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Genomic insights into a new acidophilic, copper-resistant *Desulfosporosinus* isolate from the oxidized tailings area of an abandoned gold mine

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**One sentence summary:** A novel *Desulfosporosinus* sp. I2 has been isolated from oxidized mining waste in Kuzbass; its genome was sequenced and analyzed.

**Keywords:** acidophilic sulfate-reducing bacteria, metal resistance, polyphosphates, *Desulfosporosinus*
ABSTRACT

Microbial sulfate reduction in acid mine drainage is still considered to be confined to anoxic conditions, although several reports have shown that sulfate-reducing bacteria (SRB) occur under microaerophilic or aerobic conditions. We have measured sulfate reduction rates of up to 60 nmol S cm⁻³ day⁻¹ in oxidized layers of gold mine tailings in Kuzbass (SW Siberia). A novel, acidophilic, copper-tolerant *Desulfosporosinus* sp. I2 was isolated from the same sample and its genome was sequenced. The genomic analysis and physiological data indicate the involvement of transporters and additional mechanisms to tolerate metals, such as sequestration by polyphosphates. *Desulfosporinus* sp. I2 encodes systems for a metabolically versatile life style. The genome possessed a complete Embden-Meyerhof pathway for glycolysis and gluconeogenesis. Complete oxidation of organic substrates could be enabled by the complete TCA cycle. Genomic analysis found all major components of the electron transfer chain necessary for energy generation via oxidative phosphorylation. Autotrophic CO₂ fixation could be performed through the Wood-Ljungdahl pathway. Multiple oxygen detoxification systems were identified in the genome. Taking into account the metabolic activity and genomic analysis, the traits of the novel isolate broaden our understanding of active sulfate reduction and associated metabolism beyond strictly anaerobic niches.

INTRODUCTION

Sulfate-reducing bacteria (SRB) play a vital role in metal detoxification in natural and engineered environments. Hydrogen sulfide, produced by SRB in the course of sulfate respiration, precipitates metals as highly insoluble sulfides. This bioprocess and precipitation can be used in bioreactors to treat metal-contaminated waste streams. Facilitation of microbial sulfate reduction in natural and constructed wetlands is a less costly technique to treat metal-containing effluents as compared to bioreactor processes and chemical and physical abatement. The approach can be applied to treat acid mine drainage (AMD), acidic metal-loaded water produced during coal and metal mining. Major obstacles for microbial treatment are the low pH and high concentration of potentially toxic metals in AMD (Dopson and Johnson 2012, Ñancuchoe and Johnson 2014).

SRB have been traditionally regarded as strictly anaerobic organisms. Although our understanding of the aerotolerance of this guild of prokaryotes has changed due to the recent findings in SRB ecology, physiology and biochemistry (reviewed in Cypionka, 2000; Ramel et al. 2015), various reports on AMD treatment still consider that sulfate reduction is mostly associated with anaerobic conditions (Meier, Piva, Fortin 2012; Sánchez-Andrea et al. 2012,
The oxidation of dissolved Fe$^{2+}$ to Fe$^{3+}$ is a key biogeochemical process in the metal sulfide-containing wastes. Ferric iron is a more effective chemical oxidant than oxygen. Highly oxidized upper layers of sulfide tailings are rarely regarded as SRB habitats. However, sulfate reduction based on experiments with $^{35}$SO$_4^{2-}$ has been demonstrated in oxic sediments of AMD-impacted wetlands in the Norilsk area, northern Siberia (Karnachuk et al. 2005).

To date, few pure cultures of acidophilic sulfate-reducers have been isolated. Only two acidophilic SRB, Desulfosporosinus acidiphilus and Desulfosporosinus acididurans, have been validly described (Alazard et al. 2010; Sánchez-Andrea et al. 2015). Deeply-branched on the phylogenetic tree, thermophilic Thermodesulfobium narugense isolated from a hot spring had a slightly acidic pH optimum between 5.5 and 6.0 for growth (Mori et al. 2003). Several Desulfosporosinus isolates have been shown to tolerate low pH values (Abicht et al. 2011; Karnachuk et al. 2009, 2015a,b).

Acidophilic D. acididurans was originally isolated from acidic sediments of Rio Tinto and tolerated relatively high concentrations of aluminum and ferrous iron, but was inhibited by 1 mM copper (Sánchez-Andrea et al. 2015). The tolerance to metal ions has not been reported for D. acidiphilus, isolated from AMD-impacted sediments in France (Alazard et al. 2010), or for T. narugense (Mori et al. 2003). Acidophilic Desulfosporosinus sp. OT, resistant up to 236 mM copper, was isolated from a gold mine site in Kuzbass, southwestern Siberia and its draft genome has been published (Abicht et al. 2011). The draft genome of the only deltaproteobacterial acid-tolerant Desulfovibrio sp. TomC has also been published (Karnachuk et al. 2015c). Our recent findings showed that strain TomC is sensitive to Cu$^{2+}$ at concentrations higher than 1.5 mM (O.V. Karnachuk et al., unpublished). A neutrophilic Desulfovibrio sp. A2 could tolerate up to 12.6 mM Cu$^{2+}$ (Mancini et al. 2011). At present, four complete and three draft Desulfosporosinus genomes have been announced (Abicht et al. 2011; Pester et al. 2012; Abu Laban et al. 2015; Petzsch et al. 2015a), but the metabolism and other traits have not been analyzed.

The purpose of this study was to measure sulfate reduction in oxic layers of a tailings site of an abandoned gold mine and to characterize sulfate reducers at this site. A copper-tolerant, acidophilic SRB was isolated from the same sample that was used for the in situ sulfate reduction rate measurements and its genome was sequenced. Genome data are analyzed to provide insights into the metabolism of the isolate and outline putative mechanisms of its resistance to heavy metals, low pH, and oxidative stress.
MATERIALS AND METHODS

Site description

The Kuznetsk mining area (commonly known as the “Kuzbass”) is located in southwestern Siberia, Russia, and is one of the world’s main sources of coal. Iron and non-ferrous metal sulfides are also mined in the region. Gold mining in the Mariinskaya Taiga (“Martaiga”) area of the Kuzbass dates back to the early part of the 19th century. Geologically, the region comprises a bedrock terrain of the Kuznetsk Alatau mountain belt (Figure 1; Banks et al. 2008). The mining area is associated with quartz-sulfide ore deposits with a high content of arsenopyrite.

Underground mines at the Berikul site were reportedly worked down to 500 m deep in a polymetallic sulfide-quartz-pyrite/arsenopyrite-gold deposit, but were mined only for gold. Ore material from the mines were transported to the processing works on the west bank of the Mokrii Berikul River. The processing works included milling, flotation, and cyanidation. The waste materials (tailings from all the stages) were deposited on the river bank and separated by a dam. The tailings have been present since 1952 and comprise 40-45% fine-grained sulfide (dominated by pyrite, with minor arsenopyrite and smaller amounts of pyrrhotite, sphalerite, chalcopyrite, galena, quartz, albite, chloride, muscovite, and dolomite) as reported by Sidenko et al. (2005). The mine was closed in 1994, and parts of the mine waste materials and tailings were removed from the site in the late 1990’s – early 2000’s and covered by a layer of coarse-grained mine waste rock. The remaining materials in the tailings still undergo oxidation, as evidenced by many orange-yellow pools of accumulated leachate. Highly acidic, metal-laden leachate from the tailings has been a matter of environmental concern over the years (Bortnikova et al. 2001; Gieré, Sidenko, Lazareva 2003; Sidenko et al. 2005).

Sampling, field measurements, water and sediment analyses

The samples were collected on August 26, 2007 from two different pools in the tailings area (Table 1). The sampling site Xa201 was at the foot of a mine waste pile, while Xa202 was located on the flood plain of the Mokrii Berikul River. The upper layer of the sediment was grab sampled, while deeper layers were sampled from sediment cores retrieved with a Plexiglas tube. Redox potentials were measured on-site by inserting the electrodes (HANNA HI8314F) into the water-saturated sediments.

Water for chemical analysis was filtered into 50 ml polyethylene flasks, using a Millipore filter (0.45 μm pore size) and a hand-held polypropylene syringe. No acidification was carried
out in the field. For the pH and other field measurements, electrodes were submerged to the maximum extent below to water surface (typically 5-10 cm). Two samples of filtered water from each site were transported to the analytical laboratory of the Hydrochemical Engineering Research and Outreach (HERO) Group at the University of Newcastle for chemical analysis. Prior to analysis, samples were stored at 4 °C except for brief periods of transportation. Anion concentrations were determined by ion chromatography (Dionex DX320 for Gradient Anion Analysis) from split samples from both sites. Other split samples were acidified with concentrated ultrapure HNO$_3$ in order to re-dissolve any precipitated or sorbed metals and analyzed using inductively coupled plasma-atomic emission spectrometry (Varian Vista MPX with a simultaneous charged coupled device).

The mineralogical composition of the upper layers of Xa201 and Xa202 sediments was characterized by X-ray diffraction (XRD) using a Shimadzu XRD-6000 diffractometer as previously described (Ikkert et al. 2013). For scanning electron microscopy (SEM), air dried sediments were examined under a Philips SEM 515 scanning electron microscope. Energy dispersive spectrometry (EDS; EDAX Inc., Mahwan, NJ) was performed at a voltage of 30 kV and a working distance of 12 mm. For transmission electron microscopy (TEM) ultrathin sections of the cells were prepared as previously described (Ikkert et al., 2013). Sections were viewed under a JEM-100 CXII electron microscope (JEOL, Tokyo, Japan) at 80 kV.

**Sulfate reduction rate measurements**

For the measurement of sulfate reduction rates, aliquots of sediments were withdrawn using sterile plastic syringes, with the tips clipped off, from the middle of the cores or grab samples. Samples in syringes were tightly end-sealed with butyl rubber stoppers. Aliquots (100 μl) of Na$_2^{35}$SO$_4$ (15 μCi, Isotop, Obninsk, Russia) in de-gassed sterile 0.002% resazurin solution were injected into syringes vertically along the center of the core through the butyl rubber stoppers as described earlier (Karnachuk et al. 2006). All sulfate reduction rate measurements were carried out in triplicates. The samples were incubated in the dark at in situ temperature for 24 h. 1 ml of 2 M KOH was used to terminate the incubation and to fix sulfide. The total amount of reduced $^{35}$S was measured as the sum of the acid volatile fraction and chromium-reducible sulfur as described previously (Karnachuk et al. 2005, 2006).

**SRB enrichment and pure culture isolation**
Enrichment cultures were initiated with inoculations from the Xa201 surface layer sediments within 3-4 days after the sampling. Samples were stored at 4 °C prior to inoculation. Widdel and Bak medium (Widdel and Bak 1992) modified for copper-tolerant SRB isolation (Karnachuk et al. 2008) was used to set up initial enrichments with glycerol as an electron donor. The pH was adjusted to 2.0 with 1 M H$_2$SO$_4$ solution. No special buffer solutions were used to maintain an acidic pH. The enrichment cultures were incubated at 28 °C. The final pH values of the enrichments were between 4 and 5. A pure culture was isolated by repeated serial dilutions with glycerol as an electron donor following heat treatment at 95 °C for 20 min.

For substrate utilization tests the isolate was grown in sealed, headspace-free test tubes. Electron donors were tested at final concentrations of 25 mM ethanol, 18 mM lactate, 13.5 mM butanol, 8 mM nicotinic acid, 11 mM glycerol, 5 mM citrate, 4.5 mM succinate, 3 mM sucrose, 1 mM palmitate, 9 mM each of acetate and fumarate, 7.5 mM each of formate and malate, 7 mM each of pyruvate and butyrate, 5 mM each of fructose and glucose. Peptone (1%) was also used as an electron donor. Carbohydrate stock solutions were filter-sterilized. The cultures were subcultured at least five times to confirm that they utilized the test compounds.

DNA isolation, genome sequencing, assembly, and annotation

For DNA isolation the isolate was grown at 28 °C in freshwater Widdel and Bak medium modified with glycerol and sulfate for copper-resistant SRB (Karnachuk et al. 2008). Cells were collected by centrifugation, resuspended in TE buffer (10 mM Tris; 1 mM EDTA, pH 8.0) and lysed with SDS and Proteinase K. DNA was purified by CTAB and phenol:chloroform:isoamyl alcohol extractions and precipitated with ethanol.

Genomic DNA was sequenced with a Roche Genome Sequencer (GS FLX) using the XL+ protocol. About 186 MB of a shotgun library sequences were produced with an average read length of 704 bp. The GS FLX reads were assembled using Newbler Assembler version 2.9 (454 Life Sciences, Branford, CT). The draft genome of Desulfosporosinus sp. I2 consists of 318 contigs longer than 500 bp, with a total length of 5,311,733 bp.

Coding genes were annotated using RAST server (Brettin et al. 2015) and manually curated. The tRNAscanSE tool (Lowe and Eddy, 1997) was used to find tRNA genes, whereas ribosomal RNA genes were found by RNAMMER (Lagesen et al. 2007). The annotated genome sequence of Desulfosporosinus sp. I2 has been deposited in the GenBank database under accession no. NZ_JYNH00000000.

RESULTS
Water and sediment analyses and sulfate reduction rates

Both tailings leachates were highly acidic, pH 2.5 at the Xa201 site and pH 3.6 at the Xa202 site (Table S1). Sulfate was the major anion with the concentration of 5,290 mg SO$_4^-$ l$^{-1}$ in Xa201 and 7,790 mg SO$_4^-$ l$^{-1}$ in Xa202 (Table S2). The water samples were also rich in dissolved iron, with concentrations of up to 2,290 mg Fe l$^{-1}$ in Xa202. The samples also contained high concentrations of other dissolved metals; e.g., 242 mg l$^{-1}$ Al (Xa202), 162 mg l$^{-1}$ Zn (Xa201), 12.5 mg l$^{-1}$ Cu (Xa201), 5 mg l$^{-1}$ Ni (Xa202), and 2.8 mg l$^{-1}$ Cd (Xa201). The concentration of arsenic was 178 mg l$^{-1}$ in the Xa201 leachate and 6.5 mg l$^{-1}$ in Xa202.

Muscovite, albite, and quartz were the main Si-minerals derived from the gangue material in Xa201 and Xa202 sediments (Figure S1). Crystalline Fe-sulfides or other sulfide minerals were not detected in either sample. Arsenic sulfides, including orpiment (As$_2$S) and realgar (AsS) were present as minor phases in Xa202 sediment. Various forms of secondary sulfates, including a jarosite-type phase and melanterite, were also detected in the sediment samples.

SEM-EDS analysis showed that two major types of fine-sized grains occurred in Xa201 and Xa202 sediments. One type of grain was smooth-surfaced (Figure S2A) and exhibited two major EDS peaks of Si and O, suggesting that this type represents quartz. The other type of the sediment grain was intensively weathered and covered by filaments and spheres (Figure S2B, C, D, E, and F). The EDS spectra showed enrichment in C, which implies that the filaments may be mineralized microbial cells.

The intense orange-yellow color and positive Eh values measured in the top and subsurface layers (Table 1) were a clear indication of an oxic in the top layers of the pool sediments. The highest sulfate reduction rate, 60.0 nmol S cm$^{-3}$ day$^{-1}$, was detected in the subsurface layer of Xa202, whereas the lowest value 1.69 nmol S cm$^{-3}$ day$^{-1}$ was measured in the top layer of this pool (Table 1). The sulfate reduction rate observed in Xa201 sediment was the same order of magnitude and showed little variation with depth, reaching 17.6 nmol S cm$^{-3}$ day$^{-1}$ in the upper layer and 8.72 nmol S cm$^{-3}$ day$^{-1}$ in the lower 0.5-3 cm horizon.

**SRB pure culture isolation and characterization**

The initial SRB enrichment was grown in the presence of 200 mg Cu l$^{-1}$. The strain, designated I2, was obtained by repeated serial dilutions with glycerol as an electron donor following heat treatment at 95$^\circ$ C for 20 min.
Strain I2 is slightly curved motile rods, 3-6 μm long and 0.5-1.0 μm wide (Figure 2). Cells stained Gram-negative, although the absence of a discreet outer membrane indicates a Gram-positive wall structure. Paraterminal oval spores slightly swelling the cell are often present. Electron micrographs of ultrathin sections revealed a thin peptidoglycan layer and a lighter outer, presumably an S-layer (Figure 2a). The cytoplasmic membrane has deep invaginations (Figure 2b). The extensive inner membrane excrescence occurred at the cell poles and appeared as rings on the cross-sections (Figure 2a). Large (up to 0.5 μm in diameter) electron-transparent vacuole-like globules were present in cells grown in the presence of 25 mg Cu l⁻¹ (Figure 2c,d). The globules may be surrounded with a membrane (Figure 2d). When cells were grown in the presence of 25 and 150 mg Cu l⁻¹ the globules engulfed a network of electron-dense fibrils (Figure 2e,f). The numerous observations of the fibrils in cells growing at elevated copper concentrations suggest that it was not an artifact of staining (Figure S3). The fine electron-dense fibrils also surrounded the cell wall (Figure 2f,g,h). The presence of polyphosphate in the cells was revealed both by methylene blue and Neisser staining (data not shown).

Strain I2 is psychrotolerant, growing at the temperature range 4-32 °C with the optimum at 22-28 °C. The strain did not grow under microaerophilic conditions, when cells were cultivated in stoppered vials containing 6 ml liquid medium and 6 ml ambient air in the headspace. Strain I2 could use lactate, pyruvate, malate, citrate, succinate, fumarate, butyrate, ethanol, glycerol, butanol, formate, palmitate and peptone as electron donors for sulfate reduction. The strain did not grow with fructose, glucose, sucrose, acetate or nicotinic acid in sulfate medium. Pyruvate and lactate supported fermentative growth without sulfate. Fumarate and nitrate in addition to sulfate could be used as electron acceptor. The strain did not grow with nitrite as an electron acceptor.

Strain I2 could grow at initial Cu²⁺ concentration of up to 142 mM and the pH range from 1.7 to 7.0. The pH of the medium increased during the growth and the copper concentration decreased due to CuS precipitation. The medium with initial pH 7.3 and higher did not support growth. The maximum cell numbers and the shortest lag-phases were observed at initial pH 2.6. The tolerance to other divalent metals was much lower. The initial concentration of metals allowing growth did not exceed 2.7 mM for Cd²⁺, 8.5 mM for Ni²⁺, and 8.5 mM for Co²⁺. Arsenate inhibited growth at concentrations higher than 0.2 mM (15 mg l⁻¹).

Phylogenetic analysis of the 16S rRNA gene of strain I2 places it within the phylum Firmicutes, class Peptococcaceae, genus Desulfosporosinus (Figure 3). Desulfosporosinus burensis was the closest relative with the sequence identity of 98%.

**Genome properties**
Pyrosequencing and assembly of the draft genome of *Desulfosporosinus* I2 yielded 318 contigs longer than 500 bp with N50 contig size of 30,483 bp. The size of the genome, estimated as a total length of all contigs, is about 5.3 Mbp, and the GC content of the genome is 42.04%. Five copies of the 16S-23S-5S rRNA operon and 43 tRNA genes coding for all 20 amino acids were identified. Annotation of the genome sequence revealed 5,512 potential protein-coding genes of which 3,987 (72%) can be functionally assigned. Analysis of the conserved single-copy marker genes using CheckM (Parks *et al*. 2015) estimated the completeness of this draft genome as 98%. The size of the *Desulfosporosinus* I2 genome is comparable to that of complete genomes of other *Desulfosporosinus* spp. (4.9 to 5.9 Mbp, Pester *et al*. 2012).

**Resistance to heavy metals and arsenate**

All three mechanisms of resistance to the metal stress (Nies 2003), P-type ATPases, RND-transporters, and cation diffusion facilitators (CDF) were revealed in the strain I2 genome (Table 2). Two operons comprise copper/silver ATPases CopA (UF75_1662, UF75_4258), transcriptional regulators CsoR (UF75_1661, UF75_4257), and one of the operons also contained a copper chaperon CopZ (UF75_1663). The genome contains two RND (resistance-nodulation-cell division) family efflux transporter genes (UF75_0596, UF75_1490), linked to MFP (membrane fusion protein) genes (UF75_0597, UF75_1491). In addition to the family of P-type ATPases transporting Cu and Ag ions, strain I2 possesses the second family of heavy metal P-type ATPases, transporting Zn\(^{2+}\), Cd\(^{2+}\), and Pb\(^{2+}\) (UF75_0864, UF75_1937, UF75_3100, UF75_4472). It is noteworthy that other *Desulfosporosinus* sequenced genomes possess homologous metal transporters. No duplication or extended number of metal transporters were revealed in the I2 genome.

Polypolysphates (polyP) may play role in copper detoxification in strain I2. PolyP, the linear polymers of orthophosphate, have been associated with the capacity of microorganisms to tolerate various types of stresses and to chelate metals (Seufferheld, Alvarez, Farias 2008). The polyP association with vacuole-like bodies in strain I2 resembles bacterial acidocalcisomes, polyP storage organelles that were first described in eukaryotes but subsequently confirmed for various bacteria (Seufferheld *et al*. 2003). TEM micrographs of ultrathin sections of strain I2 showed that inclusions may be surrounded by a membrane. Although there were no vacuolar pyrophosphatase (H\(^+\)-V-PPase) homologs, a proton pump characteristic for both eukaryotic and bacterial acidocalcisomes was found in the genome. The key enzyme involved in bacterial polyP synthesis is polyphosphate kinase (PPK). The *ppk* deficient mutants cannot tolerate acid stress.
Polyphosphate kinase 1 (PPK1) was not found in the I2 genome, although it contained a putative polyphosphate kinase 2 (PPK2) (UF75_4879). The latter protein had no homology in the available *Desulfosporosinus* genomes. Phylogenetic analysis shows that PPK2 from strain I2 was rather distant from all the known PPK2 and its closest relative with 30% similarity occurred in *Aminomonas paucivorans* from *Synergistetes* (Figure 4).

PolyP can be associated with the cell wall. TEM micrographs of I2 showed that electron-dense fibrils may be associated with the cell wall. The genes for the proteins responsible for synthesis of gamma-poly-glutamate capsule were found in the genome. Although the UF75_0096 is annotated as D-Ala-D-Ala dipeptidase, it contains two domains. One of them is CapA, one of three membrane-associated enzymes in *Bacillus anthracis* that is required for synthesis of gamma-polyglutamic acid (PGA), a major component of the bacterial capsule. The second is D-alanyl-D-alanine dipeptidase domain.

The sophisticated cytoplasmic organization of strain I2 observed under TEM is in agreement with the occurrence in the genome actin-like proteins, Arp, that are considered as putative early PPKs (Whitehead *et al.* 2014), including MreB (UF75_4446), Mbl (UF75_2920), and ParM (UF75_0719). The proteins involved in micro-compartment in the bacterial cell, including ethanolamine utilization protein (EutL) (UF75_4231) also known as bacterial micro-compartment (BMC) domain repeat 2, was revealed in the genome.

Despite the relatively low tolerance to arsenic, strain I2 possesses the TRX-fold arsenate reductase ArsC that catalyzes the reduction of arsenate to arsenite (UF75_2367) and arsenite efflux pump, consisting of the catalytic ArsA subunit (UF75_3206) and the membrane subunit ArsB (UF75_3204). Nearly all sequenced *Desulfosporosinus* isolates have ArsC, but the entire efflux pump operon has been found only in strain I2 and *D. youngiae*.

**Oxidative stress**

In its natural environment *Desulfosporosinus* sp. I2 may be frequently exposed to dissolved oxygen and thus should possess oxygen detoxification systems. We have identified superoxide dismutase (UF75_4309) and catalase (UF75_5085) genes in the I2 genome. These are typically found in aerobic bacteria, whereas anaerobes usually only employ superoxide reductase. The homologous catalase occurred in all six draft genomes of *Desulfosporosinus* species available in GenBank. In contrast, the superoxide dismutase homologs (UF75_4309) were found only in *D. meridei*, *D. youngiae*, and two uncharacterized strains, *Desulfosporosinus* sp. BICA1-9 and *Desulfosporosinus* sp. HMP52. However, many *Firmicutes* possess the orthologous genes.
Terminal oxygen reductases in *Desulfosporosinus* sp. I2 are represented by a *cbb3*-type cytochrome *c* oxidase and a quinol oxidase *bd* complex. The first complex comprises subunits I (UF75_0563), II (UF75_0564), and III (UF75_0565). Subunit I contains multiple transmembrane helices. The other two subunits were predicted to carry N-terminal signal peptide, indicating its export from the cytoplasm and linking to the cell membrane. Interestingly, the *cbb3*-type cytochrome *c* oxidase does not occur in any other *Desulfosporosinus* except strain I2 and shares low similarity with its closest relatives in *Deltaproteobacteria* (Figure 5). This cytochrome *c* oxidase belongs to the heme-copper transmembrane protein complexes. The *cbb3* is believed to be a modern enzyme that has evolved independently to perform a specialized function in micro-aerobic energy metabolism. The discovery a copper-containing protein in *Desulfosporosinus* spp., which until recently have been considered to be strict anaerobes, is noteworthy. The recent comparative genomic studies suggest that the use of copper by prokaryotes is strongly linked to the use of oxygen (Ridge, Zhang, Gladyshev 2008; Karnachuk *et al*. 2015a) and the evolution of copper-containing proteins followed the oxygenation of the Earth (Ochiai 1983; Saito, Sigman, Morel 2003).

The second complex, the cytochrome *bd*-type quinol oxidase, consist of two subunits (UF75_2664 and UF75_2665) also carrying transmembrane helices. The *cbb3*-type and *bd*-type oxidases differ in their affinity to oxygen (e.g., Ekici *et al*. 2012) and thus together could protect cells from oxidative stress in fluctuating environment. Moreover, both cytochrome oxidases could reduce oxygen with electrons from the quinone pool and thus they may allow respiration of *Desulfosporosinus* sp. I2 under microaerophilic conditions. Our preliminary experiments showed that strain I2 did not grow under micro-aerobic conditions, when cells were cultivated in 12 ml stoppered vials containing liquid medium and 6 ml ambient air in the headspace. Further experiments are underway to examine the growth of the strain I2 in Hungate tubes with constant sparging of 0.02% oxygen and N\(_2\) (P. Bukthyarova and G. Brasseur, unpublished).

**Low pH tolerance**

Several mechanisms that allow microorganisms to withstand high extracellular proton concentrations have been reported (Baker-Austin and Dopson 2007). Interestingly, one of the most important systems participating in the generation of an internal positive membrane potential (\(\Delta\psi\)), the K\(^+\)-transporting Kdp ATPase, was not found in strain I2. Other three acidophilic sulfate-reducers, *D. acidiphilus*, *D. acididurans*, and *Desulfosporosinus* sp. OT possess these genes. It is conceivable that the KdpABC proteins were lost for some reasons, because the regulation of the two-component histidine kinase KdpD (UF75_0351) occurred in I2 and was associated with two other K\(^+\)-transporting proteins, KtrB (UF75_0349) and TrkA.
Potassium-transport ATPases have been shown to be overrepresented in metagenomes from acidic environments (Jones et al. 2012). Additionally, the bacterium contained an acid resistance system exploiting proton-consuming decarboxylases, similar to those previously demonstrated in enterobacteria (Richard and Foster 2004; Fontenot et al. 2013), including arginine decarboxylase AdiA (UF75_RS17850), and lysine decarboxylase CadA (UF75_2412).

**Carbon and nitrogen metabolism**

Analysis of the genome *Desulfosporosinus* sp. I2 revealed genes encoding the complete Embden-Meyerhof pathway for glycolysis and gluconeogenesis: hexokinase (UF75_5178, UF75_0319), glucose-6-phosphate isomerase (UF75_5005), fructose-6-phosphate kinase (UF75_0114, UF75_3429, UF75_5006), fructose-1,6-bisphosphatase (UF75_2575), fructose bisphosphate aldolase (UF75_2577, UF75_3428), triose phosphate isomerase (UF75_3706), glyceraldehyde-3-phosphate dehydrogenase (UF75_3878, UF75_3437), phosphoenol pyruvate synthase (UF75_4789, UF75_1445, UF75_4126), 3-phosphoglycerate kinase (UF75_3436, UF75_3705), phosphoglycerate mutase (UF75_3707, UF75_3948), enolase (UF75_3708), and pyruvate kinase (UF75_3877). The inability of *Desulfosporosinus* sp. I2 to grow with glucose in sulfate medium suggests that the upper part of the Embden-Meyerhof pathway operates in the reverse direction of gluconeogenesis. The growth on glycerol is enabled by glycerol kinase (UF75_3704), the multi-subunit glycerol-3-phosphate dehydrogenase (UF75_3701-3703) and downstream enzymes.

Complete oxidation of organic substrates could be enabled by the TCA cycle, including citrate synthase (UF75_0595), aconitate hydratase (UF75_2731, UF75_3044), isocitrate dehydrogenase (UF75_3046, UF75_4848), 2-oxoglutarate oxidoreductase (UF75_0980-UF75_0983), succinyl CoA synthetase (UF75_1775, UF75_1773; UF75_2378, UF75_2379; UF75_2969, UF75_2970; UF75_3084, UF75_3085), succinate dehydrogenase (UF75_3086-UF75_3089; UF75_3017-UF75_3019), fumarate hydratase (UF75_4927-4928), and malate dehydrogenase (UF75_1777, UF75_3426).

Autotrophic CO₂ fixation could be performed through the Wood-Ljungdahl pathway (Ragsdale and Pierce 2008) including carbon monoxide dehydrogenase (UF75_0081, UF75_0341, UF75_1285, UF75_1521, UF75_1616), formate dehydrogenase (UF75_0475 (FdhD), UF75_4574 (FdhN), UF75_4575 (FdhL), UF75_4576 (FdhL)), formate-tetrahydrofolate ligase (UF75_1688), methylenetetrahydrofolate dehydrogenase (NADP+) / methenyltetrahydrofolate cyclohydrolase (UF75_0999), 5,10-methylenetetrahydrofolate
reductase (UF75_1551, UF75_3484), 5-methyltetrahydrofolate:corrinoid iron-sulfur protein methyltransferase (UF75_1608), CODH/ACS (UF75_1609-UF75_1617) and acetate kinase (UF75_0903). However, growth of *Desulfosporosinus* sp. I2 with acetate and sulfate was not observed.

The genome of *Desulfosporosinus* sp. I2 contains all genes necessary for nitrogen fixation (Gussin, Ronson, Ausubel 1986). They are closely linked in the genome (UF75_0214-UF75_0223). To our knowledge, the capability to fix nitrogen has never been shown for *Desulfosporosinus* although homologous genes occur in available *Desulfosporosinus* genomes. Nitrogen fixation was also revealed in the genome of a novel sulfate-reducing acidophilic *Firmicute* CEB3 (Petzsch *et al.* 2015b).

**Respiratory electron transfer chains**

All major components of an electron transfer chain able to generate transmembrane proton gradient are encoded in the *Desulfosporosinus* sp. I2 genome. The genome analysis revealed a putative membrane-bound complex similar to bacterial NADH:quinone oxidoreductase, respiratory complex I (Friedrich and Scheide 2000). There are two cluster of genes encoding NuoABCDHIJ subunits (UF_0875-UF_0869 and UF_4868-UF_4874), genes encoding NuoEFG (UF_1417-UF_1415), NuoKLM (UF75_5303-UF75_5305), and NuoN (UF75_5036, UF75_5172). The succinate dehydrogenase complex is encoded by a three gene operon and consists of an iron-sulfur subunit (UF_3017), a flavoprotein subunit (UF_3018) and a transmembrane cytochrome b subunit (UF_3019). The transmembrane ion gradient generated by electron transfer chains may be used for ATP synthesis by an F$_0$F$_1$-type ATPase, encoded by genes UF_2550-UF_2559.

Consistent with the ability of *Desulfosporosinus* sp. I2 to use sulfate for respiration, its genome contains the complete set of genes for dissimilatory sulfate reduction (Rabus, Hansen, Widdel 2007), including sulfate permease (UF75_2393, UF75_3177), sulfate adenylyltransferase (UF75_3182), Mn-dependent inorganic pyrophosphatase, two clusters of adenylsulfate reductase AprAB (UF75_0198/UF75_0199 and UF75_3180/UF75_3181), and dissimilatory sulfite reductase (DsrA – UF75_3747, DsrB – UF75_3746, DsrC – UF75_3007). A two-gene operon encoding the subunits QmoA (UF75_3185) and QmoB (UF75_3184) of the adenylsulfate reductase-associated electron transfer complex QmoABC is located near the sulfate adenylyltransferase gene. However, the third gene, *qmoC* (encoding a quinone-interacting membrane-bound oxidoreductase), is absent, as also reported for the *qmo* operon in *Desulfotomaculum reducens* (Junier *et al.* 2010). Genes coding for sulfite reductase-associated electron transport proteins DsrMKJOP (DsrP – UF75_2291, UF75_3008, UF75_3985; DsrO –
UF75_2292, UF75_3009, UF75_3175, UF75_3986; DsrJ – UF75_2279, UF75_3010; DsrK – UF75_3011, UF75_3750, UF75_0530; DsrM – UF75_3012, UF75_3751) are also present in the genome.

At least seven gene clusters coding for putative Complex Iron-Sulfur Molybdopterine (CISM) oxidoreductases of Psr/Psh family (reviewed by Rothery, Workun, Weiner, 2008) are present in the genome. Such oxidoreductases usually comprise molybdopterin-binding catalytic subunit A, iron-sulfur electron transfer subunit B, and the third subunit (C) linking this complex to the membrane and accepting the electrons from the quinone pool. The catalytic subunits (UF75_0346, UF75_0612, UF75_1326, UF75_2828, UF75_2884, UF75_3215, UF75_4739) are similar to the respective components of nitrate, thiosulfate, tetrathionate, sulfur, dimethyl sulfoxide and polysulfide reductases. Presumably, in Desulfosporosinus sp. I2 the CISM oxidoreductases can reduce some of these and/or other yet unknown electron acceptor(s), suggesting respiratory versatility of this bacterium.

In addition, the genome of Desulfosporosinus sp. I2 encodes NrfAH-type nitrite reductase, comprising electron transfer subunit NrfH (UF75_0062) and a catalytic subunit NrfA (UF75_0063). Both subunits are c-type cytochromes with four and five haem-binding motifs, respectively. However, the growth experiments showed that Desulfosporosinus sp. I2 did not grow with nitrite as an electron acceptor.

**Hydrogenase and formate dehydrogenase genes**

Five hydrogenases of the [NiFe]-family and two [FeFe] family enzyme are present in the Desulfosporosinus sp. I2 genome along with two formate dehydrogenases. Although the ability of this strain to grow autotrophically on hydrogen and sulfate was not investigated, growth on formate and sulfate was observed.

Two [FeFe] hydrogenases are encoded by gene clusters UF_1944-UF_1946 and UF_3768-UF_3770. The catalytic subunits UF_1944/UF_3770 contain the H-cluster domain (Vignais and Billoud 2007), and an additional N-terminal domain, which accommodates 2Fe-2S clusters. Two additional subunits, UF_1945/UF_3769 and UF_1946/UF_3768, are similar to the NuoF and NuoE subunits of NADH:quinone oxidoreductase, respectively. Their presence suggests that hydrogen reduction could be coupled to the oxidation of NAD(P)H. Another locus, UF_0436-UF_0439 contains similar NuoF and NuoE-like subunits associated with gene UF_0439 that encodes the catalytic alpha subunit of formate dehydrogenase. All these proteins lack N-terminal signal peptides or transmembrane domains, indicating their cytoplasmic localization. Subunit composition of the [FeFe] hydrogenases of Desulfosporosinus sp. I2 is
similar to that of the bifurcating hydrogenase from *Thermotoga maritima*, which simultaneously oxidizes NADH and ferredoxin to produce H₂ (Schut and Adams 2009). Similar bifurcating [FeFe]-hydrogenases and formate dehydrogenases have been identified in different sulfate-reducing Firmicutes, including *Desulfotomaculum kuznetsovii* and *P. thermopropionicum* (Visser et al. 2013; Müller et al. 2010). Such hydrogenases and formate dehydrogenases may link the oxidation of reduced ferredoxin and the unfavorable oxidation of NADH to produce hydrogen or formate.

Three group 1 [NiFe] uptake hydrogenases, each comprising small (UF_0627, UF_1035, UF_1897), large (UF_0628, UF_1034, UF_1896) and cytochrome b subunits (UF_0629, UF_1033, UF_1895), are probably linked to the cell membrane and oriented extracellularly, as evidenced by the presence of twin-arginine translocation (Tat) motifs in the small subunits and multiple transmembrane helices in the cytochrome b subunits. These enzymes can oxidize H₂ and donate the electrons to the quinone pool. They can link the oxidation of H₂ to cytoplasmic sulfate reduction, with conservation of the energy in the form of a transmembrane proton gradient. A similar function is performed by the membrane-associated formate dehydrogenase, encoded by genes UF_4571, UF_4573 and UF_4575 (subunits alpha, beta, and gamma, respectively). The enzyme catalyzes the oxidation of formate to carbon dioxide with the concomitant release of two electrons and two protons. The oxidation can be linked to sulfate respiration.

In addition, two group 4 [NiFe] hydrogenases are encoded (UF_0692-0697 and UF_0698-0703). These membrane-linked, six-subunit enzymes can dispose of the excess reducing equivalents generated by the anaerobic oxidation of organic compounds such as formate, and couple H₂ evolution and energy conservation by the generation of a transmembrane proton gradient (Vignais and Billoud 2007). The high number of hydrogenases and formate dehydrogenases in the genome of *Desulfosporosinus* sp. I2 indicates potentially high metabolic flexibility of this organism. This is important for changing growth strategies for example from sulfate respiration to syntrophic growth, although the latter mode has not been tested.

**Motility and chemotaxis**

Consistent with the observed motility of *Desulfosporosinus* sp. I2 cells, the genome contains a set of genes required for chemotaxis and flagellation. Flagellar motility is particularly important for *Desulfosporosinus* sp. I2, because it allows the cells to move towards favorable locations in terms of oxygen gradient in its natural environment. Most of the flagellar motility proteins are encoded by a single *fla* cluster (UF75_1808-1851). The flagellar motor rotation proteins are
encoded by genes UF75_4497 (MotA) and UF75_4498 (MotB). At least fifteen chemoreceptor proteins containing the transmembrane helices are encoded in the *Desulfosporosinus* sp. I2 genome. Other components of chemotaxis machinery in the genome are the signal transduction histidine protein kinase CheA (UF75_1838), its positive regulator CheW (UF75_1837, UF75_3836), response regulator methylesterase CheB (UF75_1836), CheY-P-specific phosphatase CheC (UF75_1814), chemoreceptor glutamine deamidase CheD (UF75_1813), chemotaxis protein methyltransferase CheR (UF75_0358, UF75_4501), and CheY signal receivers (UF75_1008, UF75_1824). Five copies of chemotaxis response regulators comprising a CheY-like receiver domain and a DNA-binding domain were identified (UF75_3169, UF75_3370, UF75_4113, UF75_4268, UF75_4968).

**DISCUSSION**

**Cryptic sulfate reduction may occur in the oxidized mine wastes**

The acidic pH and the high sulfate and metal concentrations of the pooled leachate, together with abundant yellow and red precipitates on rocks, and the dominance of secondary Fe(III)-hydroxysulfate minerals in the sediment, are clearly indicative of extensive sulfide oxidation in the tailings samples Xa201 and Xa202. Additionally, XRD-analysis showed very little indication of primary sulfide minerals in the sediment samples, but clearly demonstrated the presence of secondary minerals such as jarosite. These observations suggest that oxidation had resulted in the complete oxidation of primary sulfides in the upper layer of the tailings site. A positive redox potential was measured not only at the surface layer but also in the sediment interior at the depth of 3-4 cm.

The sulfate reduction rate in the oxidized Xa201 and Xa202 sediments was about the same order of magnitude as measured previously in the mining-impacted oxidized environments in Norilsk (Karnachuk *et al.* 2005). This rate may appear modest, but given the high sulfate concentrations in the oxic tailings, the absolute quantities of sulfate reduced under the oxic conditions and the accompanying precipitation of metals can reach substantial levels. Authigenic secondary sulfide minerals, recovered as a chromium-reducible sulfur fraction from the mine-impacted sediments in Norilsk, were enriched in $^{32}$S compared to the primary sulfide $\delta^{34}$S values, which testified to their biological origin (Karnachuk *et al.* 2005). At the same time, sulfides formed by SRB under oxidized conditions can undergo a relatively fast oxidation and thus cryptic sulfate reduction may occur in the upper layers of sulfide tailings.
Several previous studies have demonstrated the presence of SRB in the oxidized zone of sulfide tailings. Bruneel and co-authors (2005) discovered that the most abundant clone sequences retrieved from oxidized As-rich tailings in Cornoules (France) belonged to the class Deltaproteobacteria, which harbors many diverse SRB. In another study, a substantial number of clones related to the genera Desulfobacterium and Desulfomonile were retrieved from the fully oxygenated water of seepage from a tailings dam at the same mining site (Bruneel et al. 2006). Furthermore, culturable SRB have been regularly retrieved from the oxidized zones of various types of sulfide tailings (Fortin, Davis, Beveridge 1996, Fortin, Goulet, Roy 2000; Fortin et al. 2000; Diaby et al. 2007).

Desulfosporosinus spp. are important players in the sulfate reduction in acidic mine wastes

Our previous research of sulfate reduction at the Berikul site showed that Desulfosporosinus was the only phylotype with the known capability to reduce sulfate in the oxidized mine waste materials (Karnachuk et al. 2009). That is why the isolation procedure in this study targeted the spore-forming bacteria. Glycerol was used as a substrate for isolation because of the previous reports of possible toxicity of lactic acid and other organic acids under low pH conditions (Alazard et al. 2010; Meier, Piva, Fortin 2012; Sánchez-Andrea et al. 2014). Subsequently it was discovered that strain I2 could grow with lactic acid as an electron donor. Lactic acid was used for isolation of another acidophilic Desulfosporosinus sp. BG from mine tailings in Transbaikal area (Karnachuk et al. 2015b). There are many reports of Desulfosporosinus detection in the biotopes associated with acidic mine environments (Nevin, Finneran, Lovley 2003; Petrie et al. 2003; Suzuki et al. 2003; Kimura, Hallberg, Johnson 2006; Rowe et al. 2007; Cardenas et al. 2008; Jin et al. 2008; Madden et al. 2009; Senko et al. 2009). Many of these reports rely on cultivation rather than on the use of molecular methods. Küsel et al. (2001) isolated Desulfosporosinus sp. Lau III from the acidic sediment of a lake impacted by strip coal mining. Labrentz and Banfield (2004) described the occurrence of culturable Desulfosporosinus spp. in a sulfide-precipitating biofilm in the Piquette Zn-Pb mine. The absence of Desulfosporosinus-related sequences in the clone libraries led them to suggest that the bacteria were in the sporulating phase, which hampered DNA isolation. Some of the novel SRB Firmicutes sequences retrieved by Winch et al. (2009) via preceding enrichment phases were from acidic tailings. We have previously isolated a pure culture Desulfosporosinus sp. DB from the Berikul tailings (Karnachuk et al. 2009). These observations imply that Desulfosporosinus may represent an important player in the sulfate reducing community in the acidic environments. It is conceivable that Desulfosporosinus-related organisms in the sulfide tailings represent a “rare
biosphere,” a phenomenon described for marine ecosystems (Sogin et al. 2006), when a low-abundance but active component of the community is masked by dominant populations.

**Genomic insight does not reveal additional metal transporters in metal-tolerant**

*Desulfosporosinus sp. I2*

*Desulfosporosinus* sp. I2 habitat contained high concentrations of arsenic and the major mechanisms to tolerate it were present in the genome. However, the strain could not grow in the presence of more than 0.2 mM (15 mg l\(^{-1}\)) As, which is a lower concentration than known for the truly resistant strains. It is possible that As is effectively precipitated in the form of sulfides by SRB. Two different arsenic sulfides, orpiment and realgar, were found in the sediment samples from the tailings. It is possible that they were secondary sulfides formed due to As precipitation.

*Desulfosporosinus* sp. I2 could tolerate high copper levels of up to 142 mM. These levels are much higher than those reported for many metal-tolerant strains. For example, the model metal-tolerant *Cupriavidus metallidurans* has been reported to grow in the range of 1.5 – 12.5 mM Cu (Chen et al. 2008). A copper-resistant *Acidithiobacillus ferrooxidans*, an iron-oxidizing acidophile, could be adapted to up to 800 mM CuSO\(_4\) (Navarro et al. 2009; Orellana and Jerez 2011). The presence of additional metal transporters is suggested as a general mechanism to withstand high metal concentrations. The genomic islands combined different metal-transporters have been reported for *C. metallidurans* (van Houdt et al. 2009) and *A. ferrooxidans* (Navarro et al. 2009; Orellana and Jerez 2011). The genome sequence of strain I2 did not reveal additional metal transporters as compared to the other sequenced *Desulfosporosinus* isolates. However, the level of metal tolerance among the *Desulfosporosinus* strains varied drastically, and other mechanisms may also play role in their metal resistance. One possible mechanism involves the sequestration via polyphosphates. The transcriptome analysis, which is under way in our group, will help tackle the mechanisms involved in metal tolerance.

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**Conflict of Interest.** None declared.

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Table 1. Redox potential and sulfate reduction rates in Xa201 and Xa202 sediment samples

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<th>Sampling site</th>
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<td>17.6±2.4</td>
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<td>Xa201</td>
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<td>Xa202</td>
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### RND-family efflux pumps

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### ABC-type zinc transporters

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### ZIP-family transporters

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<td>Zinc transporter ZupT, ZIP family; divalent heavy-metal cations transporter</td>
<td>Desulfosporosinus sp. HMP52</td>
<td>91%</td>
<td>247</td>
<td>WP_034599480</td>
</tr>
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</table>

### CDF family transporters
<table>
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<tr>
<th>Accession</th>
<th>Protein ID</th>
<th>Miller</th>
<th>Description</th>
<th>Species</th>
<th>Coverage</th>
<th>Length</th>
<th>Accession</th>
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<tbody>
<tr>
<td>UF75_1064</td>
<td>WP_045573500</td>
<td>332</td>
<td>Co/Zn/Cd cation transporter of the cation diffusion facilitator family transporters</td>
<td>Desulfo sporosinus sp. BICA1-9</td>
<td>84%</td>
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<td>WP_034146005</td>
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<td>UF75_3098</td>
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<td>467</td>
<td>Co/Zn/Cd cation transporter of the cation diffusion facilitator family transporters</td>
<td>Desulfo sporosinus sp. OT</td>
<td>78%</td>
<td>307</td>
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<td><strong>Chromate transporters</strong></td>
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<td>UF75_1302</td>
<td>WP_045573713</td>
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<td>Chromate transporter ChrA</td>
<td>Desulfo sporosinus orientis</td>
<td>80%</td>
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<td>UF75_1303</td>
<td>WP_045573714</td>
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<td>Chromate transporter ChrA</td>
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<td>Desulfo sporosinus sp. BICA1-9</td>
<td>89%</td>
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<td>WP_034144505</td>
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<td><strong>Arsenate resistance</strong></td>
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<td>UF75_2367</td>
<td>WP_045574645</td>
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<td>Arsenate reductase, ArsC family</td>
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<td>Arsenite efflux pump, membrane subunit ArsB</td>
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<td>97%</td>
<td>357</td>
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<td>UF75_3205</td>
<td>WP_045575391</td>
<td>108</td>
<td>DNA-binding transcriptional repressor ArsR</td>
<td>Desulfo sporosinus sp. BICA1-9</td>
<td>92%</td>
<td>108</td>
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<td>UF75_3206</td>
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<td>Arsenite efflux pump, ATPase subunit ArsA</td>
<td>Clostridium sp. BNL1100</td>
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<td>Desulfitobacterium hafniense</td>
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<td>Desulfitobacterium dichloroeliminans</td>
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<td>105</td>
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Figure 1. Location map. The white rectangle in the upper map shows the location of the study area within the Russian Federation. The mine tailings are located at the Novii Berikul' processing works.
Figure 2. TEM micrographs of ultrathin sections of strain I2 grown without copper addition (a, b); vacuole-like inclusions in cells growing with initial copper concentration of 25 mg/l (c, d); electron-dense fibrils in the inclusions (e) and associated with the cell wall (f) in cells growing with initial copper concentration of 25 mg/l; extensive fibrils in the inclusions in the cells growing with initial copper concentration of 150 mg/l (g, h). C, cytoplasmic membrane; CE, cytoplasmic membrane extensions; CI, cytoplasmic membrane invaginations; P, peptidoglycan; S, presumed S-layer; F, fibrils; VM, vacuole membrane. The scale bars are given in μm.
Figure 3. Neighbor joining 16S rRNA tree showing the phylogenetic position of Desulfosporosinus I2, validly described members of Desulfosporosinus, and several unidentified strains. Desulfotomaculum acetoxidans DSM771 (not shown) was used as an outgroup. Numbers at branching points indicate bootstrap values determined from 1,000 iterations. The scale bar represents an estimated 2% sequence divergence.
Figure 4. Phylogenetic tree showing the most closely related amino acid sequences to PPK 2 of Desulfosporosinus sp. 12. Numbers at branching points indicate bootstrap values determined from 1,000 iterations.
Figure 5. Phylogenetic tree showing the most closely related amino acid sequences to cbb3-type cytochrome c oxidase subunit I, which contains a heme-copper binuclear center and defines the membership in the superfamily. Numbers at branching points indicate bootstrap values determined from 1,000 iterations.