Desulfoviregula thermocuniculi gen. nov., sp. nov., a thermophilic sulfate-reducer isolated from a geothermal underground mine in Japan

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A thermophilic, Gram-positive, endospore-forming, sulfate-reducing bacterial strain, designated RL80JIV^T, was isolated from a geothermally active underground mine in Japan. Cells were rod-shaped and motile. The temperature and pH ranges for growth were 61–80 °C (optimum at 69–72 °C) and pH 6.4–7.9 (optimum at pH 6.8–7.3), and the strain tolerated up to 0.5 % NaCl. Strain RL80JIV^T utilized sulfate, sulfite, thiosulfate and elemental sulfur as electron acceptors. Electron donors utilized were H₂ in the presence of CO₂, and carboxylic acids. Fermentative growth occurred on lactate and pyruvate. The cell wall contained meso-diaminopimelic acid and the major respiratory isoprenoid quinone was menaquinone MK-7. Major whole-cell fatty acids were iso-C_{15:0}, iso-C_{17:0} and C_{16:0}. Strain RL80JIV^T was found to be affiliated with the thiosulfate-reducer *Thermanaeromonas toyohensis* DSM 14490^T (90.9 % 16S rRNA gene sequence similarity) and with the sulfate-reducer *Desulfotomaculum thermocisternum* DSM 10259^T (90.0 % similarity). Strain RL80JIV^T is therefore considered to represent a novel species of a new genus, for which the name *Desulfoviregula thermocuniculi* gen. nov., sp. nov. is proposed. The type strain of *Desulfoviregula thermocuniculi* is RL80JIV^T (=DSM 16036^T=JCM 13928^T).

Thermophilic sulfate-reducers have increasingly attracted interest owing to their potential in biotechnological applications, such as biodesulfurization of flue gases and biohydrometallurgical processes. The majority of thermophilic sulfate-reducers have been isolated from geothermal habitats, including oilfield waters (Rosnes *et al.*, 1991; Beeder *et al.*, 1994, 1995), hot springs (Zeikus *et al.*, 1983; Nazina *et al.*, 1989; Karnauchow *et al.*, 1992; Henry *et al.*, 1994) and geothermal groundwaters (Daumas *et al.*, 1988; Love *et al.*, 1993). Thermophilic sulfate-reducers include Gram-positive bacteria, such as *Desulfotomaculum* (Campbell & Postgate, 1965) and *Thermodesulfobium* (Mori *et al.*, 2003) within the class 'Clostridia'. Gram-negative sulfate-reducers have been found in genera such as *Thermodesulfarhabdus* (Beeder *et al.*, 1995) and *Desulfacinum* (Rees *et al.*, 1995) within the class *Deltaproteobacteria*, *Thermodesulfobacterium* (Zeikus *et al.*, 1983) and *Thermodesulfatator* (Moussard *et al.*, 2004) within the class *Thermodesulfobacteria* and *Thermodesulfovibrio* (Henry *et al.*, 1994) within the class 'Nitrospira'. Additionally, one archaeal sulfate-reducing genus, *Archaeoglobus* (Beeder *et al.*, 1994), has been described. Recently, we have reported the enrichment and isolation of

thermophilic sulfate-reducers from a geothermal underground mine in Japan (Kaksonen *et al.*, 2006a, b) and in this paper we describe a novel thermophilic sulfate-reducing bacterium, strain RL80JIV^T, isolated from the same mine.

Strain RL80JIV^T was isolated from an underground mine 250 m below ground, from a black sediment layer beneath a thin red layer on a tunnel wall that was covered with ferric iron. The *in situ* temperature of the habitat was 70–80 °C. Enrichment and isolation of strain RL80JIV^T were performed at 80 °C using modified Postgate growth medium (pH 7.0–7.5) (Kaksonen *et al.*, 2006a) with lactate as electron donor. Anaerobic roll-tubes solidified with 1.5 % Gelrite gellan gum were used for isolation. For chemotaxonomic analysis and DNA isolation the strain was cultured at 70 °C in modified DSM medium 641 containing pyruvate as electron donor. The medium was supplemented with 1 ml selenate/tungstate solution l⁻¹ (DSM medium 385), and sodium dithionite (25 mg l⁻¹) was used as the reducing agent instead of Na₂S.

The isolate was examined via phase-contrast microscopy (Zeiss Axioskop 2). Flagellum staining was performed as described by Heimbrook *et al.* (1989). Spore formation by the strain was determined microscopically, and by testing for growth after heat treatment (95 °C for 25 min). Gram

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain RL80JIV^T is DQ208689.

Table 1. Characteristics of strain RL80JIV^T in comparison with those of its closest phylogenetic relatives

Taxa: 1, strain RL80JIV^T; 2, *Thermanaeromonas toyohensis* (Mori *et al.*, 2002); 3, *Desulfotomaculum thermocisternum* (Nilsen *et al.*, 1996); 4, *Desulfotomaculum kuznetsovii* (Nazina *et al.*, 1989); 5, *Desulfotomaculum luciae* (Liu *et al.*, 1997); 6, *Desulfotomaculum thermobenzoicum* subsp. *thermobenzoicum* (Tasaki *et al.*, 1991); 7, *Desulfotomaculum thermoacetoxidans* (Min & Zinder, 1990); 8, *Desulfotomaculum geothermicum* (Daumas *et al.*, 1988); 9, *Desulfotomaculum solfataricum* (Goorissen *et al.*, 2003); 10, *Desulfotomaculum australicum* (Love *et al.*, 1993). +, Positive; –, negative; +/–, variable; NR, not reported. All shared the following characteristics: rod-shaped cells, spore formation and oxidation of lactate (10 mM). Strain RL80JIV^T utilized heptanoate, crotonate and glutamate (each 10 mM), decanoate (1 mM) and nonanoate (0.5 mM) as electron donors, but did not utilize Fe(III) (10 mM) as electron acceptor or tartrate, isobutyrate, citrate, mannose, galactose, *myo*-inositol, lactose or trehalose (each 10 mM), pentanol (5 mM), phenylacetate (3 mM), 4-hydroxybenzoate or octanoate (each 2 mM), dodecanoate or tetradecanoate (each 1 mM) as electron donors. The concentrations apply only to this study.

Characteristic	1	2	3	4	5	6	7	8	9	10
Motility	+	–	+	+	+	+	+	+	–	+
Gram stain	+	NR	NR	–	+/–	+	–	–	–	–
Gram reaction	+	–*	NR	+†	NR	+	NR	NR	+†	NR
Temperature range (°C)	61–80	55–73	41–75	50–85	50–70	40–70	45–65	37–56	48–65	40–74
Optimal temperature (°C)	69–72	70	62	60–65	NR	62	55–60	54	60	68
pH range	6.4–7.9	5.5–8.5	6.2–8.9	NR	6.3–7.8	6–8	6.0–7.5	6–8	6.4–7.9	5.5–8.5
Optimal pH	6.8–7.3	6.5	6.7	7.0–7.2	NR	7.2	6.5	7.2–7.4	7.3	7–7.4
NaCl range (%)	0–0.5	0–1	0–4.7	0–3	<2	0	0–1.5	0.2–5	0–1.5	>0
Optimal salinity (NaCl %)	0	NR	0.3–1.2	0	NR	0	0	2.4–3.4	0	NR
DNA G+C content (mol%)	60.1	49.6	56–57	49	51.4	52.8	49.7	50.4	48.3	48.1
Electron acceptors (all 10 mM)										
Sulfate	+	–	+	+	+	+	+	+	+	+
Sulfite	+	–	+	+	–	+	–	+	+	NR
Thiosulfate	+	+	+	+	+	+	+	NR	+	NR
Sulfur	+	–	–	–/+†	NR	–	+	–	+†	NR
Nitrate	–	+	–	–	NR	+	–	–	–	NR
Nitrite	–	+	NR	NR	NR	NR	–	NR	NR	NR
Electron donors‡										
H ₂ /CO ₂ (80:20, v/v)	+	NR	+	+	+	+	+	+	+	+
Formate (10 mM)	–	+	–	+	+	+	+	+	+	–
Acetate (10 mM)	–	–	–	+	–	–	+	–	+	+
Propionate (10 mM)	–	NR	+	+	NR	+	+	+	+	–
Butyrate (10 mM)	+	NR	+	+	NR	+	+	+	+	–
Pentanoate (10 mM)	+	NR	+	+	NR	+	+	NR	+†	NR
Hexanoate (2 mM)	+	NR	+	+	NR	+	NR	+	+†	NR
Hexadecanoate (1 mM)	–	NR	+	+	NR	NR	–	+	+†	+
Benzoate (5 mM)	–	NR	–	–	NR	+	–	–	–	+
Pyruvate (10 mM)	+	+	+	+	+	+	+	NR	+†	+
Malate (10 mM)	–	–	NR	+	–	+	+	NR	+†	–
Fumarate (10 mM)	+	–	NR	+	NR	+	NR	NR	+	–
Succinate (10 mM)	–	–	NR	+	NR	–	+	NR	+	–
Methanol (10 mM)	–	NR	–	+	–	–	NR	–	+	–
Ethanol (10 mM)	–	NR	+	+	+	+	–	+	+	+
Propanol (10 mM)	–	NR	+	+	NR	+	+	NR	+	NR
Butanol (10 mM)	–	NR	+	+	NR	+	+	NR	+	NR
Glucose (10 mM)	–	+	NR	–	–	–	–	NR	+	–
Fructose (10 mM)	–	+	–	–	NR	–	NR	+	+	NR
Sucrose (10 mM)	–	+	NR	NR	NR	–	–	NR	NR	NR
Cellobiose (10 mM)	–	+	NR	NR	NR	NR	–	NR	NR	NR
Fermentation of: (all 10 mM)										
Pyruvate	+	+	+	+	+	+	+	NR	+†	+
Lactate	+	+	NR	+	+	+	–	NR	+	+

*Gram-positive type cell wall observed by transmission electron microscopy.

†Data obtained from Kaksonen *et al.* (2006b).

‡With sulfate, except for *T. toyohensis*, for which thiosulfate was used.

Table 2. Whole-cell fatty acid composition of strain RL80JIV^T

ECL, Equivalent chain length; DMA, dimethyl acetal; *c*, *cis* isomer.

ECL	Fatty acid	Content (%)
13.62	iso-C _{14:0}	0.4
14.00	C _{14:0}	1.3
14.63	iso-C _{15:0}	36.2
14.71	anteiso-C _{15:0}	1.8
14.95	C _{16:0} aldehyde	0.5
15.00	C _{15:0}	1.2
15.11	iso-C _{15:0} DMA	1.3
15.63	iso-C _{16:0}	3.9
16.00	C _{16:0}	15.7
16.47	C _{16:0} DMA	1.9
16.63	iso-C _{17:0}	16.6
16.72	anteiso-C _{17:0}	1.3
17.00	C _{17:0}	0.4
17.11	iso-C _{17:0} DMA	7.1
17.20	anteiso-C _{17:0} DMA	0.6
17.72	C _{18:2} ω6,9 <i>c</i>	0.6
17.77	C _{18:1} ω9 <i>c</i>	2.3
18.00	C _{18:0}	0.7
	Total unidentified	6.3

type of the cells was determined by both Gram staining and the KOH test (Gegersen, 1978).

The effects of temperature, pH and NaCl concentration on growth were determined as previously described (Kaksonen *et al.*, 2006a). The ability of the strain to utilize various electron donors (1–20 mM) was tested in a medium containing 20 mM sulfate. The utilization of various electron acceptors (10 mM) was studied using lactate (10 mM) as the electron donor. Amorphous Fe(III) oxyhydroxide was formed by neutralizing FeCl₃ solution to a pH of 7 with NaOH. Cultures were incubated for 1–2 weeks. Electron donor utilization was determined by measuring growth (optical density at 660 nm; Shimadzu

UV-1601 spectrophotometer or Ultrospec II LKB Biochrom 4050 UV/visible spectrophotometer), or based on hydrogen sulfide production or substrate conversion as described previously (Kaksonen *et al.*, 2004). Ferrous iron was determined colorimetrically (Shimadzu UV-1601) with ferrozine (Stookey, 1970). Concentrations of sulfate, sulfite, thiosulfate, nitrate and nitrite were determined by using ion chromatography (Dionex DX-120).

Diaminopimelic acid isomers were detected in cell-wall hydrolysates by using TLC as described by Rhuland *et al.* (1955) and Kaksonen *et al.* (2006b). Respiratory isoprenoid quinones were extracted and analysed according to the methods described by Collins & Jones (1981), Monciardini *et al.* (2003) and Groth *et al.* (1996) by using HPLC and GC-MS (Kaksonen *et al.*, 2006b).

Fatty acid methyl esters of cellular fatty acids were obtained by saponification, methylation, extraction and base wash, as described by Kämpfer & Kroppenstedt (1996), Kroppenstedt (1985) and Miller (1982). The fatty acid methyl mixtures were separated on a gas chromatograph (Hewlett Packard 5890 Series II) as described previously (Kaksonen *et al.*, 2006b) and analysed by using the Microbial Identification Standard software package Sherlock version 4.5 (Sasser, 1990).

Methods for the amplification, sequencing and phylogenetic analysis of the 16S rRNA gene were performed as described previously (Kaksonen *et al.*, 2006a). Genomic DNA for G+C content determination was released by rupturing cells using a French pressure cell (Thermo Spectronic) and was purified by chromatography on hydroxyapatite (Cashion *et al.*, 1977). The DNA was hydrolysed with P1 nuclease and the nucleotides were dephosphorylated with bovine alkaline phosphatase (Mesbah *et al.*, 1989). The G+C content of the resulting deoxyribonucleosides was determined by using reversed-phase HPLC (Shimadzu Corp.) and calculated from the ratio of deoxyguanosine (dG) and thymidine (dT) (Tamaoka & Komagata, 1984; Mesbah *et al.*, 1989).

Fig. 1. Phylogenetic tree generated using distance-matrix and neighbour-joining methods based on the 16S rRNA gene sequences of strain RL80JIV^T (1436 bp between *Escherichia coli* positions 28 and 1453) and related taxa. *Archaeoglobus veneficus* SNP6^T (Y10011) was used as an outgroup (not shown). Numbers at nodes represent bootstrap percentages based on 1000 samplings. Bar, 0.05 nucleotide changes per position.

Cells of strain RL80JIV^T were straight or slightly curved rods, 0.8–1 µm in diameter and 2–6 µm in length. The strain formed spherical spores which germinated after a heat shock treatment at 95 °C for 25 min. The spores were located centrally or subterminally and sporulation caused swelling of the cells, creating a lemon-shaped appearance. Cells were motile with two or more flagella and Gram-positive as determined by both Gram staining and the KOH test. Data for temperature, pH and NaCl range for growth of strain RL80JIV^T are given in Table 1. The temperature at the sampling point in the mine was 70–80 °C, which is also within the growth range of strain RL80JIV^T. Strain RL80JIV^T was able to use sulfate, sulfite, thiosulfate and elemental sulfur as electron acceptors. RL80JIV^T utilized H₂ in the presence of CO₂ and various carboxylic acids or their sodium salts as electron donors (Table 1). A number of electron donors (e.g. lactate, pyruvate, crotonate, pentanoate and hexanoate) were oxidized to acetate, whereas no acetate accumulated during the oxidation of others (e.g. fumarate and glutamate). The strain fermented lactate and pyruvate.

The cell wall of strain RL80JIV^T contained *meso*-diaminopimelic acid and the major respiratory isoprenoid quinone was menaquinone MK-7. The whole-cell fatty acid composition of strain RL80JIV^T is given in Table 2. Significant proportions of saturated fatty acids were found; the major fatty acids were iso-C_{15:0}, iso-C_{17:0} and C_{16:0} (Table 2).

Phylogenetic analysis (Fig. 1) revealed that strain RL80JIV^T clustered with the thiosulfate-reducing bacterium *Thermanaeromonas toyohensis* DSM 14490^T (90.9% 16S rRNA gene sequence similarity), an isolate from a similar geothermal mine environment (Mori *et al.*, 2002), and with the sulfate-reducer *Desulfotomaculum thermocisternum* DSM 10259^T (90.0% sequence similarity). The G+C content of the total DNA of strain RL80JIV^T (60.1 mol%) (Table 1) was higher than that of *T. toyohensis* (49.6 mol%) and those reported for thermophilic *Desulfotomaculum* species (47–57 mol%) (Castro *et al.*, 2000; Goorissen *et al.*, 2003; Liu *et al.*, 1997), thereby differentiating strain RL80JIV^T from *T. toyohensis* and species of the genus *Desulfotomaculum*.

Strain RL80JIV^T exhibits many features typical for the sulfate-reducing members of the genus *Desulfotomaculum* and the thiosulfate-reducing members of the genus *Thermanaeromonas*, such as rod-shaped, Gram-positive, spore-forming cells and MK-7 as the major menaquinone. However, based on 16S rRNA gene sequence analysis, the strain is only distantly related to representatives of the two genera. Moreover, the DNA G+C content of strain RL80JIV^T is comparatively high and its closest phylogenetic relative, *T. toyohensis*, does not reduce sulfate. The phylogenetic and phenotypic distinctiveness of strain RL80JIV^T indicates that it represents a novel species of a new genus, for which the name *Desulfoviregula thermocuniculi* gen. nov., sp. nov. is proposed.

Description of *Desulfoviregula* gen. nov.

Desulfoviregula (De.sul.fo.vir'gu.la. L. pref. *de* from; L. n. *sulfur* sulfur; L. fem. n. *virgula* twig or rod, N.L. fem. n. *Desulfoviregula* a rod that reduces sulfur compounds).

Cells are rod-shaped, spore-forming, Gram-positive and thermophilic. Sulfate and other sulfur compounds are used as electron acceptors. Optimal growth occurs at neutral pH. The cell wall contains *meso*-diaminopimelic acid and MK-7 is the major menaquinone. Major cellular fatty acids are iso-C_{15:0}, iso-C_{17:0} and C_{16:0}. The genomic DNA G+C content of the type species is 60.1 mol% (as determined by HPLC). Phylogenetic position based on 16S rRNA gene sequence analysis is within the Gram-positive bacteria. The type species is *Desulfoviregula thermocuniculi*.

Description of *Desulfoviregula thermocuniculi* sp. nov.

Desulfoviregula thermocuniculi (ther.mo.cu.ni'cu.li. Gr. adj. *thermos* hot; L. n. *cuniculus* mine; N.L. gen. n. *thermocuniculi* of a hot mine).

Has the following properties in addition to those given in the description of the genus. Cells (0.8–1 × 2–6 µm) are motile. Growth occurs at 61–80 °C (optimum 69–72 °C), pH 6.4–7.9 (optimum pH 6.8–7.3) and NaCl concentrations of 0–0.5% (optimum 0% NaCl). Sulfate, sulfite, thiosulfate and elemental sulfur are used as electron acceptors, but Fe(III), nitrate and nitrite are not. H₂ in the presence of CO₂ and various carboxylic acids or their sodium salts (Table 1) are used as electron donors. Fermentative growth occurs on lactate and pyruvate.

The type strain, RL80JIV^T (=DSM 16036^T =JCM 13928^T), was isolated from a geothermally active underground mine in Japan.

Acknowledgements

This work was supported by the Finnish Funding Agency for Technology and Innovation, Outokumpu Oyj, Finland, Finnish Graduate School in Environmental Science and Technology, Academy of Finland and European Commission (BioMinE contract 500329 and a grant for the DSMZ large-scale facility). Annukka Hämäläinen, Esther Schüller, Anika Vester and Marlen Jando are thanked for their technical assistance.

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