Rhodanobacter denitrificans sp. nov., isolated from nitrate-rich zones of a contaminated aquifer

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Bacterial strains 2APBS1[T] and 116-2 were isolated from the subsurface of a nuclear legacy waste site where the sediments are co-contaminated with large amounts of acids, nitrate, metal radionuclides and other heavy metals. A combination of physiological and genetic assays indicated that these strains represent the first member of the genus *Rhodanobacter* shown to be capable of complete denitrification. Cells of strain 2APBS1[T] and 116-2 were Gram-negative, non-spore-forming rods, 3–5 µm long and 0.25–0.5 µm in diameter. The isolates were facultative anaerobes, and had temperature and pH optima for growth of 30 °C and pH 6.5; they were able to tolerate up to 2.0 % NaCl, although growth improved in its absence. Strains 2APBS1[T] and 116-2 contained fatty acid and quinone (ubiquinone-8; 100 %) profiles that are characteristic features of the genus *Rhodanobacter*. Although strains 2APBS1[T] and 116-2 shared high 16S rRNA gene sequence similarity with *Rhodanobacter thiooxydans* LCS2[T] (>99 %), levels of DNA–DNA relatedness between these strains were substantially below the 70 % threshold used to designate novel species. Thus, based on genotypic, phylogenetic, chemotaxonomic and physiological differences, strains 2APBS1[T] and 116-2 are considered to represent a single novel species of the genus *Rhodanobacter*, for which the name *Rhodanobacter denitrificans* sp. nov. is proposed. The type strain is 2APBS1[T] (=DSM 23569[T]=JCM 17641[T]).

The genus *Rhodanobacter* was proposed by Nalin *et al.* (1999) to accommodate yellow-pigmented, Gram-negative, non-spore-forming, aerobic bacteria belonging to the family *Xanthomonadaceae*, order *Xanthomonadales* and class *Gammaproteobacteria* of phylum *Proteobacteria*. At the time of writing, the genus *Rhodanobacter* comprised nine recognized species, mainly isolated from surficial soils (Nalin *et al.*, 1999; Im *et al.*, 2004; Weon *et al.*, 2007; Bui *et al.*, 2010; An *et al.*, 2009; De Clercq *et al.*, 2006; Wang *et al.*, 2011). Anaerobic metabolism has not been studied in detail for the genus *Rhodanobacter*, and none of its recognized species has been shown to be capable of complete denitrification.

Quantitative and semi-quantitative molecular surveys of acidic, nitrate-rich subsurface environments have revealed extremely high abundances and activity of bacteria of the genus *Rhodanobacter* (Green *et al.*, 2010). The abundance and activity of *Rhodanobacter* spp. in the subsurface indicates the survival potential of these organisms in harsh subsurface conditions of elevated groundwater ionic strength, proton concentration, and nitrate and metal concentrations. In other systems, including soils impacted by agricultural waste and denitrifying bioreactors, *Rhodanobacter* spp. have been detected at pH levels near 4 (Van Den Heuvel *et al.*, 2010; Weon *et al.*, 2007). Strains of *Rhodanobacter* have been isolated and described to improve our understanding of the physiological mechanisms of nitrate attenuation in the subsurface.

Strains 2APBS1[T] and 116-2 were isolated from the Oak Ridge Integrated Field Research Challenge (OR-IFRC) site, a model nuclear legacy site that contains variable but high levels of nitrate (10–1000 mM) and acidity, along with uranium, technetium and other heavy metals (Watson *et al.*, 2004). Procedures used for sampling, anoxic enrichment and isolation have been described in detail by

Green et al. (2010). Strain 2APBS1[T] was isolated by anoxic enrichment of sediment from moderately contaminated Area 2 (pH 6–7, nitrate <2 mM) of the OR-IFRC site by using nitrate as an electron acceptor and a combination of acetate, propionate and butyrate as electron donors in minimal medium. Strain 116-2 was isolated from the most highly contaminated Area 3 (pH 3–4, nitrate at tens to hundreds millimolar) of the OR-IFRC site by direct plating of diluted sediments onto complex solid media of potato dextrose. Denitrification potential and the presence of a complete pathway of denitrification in the two strains were verified by using stable isotope tracer techniques and measurements of produced N_2O and N_2 by GC and membrane inlet MS (An et al., 2009; Green et al., 2010). For strains 116-2 and 2APBS1[T], a linear correlation was observed between nitrate consumption and protein production, and a mass balance of nitrogen species indicated >80 % nitrate was converted to gaseous nitrogen ($N_2 +$ N_2O) (thus satisfying criteria for identifying respiratory denitrifiers according to Mahne & Tiedje, 1995).

Growth response and colony morphology were observed on R2A agar, nutrient agar, Luria–Bertani (LB) agar and trypticase soy agar media at 30 °C. Growth at 5–40 °C was tested in R2A broth by using a temperature gradient block fabricated at Florida State University. The optimum temperature for growth was determined by plotting OD_{600} against temperature. The optimum pH and pH ranges for growth were also determined in R2A broth supplemented with different pH buffers [homopiperazine-N,N'-bis-2(ethanesulfonic acid) (HOMOPIPES), PIPES and HEPES; Sigma-Aldrich] and incubated at 30 °C (increments of 1 pH unit). Growth in the presence of NaCl, nitrate and nitrite was tested in R2A broth media and employed OD_{600} measurements. Catalase and oxidase tests were conducted by using 3 % H_2O_2 and oxidase reagents (N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride), respectively. Motility and gelatinase activity were tested in R2A media supplemented with 0.3 % molecular grade agar (Sigma-Aldrich) and 12 % gelatin, respectively, while a urease test was performed in Difco urea broth with phenol red as pH indicator. Carbohydrate assimilation tests were conducted in synthetic groundwater media (Green et al., 2010) supplemented with 2 % final concentrations of filter-sterilized solutions of different carbohydrates. Along with standardized microbiological protocols, enzyme activity and substrate assimilation were also assessed by using the API ZYM and API 20NE microtest systems (bioMérieux) according to the manufacturer's instructions. Utilization of different electron acceptors [NO_3^-, NO_2^-, N_2O, fumarate and iron(III)] and electron donors (ethanol, butyrate, acetate, lactate and propionate) was tested in triplicate cultures under strictly anoxic conditions, as described by Green et al. (2010).

For analysis of fatty acid methyl esters, cells were cultivated on low-salt (1 g NaCl l^{-1}) trypticase soy agar plates and harvested at an early stage (72 h) of growth. Fatty acid methyl esters were extracted and then analysed by using the standard protocol of the Sherlock Microbial Identification System (MIDI) (Miller, 1982; Sasser, 1990). Identification and comparison of peaks were carried out by using the MIDI aerobic database (RTSB6 v. 6.10). Quinone and polar lipid analyses were performed by the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) as described by Tindall et al. (2010). The TLC plate was stained with 5 % molybdophosphoric acid in order to visualize lipids.

16S rRNA gene and internal transcribed spacer (ITS) sequence data for strain 2APBS1[T] were recovered from the draft genome sequence for this organism (http://gp-edge4.jgi-psf.org:1080/rho_2/rho_2.home.html). Genomic DNA was extracted according to the 'DNA Isolation Bacterial CTAB' protocol defined by the Joint Genome Institute (JGI). Genomic DNA was transported to the JGI for genome sequencing, and the 16S rRNA gene sequence (single copy per genome) was recovered from one of the large contigs produced by the JGI. This sequence shares 100 % sequence similarity to a partial (1419 bases) sequence of the 16S rRNA gene previously recovered from this organism (GenBank accession number FJ851443). The full sequence, with the ITS region, was submitted to the National Center for Biotechnology Information (NCBI) with accession number JF719060. The DNA GC content of strain 2APBS1[T] was determined from the draft genome sequence. The nearly complete 16S rRNA gene sequence of strain 116-2 was obtained by using the procedures described by Green et al. (2010). Sequencing reactions from each organism were assembled into a single contig via the software package Sequencher (Gene Codes). The full-length sequence, containing almost all of the 16S rRNA gene and the complete ITS region, for strain 116-2 was submitted to NCBI with accession number JF719061.

16S rRNA gene sequences of the type strains of all recognized Rhodanobacter species, in addition to a range of closely related species, were initially aligned by using the Greengenes NAST multiple sequence alignment server (DeSantis et al., 2006). This alignment was imported into the software package ARB (Ludwig et al., 2004), and the alignments were manually refined. The aligned sequences were exported, but with portions of the 5′ and 3′ ends of the rRNA molecules discarded to prevent bias associated with commonly used primer sites for rRNA gene sequencing and a variable region at the 5′ end that could not be reliably aligned (positions 76–90). The remaining regions spanned the 16S rRNA gene from position 97 to 1462 (Escherichia coli numbering), and after complete deletion of positions that were not shared by all sequences, 1345 base positions were used for comparative analysis.

The phylogeny of the new isolates was inferred by producing a bootstrapped neighbour-joining tree within the software package MEGA v5.03 (Tamura et al., 2007). Evolutionary distances were computed by using the maximum composite likelihood method (Tamura et al., 2004), and all positions containing gaps and missing data

were eliminated. Similarly, a bootstrapped maximum-likelihood tree was also generated within MEGA, and the branching order was compared with that of the initial tree. Additionally, Bayesian analyses were performed on the aligned sequence data (MrBayes version 3.1; Ronquist & Huelsenbeck, 2003) by running five simultaneous chains (four heated, one cold) for two million generations, sampling every 1000 generations. The selected model was the general time-reversible one by using empirical base frequencies and estimating the shape of the gamma distribution and proportion of invariant sites from the data. The split-differential had dropped to below 0.01 at the end of the run. The resulting 50 % majority-rule consensus tree (after discarding 25 % of the generations as burn-in) was determined to calculate the posterior probabilities for each node.

For DNA–DNA hybridization analysis, genomic DNA was isolated from R2A agar-grown cells of strain 2APBS1[T], strain 116-2, *Rhodanobacter thiooxydans* DSM 18863[T], *Rhodanobacter ginsenosidimutans* DSM 21013[T], *Rhodanobacter fulvus* DSM 18449[T], *Rhodanobacter spathiphylli* DSM 17631[T], *Rhodanobacter soli* KCTC 22620[T] and *Rhodanobacter terrae* DSM 19241[T] by using a French pressure cell (Thermo Spectronic) and was purified by using chromatography on hydroxyapatite as described by Cashion *et al.* (1977). DNA–DNA hybridization was performed by the DSMZ as described by De Ley *et al.* (1970) under consideration of the modifications described by Huß *et al.* (1983), by using a model Cary 100 Bio UV/Vis spectrophotometer equipped with a Peltier-thermostatted 6×6 multi-cell changer and a temperature controller with an *in situ* temperature probe (Varian).

Strains 2APBS1[T] and 116-2 formed visible yellow colonies at 30 °C (1.5–2 mm, smooth) on R2A agar within 48 h. No growth or very limited growth was observed below 15 °C and at 40 °C. The temperature optimum for the two strains was 30 °C, similar to most described *Rhodanobacter* species (Table 1). Cells of the two strains were motile, similar to *R. soli* and *R. fulvus*, but this feature is lacking in many other members of the genus. The two strains produced a number of extracellular enzymes, including acid phosphatase and β-glucosidase. Strains 2APBS1[T] and 116-2 differed in carbon assimilation capabilities, with strain 2APBS1[T], but not 116-2, capable of glycogen, arabinose, melibiose and salicin uptake (Table 1). Both strains were capable of acetate assimilation, a rare feature for members of the genus *Rhodanobacter*.

Overall, the fatty acid profiles of strains 2APBS1[T] and 116-2 were consistent with those of recognized *Rhodanobacter* species (Table S1 available in IJSEM Online), but some distinct differences were observed. In comparison with recognized *Rhodanobacter* species, strains 2APBS1[T] and 116-2 contained more branched-chain fatty acids, a somewhat higher relative abundance of iso-$C_{15:0}$ and iso-$C_{17:1}\omega9c$ fatty acids, and lower iso-$C_{11:0}$ content. Strains 2APBS1[T] and 116-2 contained low but detectable levels of

$C_{13:0}$ 2-OH, iso-$C_{15:0}$ 3-OH, iso-$C_{16:0}$ 3-OH and iso-$C_{17:0}$ 3-OH, while these were absent from recognized members of the genus *Rhodanobacter*. We also detected a peak of an unknown fatty acid (ECL 11.799; equivalent chain-length) in the profile of strain 2APBS1[T], but the quantity of this component was negligible. Unlike the fatty acid profiles of recognized *Rhodanobacter* species, the annotation of summed feature 3 has been changed from $C_{16:1}\omega7c$ and/or iso-C_{15} 2-OH to $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$ in the case of strains 2APBS1[T] and 116-2 (Table S1), which implies that summed feature 3 comprises $C_{16:1}\omega7c$, iso-C_{15} 2-OH and/or $C_{16:1}\omega6c$. Further resolution of summed fatty acid features is beyond the scope of this study. Strain 2APBS1[T] contained ubiquinone-8 (Q-8; 100 %) and a distinct polar lipid profile (Fig. S1).

Strains 2APBS1[T] and 116-2 clustered together in a discrete clade in our phylogenetic analysis of 16S rRNA gene sequences of *Rhodanobacter* species (Fig. 1). The rRNA gene sequences of these organisms were highly similar to that of *R. thiooxydans* LCS2[T], with strain 2APBS1[T] sharing 99.2 % similarity (across 1484 positions), and strain 116-2 sharing 98.7 % similarity (across 1481 positions). Strains 2APBS1[T] and 116-2 shared 99.4 % 16S rRNA gene sequence similarity (across 1522 positions), and almost all of the sequence divergence was within a hypervariable region from position 76 to 90 (*E. coli* numbering) of the 16S rRNA gene. Levels of DNA–DNA relatedness between strain 2APBS1[T] and the type strains of closely related species of the genus *Rhodanobacter* are presented in Table S2. Strain 2APBS1[T] showed the highest DNA–DNA relatedness (86 %) to strain 116-2, followed by *R. soli* KCTC 22620[T] (56.7 %) and *R. thiooxydans* DSM 18863[T] (41.1 %). According to the recommendations of Wayne *et al.* (1987), the DNA–DNA hybridization threshold for delineating bacterial species is 70 %. Thus, based on DNA–DNA hybridization data, strains 2APBS1[T] and 116-2 are considered to represent a single novel species of the genus *Rhodanobacter*.

Strains 2APBS1[T] and 116-2 used nitrate, nitrite and nitrous oxide as electron acceptors under anaerobic conditions, and produced nitrogen and nitrous oxide (Table S3), thereby demonstrating the ability to perform complete denitrification. *R. thiooxydans*, isolated from a sulfur-particle biofilm, was initially described as having the capacity to reduce nitrate to nitrite, but was unable to reduce nitrite (Lee *et al.*, 2007). Recently, however, Van Den Heuvel *et al.* (2010) indicated that *R. thiooxydans* mediates N_2O as well as NO_3^- reduction, a finding that we confirmed in this study for strains 2APBS1[T] and 116-2 (Table S3). Thus, in addition to strains 2APBS1[T] and 116-2, *R. thiooxydans* (the most closely related species of the genus *Rhodanobacter*) is also probably capable of denitrification. DNA–DNA hybridization analyses demonstrate, however, that the two new isolates described in this study represent a species that is distinct from *R. thiooxydans*. *R. thiooxydans* and strains 2APBS1[T] and 116-2 form a distinct clade of denitrifying members of the genus *Rhodanobacter*; no other recognized member of

Table 1. Differential phenotypic features among strain 2APBS1[T], strain 116-2 and the type strains of closely related *Rhodanobacter* species

Strains: 1, 2APBS1[T]; 2, 116-2; 3, *R. thiooxydans* DSM 18863[T] (data from Lee *et al.*, 2007, except where indicated); 4, *R. soli* KCTC 22620[T] (Bui *et al.*, 2010); 5, *R. fulvus* DSM 18449[T] (Im *et al.*, 2004); 6, *R. spathiphylli* DSM 17631[T] (De Clercq *et al.*, 2006); 7, *R. terrae* DSM 19241[T] (Weon *et al.*, 2007); 8, *R. ginsenosidimutans* DSM 21013[T] (An *et al.*, 2009). −, Negative; +, positive; + +, strong positive; + + +, very strong positive; W, weak positive; ND, no data.

Characteristic	1	2	3	4	5	6	7	8
DNA G+C content (mol%)	67.5	ND	64.6	65.4	65.3	67.6	62.5	65.6
Motility	+	+	−	+	+	−	−	−
Cell size (μm)								
Length	3–5	ND	1.5–3	2.3	0.7–1	1.5–4	2.0–5.0	1.1–1.3
Width	0.25–0.5	ND	0.6–0.8	0.5	0.2–0.5	0.8	0.5	0.4–0.6
Optimum growth temperature (°C)	30	30	30*	30	25	30	30	28
Gelatin hydrolysis	+	−	−*	+	−	−	W	−
Urea hydrolysis	−	−	−*	+	−	+	+	+
Enzyme activity (ZYM test)								
Valine arylamidase	+	−	+*	+	−	+	W	−
Cystine arylamidase	−	+	+*	+	−	W	−	−
Acid phosphatase	+	+	W*	+	+	+	W	−
Naphthol-AS-BI phosphohydrolase	+	+	+*	+	+	W	+	−
Esterase (C8)	+	+	+*	+	+	W	+	W
α-Galactosidase	+	−	−*	−	+	−	−	−
β-Galactosidase	−	−	−*	−	W	−	+	−
α-Glucosidase	W	+	W*	−	W	−	−	W
β-Glucosidase	+	+	+*	−	+	−	+	+
N-Acetyl-β-glucosaminidase	+	+	+*	−	+	−	W	−
β-Glucuronidase	−	−	+*	ND	ND	ND	ND	ND
Trypsin	−	−	+	ND	ND	ND	ND	ND
Assimilation of:								
Mannose	−	−	−*	−	+	−	W	+
Glucose	+	+	+*	W	+	+	+	+
Maltose	+ +	+ +	+ +*	W	+	+	W	+
D-Ribose	+	+	W	−	−	−	−	−
Glycogen	+ +	−	W	−	−	−	W	−
L-Arabinose	−	−	−*	−	−	−	+	−
Melibiose	+	−	−	−	+	−	−	+
Salicin	+	−	−	−	+	−	−	+
Sodium acetate	+ + +	+ +	−*	W	−	+	−	−
Malic acid	−	−	−	−	−	−	+	−
N-Acetylglucosamine	+ +	+ +	+ +*	−	+	+	+	+
3-Hydroxybutyric acid	+	+	−*	+	W	+	−	−
Quinone	Q-8	ND	Q-8	Q-8	Q-8	ND	ND	Q-8

*Data generated from this study along with all tests conducted on strains 2APBS1[T] and 116-2.

the genus appears to be capable of nitrate respiration. Furthermore, mass balance data indicated that strain 2APBS1 converted >80 % of added nitrate to gaseous nitrogen in pure culture, satisfying the criteria for true denitrifiers as described by Mahne & Tiedje (1995). Thus, based on distinctive 16S rRNA gene sequences, complemented by DNA–DNA hybridization values, and the novel physiological capability of denitrification, we demonstrate that strains 2APBS1[T] and 116-2 are members of a novel species of the genus *Rhodanobacter*. Phylogenetic analyses of 16S rRNA gene sequences, chemotaxonomic data, specifically

iso-$C_{15:0}$, iso-$C_{17:1}\omega 9c$ and iso-$C_{16:0}$ as major fatty acids, and the presence of Q-8 as the sole quinone support the classification of strains 2APBS1[T] and 116-2 in the genus *Rhodanobacter*. Acetate is also a well-utilized carbon source for denitrifying members of this lineage, whereas it is poorly used or not used at all by non-denitrifying members of the genus.

Based on the data presented here, strain 2APBS1[T] (isolated from a neutrophilic, moderately contaminated subsurface) is considered to represent a novel species of the genus *Rhodanobacter*, for which the name *Rhodanobacter*
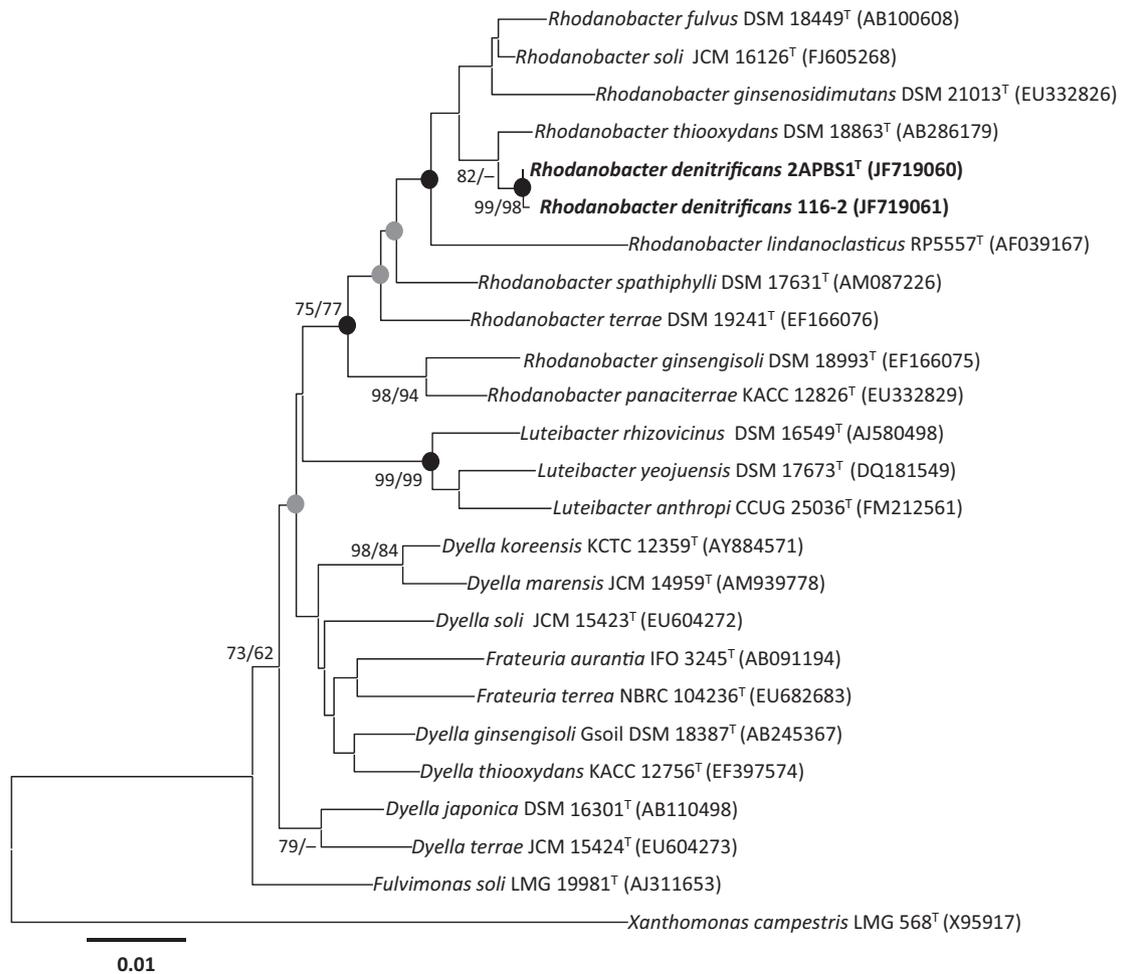
Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences of members of the *Xanthomonadaceae* in the *Gammaproteobacteria*, including strains 2APBS1[T] and 116-2 isolated from the subsurface of the OR-IFRC site (bold type). The tree was constructed by using the maximum composite likelihood method, implemented within the software package MEGA, based on manually aligned sequences. Only sites shared by all sequences were compared, yielding an alignment of 1345 base positions. Multiple tree-creation methods were employed, including bootstrapped neighbour-joining (1000 iterations), bootstrapped maximum-likelihood (1000 iterations) and Bayesian analysis (as described in the text). Nodes supported by neighbour-joining (NJ) and maximum-likelihood (ML) bootstrap values of greater than 70 % are indicated by numbers (NJ/ML). Nodes supported by Bayesian analysis with posterior probability values of greater than 70 % (grey circles) or 95 % (filled circles) are indicated. Bar, 0.01 substitutions per nucleotide position.

denitrificans sp. nov. is proposed. Strain 116-2, isolated from an acidic and more highly contaminated area of the same site, is a different strain of the species, and the two strains have adapted to distinct geochemical conditions.

Description of *Rhodanobacter denitrificans* sp. nov.

Rhodanobacter denitrificans (de.ni.tri′fi.cans. N.L. v. *denitrificare* to denitrify; N.L. part. adj. *denitrificans* denitrifying).

Facultatively anaerobic, and capable of complete denitrification; grows anoxically by using nitrate (NO_3^-), nitrite (NO_2^-) and nitrous oxide (N_2O) as sole electron acceptors. Slow-growing, Gram-negative, non-spore-forming, motile,

rod-shaped bacterium that forms yellow, smooth colonies (1.5–2.0 mm) on R2A agar after 7 days of incubation at 30 °C. Grows poorly on nutrient agar, LB agar and tripticase soy agar with elevated NaCl, but good growth is observed with 100 mg NaCl l^{-1}. Tolerates up to 2 % NaCl. Growth occurs at pH 4–8, optimally at pH 6.5. The temperature range for growth is 10–35 °C with an optimum at 30 °C. Negative for urease, but positive for gelatinase, catalase and oxidase activity. Contains iso-$C_{15:0}$ and iso-$C_{17:1}\omega9c$ as the predominant saturated and unsaturated fatty acids, followed by iso-$C_{16:0}$, iso-$C_{11:0}$ and iso-$C_{11:0}$ 3-OH. Assimilates glucose, maltose, D-ribose, glycogen, L-arabinose, salicin, sodium acetate, *N*-acetylglucosamine and 3-hydroxybutyric acid. Positive for activity of valine

arylamidase, cystine arylamidase, acid phosphatase, alkaline phosphatase, naphthol-AS-BI phosphohydrolase, esterase (C8), α-glucosidase, β-glucosidase and N-acetyl-β-glucosaminidase, but negative for cystine arylamidase, β-galactosidase, β-glucuronidase, trypsin, α-mannosidase and fucosidase. Ubiquinone-8 (Q-8) is the only quinone, and the polar lipid profile comprises diphosphatidylglycerol, phosphatidylglycerol, phosphatidylglycerolethanolamine, one phospholipid and one aminolipid. The G + C content of the genomic DNA of the type strain is 67.5 mol% as derived from a draft genome sequence.

The type strain, 2APBS1^T (=DSM 23569^T=JCM 17641^T), was isolated from nitrate- and uranium-contaminated subsurface sediment of Area 2 of the OR-IFRC site, Oak Ridge, TN, USA. Strain 116-2 (=DSM 24678=JCM 17642), isolated from Area 3 of the OR-IFRC site, is a second strain of the species.

Acknowledgements

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