be used by *Desulfitobacterium* species. The hallmark respiration of *Desulfitobacterium* species has been their ability to reduce chlorinated compounds (Mackiewicz & Wiegel, 1998) but, even in subsurface environments contaminated with chlorinated compounds, Fe(III) is likely to be more abundant than the chlorinated compounds. Extracellular quinones might also be quantitatively significant electron acceptors for *D. metallireducens* in many environments because, even when the concentration of extracellular quinones is low, they can undergo multiple cycles of reduction and oxidation as they act as electron shuttles to Fe(III) oxides (Lovley, 2000; Lovley *et al.*, 1996). Thus, the ability to reduce Fe(III) and extracellular quinones may be an important attribute that contributes to the

Table 1. Comparison of strain 853-15A^T with the four other *Desulfitobacterium* species

Data for reference strains were taken from Bouchard *et al*. (1996) (*D. frappieri*), Christiansen & Ahring (1996) (*D. hafniense*), Sanford *et al*. (1996) (*D. chlororespirans*) and Utkin *et al*. (1994) (*D. dehalogenans*). ND, Not determined; NT, not tested; 3-Cl-4-OHPA, 3-chloro-4-hydroxyphenylacetate. All strains are positive for utilization of pyruvate as an electron donor and thiosulfate as an electron acceptor and negative for utilization of sulfate as an electron acceptor.

Trait	Strain 853-15A^T	*D. frappieri* PCP-1^T	*D. hafniense* DCB-2^T	*D. chlororespirans* Co23^T	*D. dehalogenans* JW/IU-DC1^T
Gram stain	+	+	−	−	+
Temperature optimum (°C)	30	38	37	37	38
Spores	−	+	+	+	−
Motility	−	−	+	+	+
Cell morphology	Curved rod	Straight rod	Straight rod	Curved rod	Straight or curved rod
Pyruvate fermentation	−	+	+	+	+
Electron donors:					
Lactate	+	−	−	+	+
Acetate + H_2	−	−	ND	+	+
Formate	+	−	−	+	+
Yeast extract	−	−	+	ND	+
Butyrate	+	−	−	+	−
Electron acceptors:					
Fe(III)	+	NT	+*	NT	NT
AQDS	+	NT	NT	NT	NT
Sulfite	−	+	+	+	+
Nitrate	−	+	+	−	+
3-Cl-4-OHPA	+	−	+	+	+

* With pyruvate as an electron donor.

survival of some *Desulfitobacterium* species in the subsurface.

Description of *Desulfitobacterium metallireducens* sp. nov.

Desulfitobacterium metallireducens (me.tal.li.re.du′cens. L. n. *metallum* metal; L. part. adj. *reducens* converting to a different state; N.L. adj. *metallireducens* reducing metal, referring to the ability to couple growth to respiration of several metals).

Cells are Gram-positive, non-motile, slightly curved rods, approximately 0·5 × 2–5 μm. Colonies on purified agar are round, smooth, domed, white and entire. Optimum temperature and pH are respectively 30 °C and 7·0. Obligate anaerobe that respires with Fe(III), Mn(IV), sulfur, thiosulfate, 3-chloro-4-hydroxyphenylacetate, trichloroethylene, tetrachloroethylene, AQDS and humic acids; Cr(VI) and selenite are also reduced. Sulfate, sulfite, nitrate, nitrite, O_2, selenate, cobalt EDTA and fumarate are not reduced. Electron donors for respiration include lactate, formate, ethanol, butanol, butyrate, pyruvate and malate. Does not utilize yeast extract, acetate, H_2, methanol, 2-propanol, benzoate, peptone, Casamino acids, isobutyrate, valerate, benzyl alcohol, salicylic acid, phenol, benzene, glucose, fructose, fumarate, glycerol, nicotinate or caproate.

Isolated from uranium-contaminated, shallow aquifer sediment. The type strain is strain 853-15A^T (= ATCC BAA-636^T).

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