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H. Sass
Cardiff University

S. Ramamoorthy
Cardiff University

C. Yarwood
University of Montana

H. Langner
University of Montana

P. Schumann
Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

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Authors

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Desulfovibrio idahonensis sp. nov., sulfate-reducing bacteria isolated from a metal(loid)-contaminated freshwater sediment

H. Sass,[1] S. Ramamoorthy,[2] C. Yarwood,[2] H. Langner,[3] P. Schumann,[4] R. M. Kroppenstedt,[4] S. Spring[4] and R. F. Rosenzweig[2]

Correspondence
R. F. Rosenzweig
Frank.Rosenzweig@mso.umt.edu

[1]School of Earth, Ocean and Planetary Sciences, Cardiff University, Main Building, Park Place, Cardiff CF10 3YE, UK

[2]Division of Biological Sciences, University of Montana, Missoula, MT 59812-4824, USA

[3]Department of Geology, University of Montana, Missoula, MT 59812, USA

[4]DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Inhoffenstraße 7B, D-38124 Braunschweig, Germany

Two novel sulfate-reducing bacteria, strains CY1[T] and CY2, were isolated from heavy-metal-contaminated sediments of Lake Coeur d'Alene, Idaho, USA. Strains CY1[T] and CY2 were found to contain c-type cytochromes and to reduce sulfate, sulfite, thiosulfate, elemental sulfur, DMSO, anthraquinone disulfonate and fumarate using lactate as an electron donor. In a comparison of 16S rRNA gene sequences, CY1[T] and CY2 were found to be 100 % identical, but only 97 and 92.4 % similar, respectively, to the type strains of *Desulfovibrio mexicanus* and *Desulfovibrio aminophilus*. Unlike these species, however, CY1[T] was neither able to disproportionate thiosulfate nor able to use yeast extract or amino acids as electron donors. These data, considered in conjunction with differences among strain CY1[T] and the two related type strains in chemotaxonomy, riboprint patterns, temperature and pH optima, support recognition of a distinct and novel species within the genus *Desulfovibrio*, *Desulfovibrio idahonensis* sp. nov., with the type strain CY1[T] (=DSM 15450[T] =JCM 14124[T]).

The genus *Desulfovibrio* ranks among the most speciose, phenotypically diverse genera in the *Proteobacteria*. Diagnostic traits include dissimilatory sulfate reduction, absence of sporulation, presence of polar flagella and the presence of desulfoviridin and cytochrome c (Postgate & Campbell, 1965). Ribosomal gene sequence analyses indicate that the group's origin lies deep in the *Deltaproteobacteria* (Devereux et al., 1990). To date, names of some 57 *Desulfovibrio* species have been validly published (http://www.dsmz.de/microorganisms/bacterial_nomenclature_info.php?genus=Desulfovibrio; four of these species have since been reclassified), enriched from such diverse environments as sediments (Bale et al., 1997; Sass et al., 1998), wastewater sludge (Baena et al., 1998; Hernandez-Eugenio et al., 2000) and animal intestines

(Goldstein et al., 2003). Desulfovibrios facilitate metal corrosion (Neria-Gonzalez et al., 2006) and have been repeatedly isolated from oil and gas production facilities (Miranda-Tello et al., 2003; Magot et al., 2004). *Desulfovibrio* ribosomal gene sequences have been retrieved from submerged mine timbers (Labrenz & Banfield, 2004), gas hydrate mounds (Mills et al., 2003) and floating macrophyte rhizospheres (Acha et al., 2005), while desulfovibrio-specific dissimilatory sulfite reductase genes (*dsrAB*) have been recovered from temperate sulfide-rich streams (Elshahed et al., 2003) and frozen Antarctic lakes (Karr et al., 2005).

As a group, desulfovibrios tolerate extreme ranges of temperature (Bale et al., 1997; Vandieken et al., 2006), pH (Abildgaard et al., 2006; Fröhlich et al., 1999) and salinity (Sass & Cypionka, 2004; Ito et al., 2002), and productively use a multitude of electron donors, from aromatic compounds (Reichenbecher & Schink, 1997) and halogenated hydrocarbons (Sun et al., 2000) to amino acids (Baena et al., 1998; Hernandez-Eugenio et al., 2000). In addition to dissimilatory sulfate reduction, certain *Desulfovibrio* species use alternative terminal electron acceptors such as U(VI) (Payne et al., 2002), Fe(III)

(Vandieken *et al.*, 2006) and Cr(VI) (Humphries & Macaskie, 2005), though these electron acceptors do not generally support growth. It has long been known that sulfidogenic microbes biomineralize aqueous-phase metals and metalloids (Tuttle *et al.*, 1969). Now, this activity is being harnessed to bioremediate soils and sediments contaminated by mining and radionuclide processing (Lovley, 2001). Because the genomes of several desulfovibrios have been fully sequenced (Heidelberg *et al.*, 2004; http://cmr.tigr.org/tigr-scripts/CMR/GenomePage.cgi?org=ntds01), autecological studies of *Desulfovibrio* species are now possible using genome-enabled technologies (e.g. Clark *et al.*, 2006).

To understand metal(loid) cycling better in mining-impacted freshwater sediments, our group has enriched for and studied iron-, arsenic- and sulfate-reducing bacteria, as the characteristic activities of such microbes are likely to influence contaminant fate and stability (Cummings *et al.*, 2000; Niggemyer *et al.*, 2001; Ramamoorthy *et al.*, 2009). Strains CY1[T] and CY2 were isolated from the highest positive tubes of an MPN series inoculated with sediment (pH 6.8–7.2) from Lake Coeur d'Alene, Idaho, USA, a freshwater system historically impacted by lead, zinc and antimony mining (Ramamoorthy *et al.*, 2009). For enrichment and isolation, we used selective dithionite-reduced medium with sodium lactate (10 mM) and Na_2SO_4 (10 mM) (Widdel & Pfennig, 1977; Widdel, 1980). Bacteria were isolated by three passages in 1.5 % agar shake tubes.

Gram staining, oxidase and catalase tests, electron microscopy and assessment of spore formation were performed as described previously (Magee *et al.*, 1975; Cappuccino & Sherman, 1998; Ramamoorthy *et al.*, 2006). Cells were tested for desulfoviridin as described by Postgate (1959); *Desulfovibrio desulfuricans* ATCC 27774 was used as a positive control and *Escherichia coli* K-12 as a negative control. Cytochromes were analysed by recording redox difference and CO difference spectra; separation and identification of *b*-type and *c*-type cytochromes were performed as described by Widdel (1980).

Cells of strains CY1[T] and CY2 were Gram-negative, curved rods with a single polar flagellum (see Supplementary Fig. S1, available in IJSEM Online). Cells of strain CY1[T] were 1.3–2.5 µm long and 0.5–0.65 µm wide, whereas cells of strain CY2 were 1.0–3.3 µm long and 0.4–0.6 µm wide (Table 1). Endospores were not formed. Strains CY1[T] and CY2 produced black, round colonies in shake tubes amended with $Fe(NH_4)_2(SO_4)_2$. CY1[T] and CY2 were catalase- and oxidase-negative and desulfoviridin-positive (λ_{max} 631 nm). They also possessed *c*-type cytochromes (λ_{max} 553 nm).

Electron donors were tested using sulfate (5 mM) as the terminal electron acceptor, while electron acceptors were tested with sodium lactate (10 mM) as the electron donor. Organisms were considered positive for utilization of an electron donor or acceptor if 2.5 to 3 doublings occurred

after three successive passages. Negative controls consisted of (i) live cells in medium without lactate or sulfate, (ii) autoclaved cells added to complete medium (Madigan *et al.*, 1997) and (iii) live cells filtered through a 0.2 µm sterile filter, with the filtrate added to complete medium. Reduction of sulfate, sulfite, sulfur and thiosulfate to sulfide was tested by adding 0.5 % $Fe(NH_4)_2(SO_4)_2$ to culture tubes. For accurate determination of sulfide (as hydrogen sulfide, H_2S) concentrations, the spectrophotometric methylene blue assay of Cline (1969) was used. Reduction of anthraquinone disulfonate (AQDS) was measured photometrically at 450 nm, as described by Lovley *et al.* (1996). As CY1[T] and CY2 were isolated from metal(loid)-contaminated sediments, we tested for reduction of Mn(IV), As(V), Fe(III) and Se(VI) presented respectively as MnO_2, $Na_2HAsO_4 . 7H_2O$, $Fe_4(P_2O_4)_3$ and Na_2SeO_4. MnO_2 reduction was scored by the characteristic colour change from black MnO_2 to white $MnCO_3$, while formation of red elemental selenium precipitate was scored as indicating Se(VI) reduction. Reduced arsenic [as arsenite, As(III)] was quantified photometrically from the A_{865} using the procedure originally described by Johnson & Pilson (1972) and later modified by Niggemyer *et al.* (2001). Sulfate, thiosulfate, lactate and acetate concentrations were determined using ion chromatography (Ramamoorthy *et al.*, 2006). Culture density was estimated photometrically as OD_{420}. Fluorimetry after staining with SybrGreenI (Martens-Habbena & Sass, 2006) or direct cell counting was used to confirm growth on selenate, manganese oxide and Fe(III). Cells were stained with 10 µg 4′,6-diamidino-2-phenylindole (DAPI; Sigma) ml^{-1}, and cells were then counted by epifluorescence microscopy using a Zeiss Axioskop. Growth yield was defined as bacterial dry mass obtained from a given amount of substrate, as described by Cypionka & Pfennig (1986).

Desulfovibrio strains CY1[T] and CY2 were able to use hydrogen, formate, lactate, pyruvate, fumarate, succinate and malate as electron donors (Table 1) and carbon sources and grew fermentatively on pyruvate in the absence of sulfate. In contrast to their closest relative, *Desulfovibrio mexicanus* (Hernandez-Eugenio *et al.*, 2000), strains CY1[T] and CY2 did not grow with yeast extract or amino acids as electron donors. Both strains oxidized lactate to acetate and CO_2, identifying them as incompletely oxidizing sulfate-reducers.

In addition to sulfate, CY1[T] and CY2 could use fumarate, thiosulfate, elemental sulfur and sulfite as terminal electron acceptors for growth. Strains CY1[T] and CY2 and *Desulfovibrio mexicanus* DSM 13116[T] grown at 30 °C showed the highest growth yield with DMSO as electron acceptor, followed by thiosulfate and sulfate (Table 2). Manganese oxide as an electron acceptor supported growth of strain CY1[T] only. Ferric iron was reduced by all three strains but supported little growth.

Strain CY1[T] failed to disproportionate sulfur presented in the form of thiosulfate, bisulfite or elemental sulfur. Lack of growth and absence of sulfate and sulfide production

Table 1. Biochemical and chemotaxonomic features that distinguish strains CY1[T] and CY2 from the most closely related type strain

D. mexicanus DSM 13116[T] was isolated from a UASB reactor treating cheese wastewater; data for this strain were taken from Hernandez-Eugenio *et al.* (2000) unless indicated. In our study, all three strains used elemental sulfur (10 mM) as an electron acceptor in the presence of lactate and H_2+CO_2 and formate (10 mM) as an electron donor (with 2 mM sodium acetate as carbon source) in the presence of sulfate, and none of the strains tested used acetate (10 mM), propionate (5 mM), butyrate (5 mM), benzoate (1 mM), methanol (10 mM), propanol (5 mM), butanol (5 mM), ethylene glycol (10 mM), 1,2-propanediol (5 mM), glycerol (5 mM), glutamate (5 mM), isoleucine (5 mM), ethanolamine (10 mM) or choline (10 mM) when each was presented as an electron donor. None of the strains tested used As(III), Se(VI) or nitrate as terminal electron acceptor in our study. ND, Not determined; ++, strongly positive ($OD_{420}>0.250$; this study); +, positive (in this study, $0.1<OD_{420}\leqslant0.250$); −, negative (in this study, $OD_{420}<0.1$).

Characteristic	CY1[T]	CY2	*D. mexicanus* DSM 13116[T]
Morphology	Curved rods	Curved rods	Curved rods, sometimes in chains
Cell size (μm)	0.6 × 1.3–2.5	0.5 × 1–3.3	0.5 × 1.7–2.5
Flagella	Single, polar	Single, polar	Non-motile
Salinity range (% NaCl)	0–0.75	0–0.75	0–1.5
Temperature range (optimum) (°C)	10–37 (28)	10–37 (32)	20–40 (37)
pH range (optimum)	6.5–7.5 (6.5)	6.5–7.5 (7.0)	6.3–8.2 (7.2)
Growth rate at 30 °C (h^{-1})	0.05	0.05	0.23
Electron acceptors in the presence of lactate			
Sulfate (10 mM)	++	++	+
Sulfite (5 mM)	++	++	+
Thiosulfate (5 mM)	++	++	+
Fumarate (20 mM)	++	++	−
DMSO (10 mM)	++	++	+*
AQDS (4 mM)	+	+	−*†
Manganese oxide (20 mM)	+	−	−*
Electron donors in the presence of sulfate‡			
Fumarate (10 mM)	++	++	−
Lactate (10 mM)	++	++	+
Malate (1 mM)	+	+	−
Pyruvate (10 mM)	++	++	+
Succinate (1 mM)	+	+	−
Cysteine (5 mM)	−	−	+
Serine (10 mM)	−	−	++
Alanine (10 mM)	−	−	+*
Casamino acids (0.1 %)	−	−	+
Yeast extract (0.2 %)	−	−	+*
Disproportionation§	−	−	+ (Thiosulfate)
DNA G+C content (mol%)	63.5	ND	66.0

*Data from this study.

†AQDS reduction, but no growth.

‡Electron donor assays for *D. mexicanus* DSM 13116[T] were conducted in the presence of 20 mM thiosulfate (Hernandez-Eugenio *et al.*, 2000) or 10 mM sulfate (this study).

§With 2 mM sodium acetate as carbon source.

Table 2. Growth yields of strains CY1[T] and CY2 and *D. mexicanus* DSM 13116[T] grown with lactate and different electron acceptors

Values are g dry weight per mol lactate.

Strain	Sulfate	Thiosulfate	DMSO	Fe(OH)$_3$	MnO$_2$
CY1[T]	6.8 ± 5.8	12.5 ± 1.4	13.6 ± 10.4	1.8 ± 0.8	5.9 ± 0.9
CY2	5.5 ± 0.8	6.0 ± 0.5	6.5 ± 1.6	2.9 ± 1.4	0.6 ± 0.3
D. mexicanus DSM 13116[T]	5.9 ± 3.8	5.3 ± 1.3	10.8 ± 7.0	1.3 ± 0.6	0

were inferred from the absence of change in OD_{420} and the absence of black FeS precipitate following addition of 0.05 % $Fe(NH_4)_2(SO_4)_2$ at the end of each experiment.

Temperature ranges were estimated by culturing cells in basal medium amended with 10 mM sodium lactate and 5 mM sodium sulfate at 4, 10, 15, 21, 28, 30, 32, 37, 42 and 45 °C. To determine pH optima, the bicarbonate buffer was replaced with 20 mM (each) HEPES, PIPES, MES and Tris and the initial medium pH was set using 1 M HCl or NaOH. Population growth at 30 °C and pH 6.0, 6.5, 7.0 and 7.5 was estimated by following the change in optical density; growth rates were calculated from the linear portion of a semi-logarithmic plot of OD against time. Oxygen sensitivity was tested by transferring late-exponential phase cells grown on lactate and sulfate into sterile flasks and shaking them under air at 30 °C for 24 h, whereafter they were reintroduced into anoxic growth medium.

While strains $CY1^T$ and CY2 could both respire sulfate at 10–37 °C, only $CY1^T$ could grow at 4 °C. Strains $CY1^T$ and CY2 attained μ_{max} of 0.05 h^{-1} at 28 and 32 °C, respectively. Sulfidogenic growth of $CY1^T$ and CY2 was confined to the range pH 6.5–7.5. Strain $CY1^T$ grew fastest at the lowest pH tested, 6.5, compared with pH 7.0 for CY2. Strains $CY1^T$ and CY2 were also incapable of growth after exposure to atmospheric oxygen for 24 h.

The bacterial strains were assayed for their tolerance to environmental contaminants that are relevant in Lake Coeur d'Alene. Specifically, they were inoculated into medium containing lactate (10 mM) and Na_2SO_4 (10 mM) and exposed to incremental levels of $Na_2HAsO_4 . 7H_2O$ (up to 10 mM), $CdSO_4$ (up to 10 mM), K_2CrO_4 (up to 10 mM) or $ZnSO_4$ (up to 50 mM). The maximum metal(loid) concentrations were chosen near the saturation point calculated using the geochemical equilibrium-modelling program MINTEQA2 (Allison *et al.*, 1991), in order to avoid any precipitates other than the sulfides. Prior to inoculation in contaminant-amended media, cells were washed in order to remove all previously produced sulfide. Isolates were considered metal-tolerant if the medium turned black as a result of formation of FeS precipitate following addition of 0.05 % $Fe(NH_4)_2(SO_4)_2$ and the cell density increased more than threefold as determined by epifluorescence microscopy after staining with DAPI.

Both strains were incapable of growth in the presence of chromium, even at the lowest concentration tested (0.4 mM). On the other hand, strain $CY1^T$ was able to grow in the presence of all concentrations of As, Cd and Zn tested (up to 10, 10 and 50 mM, respectively). Strain CY2 was capable of growth at lower concentrations of As, Cd and Zn, but its growth was inhibited by each metal at the highest concentrations tested (not shown).

Cell walls and isoprenoid quinones were analysed as described previously (Ramamoorthy *et al.*, 2006). Cellular

fatty acid patterns were determined from cells grown to stationary phase in DSMZ medium 641 (http://www.dsmz.de/media). Fatty acid methyl esters were obtained from 40 mg wet weight of cells by saponification, methylation and extraction and fatty acid methyl esters were analysed as described previously (Kämpfer & Kroppenstedt, 1996; Kroppenstedt, 1985; Miller, 1982).

Cellular fatty acid profiles for the novel strains and *D. mexicanus* DSM 13116^T are displayed in Table 3. In strains $CY1^T$ and CY2, anteiso-15 : 0 (15.6 and 15.4 %, respectively) and 16 : 0 (15.3 and 15.6 %) are the predominant fatty acids. The diagnostic amino acid of $CY1^T$ was *meso*-diaminopimelic acid and the predominant isoprenoid quinone was MK-6(H_2).

Following PCR using universal primers 8-27F and 1541R, purification of nearly full-length (1529 bp) bacterial 16S rRNA gene fragments and sequence analysis were performed as described previously (Rainey *et al.*, 1996; Ramamoorthy *et al.*, 2006). Almost-complete 16S rRNA gene sequence of strains $CY1^T$ and CY2 were aligned with sequences included in the ARB database or those obtained from the EMBL nucleotide sequence database (http://www.ebi.ac.uk) using tools implemented in the ARB package. The resulting alignment was visually inspected and potential errors were corrected manually. Evolutionary distances were calculated based on the algorithms of Jukes & Cantor (1969) using neighbour-joining and parsimony methods of tree reconstruction as implemented in the ARB program package. The databases of 16S rRNA gene sequences used in this study, as well as the phylogenetic programs, are available via the Internet at http://www.arb-home.de. The G + C content was determined by reversed-phase HPLC of nucleosides according to Mesbah *et al.* (1989). Ribotyping of cultures was performed as described previously using the Qualicon RiboPrinter system (DuPont) with *Pvu*II or *Eco*RI as restriction enzymes (Bruce, 1996).

Fig. 1 depicts phylogenetic relationships of $CY1^T$ to other *Desulfovibrio* species. Similarity values based on almost-complete 16S rRNA gene sequences indicate that strains $CY1^T$ and CY2 are 100 % identical to one another, but only 97.0 and 92.4 % similar to *Desulfovibrio mexicanus* DSM 13116^T and *Desulfovibrio aminophilus* ALA-3^T, respectively. Ribotype patterns derived from restriction endonuclease digestion of chromosomal DNA followed by hybridization to probes for sequences that encode the 5S–16S–23S rRNA operon (Barney *et al.*, 2001) have been shown to discriminate among closely related bacterial strains (Scott *et al.*, 2003). The ribotype pattern for $CY1^T$ using *Pvu*II was nearly identical to that of CY2, but distinct from that of the type strain of the closest related species with a validly published name, *D. mexicanus* (97.0 % 16S rRNA sequence similarity) (Supplementary Fig. S2). The G + C content for strain $CY1^T$ was 63.5 mol%.

Considered together, the physiological, chemotaxonomic and genomic data strongly support recognition of strains

Table 3. Fatty acid composition of strains CY1^T and CY2 and *D. mexicanus* DSM 13116^T

Values are percentages of total fatty acids. Major components are highlighted in bold. DMA, Dimethylacetal. −, Not detected.

ECL	Fatty acid	CY1^T	CY2	*D. mexicanus* DSM 13116^T
13.62	iso-14:0	0.8	0.7	−
14.00	14:0	0.9	0.8	−
14.62	iso-15:0	9.1	7.5	5.3
14.71	anteiso-15:0	**15.6**	**15.4**	**17.3**
15.00	15:0	0.5	0.8	1.9
15.46	iso-16:1	7.8	9.1	**16.1**
15.63	iso-16:0	7.2	7.3	**15.6**
15.81	16:1 *cis*9	7.8	7.9	2.7
16.00	16:0	**15.3**	**15.6**	4.2
16.13	iso-15:0 3-OH	0.8	0.7	−
16.41	iso-17:1 *cis*7	**15.2**	**12.9**	10.3
16.52	anteiso-17:1	6.2	6.1	5.8
16.63	iso-17:0	3.5	2.9	3.4
16.72	anteiso-17:0	2.9	3.0	5.8
16.79	17:1 *cis*9	−	0.6	1.1
16.86	17:1 *cis*11	0.7	1.2	2.0
17.00	17:0	0.9	1.2	2.6
17.47	17:0 DMA	1.0	1.4	3.4
17.82	18:1 *cis*11	2.4	2.5	1.7
18.00	18:0	1.6	2.3	1.1

CY1^T and CY2 as representatives of a novel species within the genus *Desulfovibrio*, for which the name *Desulfovibrio idahonensis* sp. nov. is proposed.
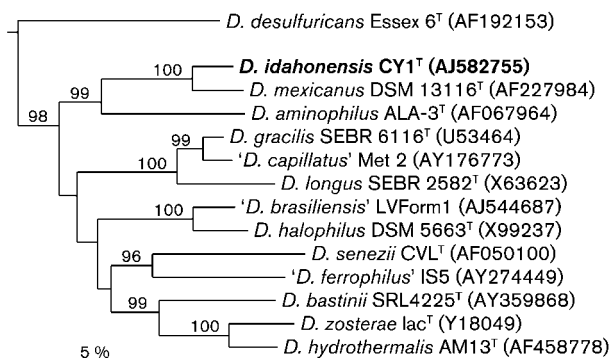

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences showing the position of the novel strains CY1^T and CY2 among members of the genus *Desulfovibrio*. The sequence of *Escherichia coli* K-12 MG1655 (GenBank accession no. L10328) was used to define the root (not shown). The neighbour-joining method of Saitou & Nei (1987) was used to reconstruct the matrix and the algorithm of Jukes & Cantor (1969) was used to calculate phylogenetic distances. Only bootstrap values above 70 % (1000 resamplings for each node) are shown at branching points. Bar, 5 % estimated sequence divergence.

Description of *Desulfovibrio idahonensis* sp. nov.

Desulfovibrio idahonensis (i.da.ho.nen′sis. N.L. gen. n. *idahonensis* of Idaho, referring to the source of isolation of the first strains).

Cells are rod-shaped and Gram-negative with single polar flagella. Individual cells are 0.4–0.6 μm wide and 1.0–3.3 μm long. Capable of using sulfate, sulfite, thiosulfate, sulfur, DMSO and fumarate as terminal electron acceptors in the presence of lactate. Arsenate, nitrate and selenate cannot be used as electron acceptors; use of manganese oxide is strain dependent. Iron(III) is reduced but does not support growth. In the presence of sulfate, cells are capable of using H_2/CO_2 plus acetate, formate, pyruvate, lactate, fumarate, succinate or malate as electron donor and carbon source. Unable to use acetate, propionate, benzoate, butyrate, methanol, propanol, butanol, ethylene glycol, 1,2-propanediol, glycerol, ethanolamine, choline, yeast extract or amino acids as electron donor and carbon source. Also capable of fermentative growth using pyruvate as energy source in the absence of any electron acceptor, i.e. as sole source of carbon and energy. The temperature and pH ranges for growth are 10–37 °C and pH 6.5–7.5. The type strain is desulfoviridin-positive and possesses *c*-type cytochromes. The diagnostic amino acid of the peptidoglycan is *meso*-diaminopimelic acid. The predominant whole-cell fatty acids are anteiso-15:0, iso-16:0, 16:0, iso-15:0 and 16:1*cis*9. The predominant isoprenoid quinone is MK-6(H$_2$). The DNA G+C content of the type strain is 63.5 mol%.

The type strain CY1^T (=DSM 15450^T =JCM 14124^T) and reference strain CY2 were isolated from sediment of Lake Coeur d'Alene, Idaho, USA.

Acknowledgements

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