**Calditerrivibrio nitroreducens** gen. nov., sp. nov., a thermophilic, nitrate-reducing bacterium isolated from a terrestrial hot spring in Japan

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A moderately thermophilic, nitrate-reducing bacterium, strain Yu37-1^T^, was isolated from hot spring water from Yumata, Nagano, Japan. Cells of strain Yu37-1^T^ were strictly anaerobic, Gram-negative, non-sporulating, motile by means of a single polar flagellum, vibrio-shaped and 1.4–2.0 μm long. The temperature and pH for optimum growth were 55 °C and pH 7.0–7.5, respectively. Strain Yu37-1^T^ grew best in basal medium without the addition of NaCl. Acetate, pyruvate, lactate, fumarate, succinate, malate, yeast extract, peptone and Casamino acids were utilized as electron donors, with nitrate as the only electron acceptor. Ammonium was the end product from nitrate. The G+C content of the genomic DNA was 35.1 mol%. Phylogenetic analysis based on the 16S rRNA gene revealed that strain Yu37-1^T^ could be accommodated in the family *Deferribacteraceae* and that its closest neighbours were members of the five genera of the family *Deferribacteraceae*, namely *Deferribacter*, *Denitrovibrio*, *Flexistipes*, *Geo vibrio* and *Mucispirillum*, with similarities of only 83.2–86.2 %. The growth temperature and salinity range for growth of strain Yu37-1^T^ differed from those of the phylogenetically related organisms. On the basis of phenotypic features and phylogenetic position, a novel genus and species are proposed, *Calditerrivibrio nitroreducens* gen. nov., sp. nov. Strain Yu37-1^T^ (=NBRC 101217^T^ =DSM 19672^T^) is the type strain of *Calditerrivibrio nitroreducens*.

Dense microbial mats and streamers often develop in hot spring water streams. Microbiological diversity in such hot springs has been investigated phylogenetically by both culture-dependent and -independent approaches (Barns et al., 1994; Hiraishi et al., 1999; Hugenholtz et al., 1998; Nakagawa & Fukui, 2002, 2003; Ward et al., 1990; Yamamoto et al., 1998). By culture-independent quinone profile analyses, the dense microbial mats in Japanese hot springs such as Yumata and Nakanoyu have been shown to be inhabited by so-called ‘sulfur-turf bacteria’, *Chloroflexus* species, cyanobacteria and purple phototrophic bacteria (Hiraishi et al., 1999). Moreover, microbial streamers have been analysed by a 16S rRNA gene-based phylogenetic approach and shown to contain bacteria related to *Sulfurihydrogenibium* and *Thermodesulfobacteria* (Nakagawa & Fukui, 2003).

On the other hand, isolation of novel thermophilic microorganisms from Japanese hot springs has been rare due to the difficulty in cultivation, although thermophilic archaea and bacteria such as *Vulcanisaeta distrubita* (Itoh et al., 2002), *Sulfurihydrogenibium subterraneum* (Takai et al., 2003a) and *Methylothermus thermalis* (Tsubota et al., 2005) have been isolated from terrestrial hot springs. We attempted to isolate a variety of anaerobic microorganisms from terrestrial hot springs at Yumata in Japan, the microbiological diversity of which have been studied previously (Hiraishi et al., 1999; Nakagawa & Fukui, 2003). In this paper, the isolation of a moderately thermophilic, nitrate-reducing bacterium is described. On the basis of its morphological, biochemical, physiological, chemotaxonomic and phylogenetic properties, it is proposed that this bacterium represents a novel species in a new genus.

Water samples were collected from a sulfide-rich hot spring at Yumata, Nagano, Japan (36° 23’ 10’’ N 137° 45’ 40’’ E). The temperature and pH of the water sample were 60 °C and pH 7.6, respectively. The samples were kept in nitrogen-gas-flushed vials sealed with tight-fitting butyl rubber stoppers until transfer to fresh medium.

For the enrichment, 1 ml hot spring water was used to inoculate 20 ml NAB medium in a vial sealed with a

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Calditerrivibrio nitroreducens* Yu37-1^T^ is AB364234.
tight-fitting butyl rubber stopper. NAB medium was composed of 0.54 g NH₄Cl, 0.14 g KH₂PO₄, 0.20 g MgCl₂·6H₂O, 0.15 g CaCl₂·2H₂O, 2.5 g NaHCO₃, 0.85 g sodium nitrate, 0.82 g sodium acetate, 1.0 ml trace element solution (Sekiguchi et al., 2000) and 1 l distilled water. Prior to inoculation, the pH of the medium was adjusted to 6.8, dissolved air was removed by flushing with H₂/CO₂ (4:1, v/v; about 150 kPa), and 10 ml vitamin stock solution (50.0 g l⁻¹) 1⁻¹ were added. The enrichment culture was cultivated at 60 °C for 5 days and transferred several times to fresh NAB medium. After bacterial growth was observed in NAB medium, the culture was spread on slants of NAB medium solidified with 1.5 % (w/v) agar and colonies that appeared were picked and streaked on the same medium. The purification procedure was repeated several times to establish an axenic culture of a strain designated Yu37-1T. Further studies of strain Yu37-1T were carried out using the same medium that was used for enrichment (NAB medium).

Cells of strain Yu37-1T were vibrio-shaped and approximately 0.4–0.5 μm wide and 1.4–2.0 μm long (Fig. 1a, b). Cells usually occurred singly or in pairs. Vibrating motility was observed by phase-contrast microscopy. A single polar flagellum was observed by electron microscopic examination. The Gram reaction of the cells was negative. Spore formation was not observed using phase-contrast microscopy.

Strain Yu37-1T was a strictly anaerobic bacterium that was capable of growing in NAB medium under a N₂/CO₂ (4:1, v/v) atmosphere, but could not grow under microaerobic or aerobic conditions. It was negative for the catalase reaction. The growth temperature for strain Yu37-1T ranged from 30 to 65 °C, with an optimum at 55 °C. No growth was observed at 25 or 70 °C. The pH range for growth was pH 5.5–8.0, with an optimum at pH 7.0–7.5. No growth was observed at pH 5.0 or 8.5. Growth occurred at NaCl concentrations below 0.5 % (w/v); optimum growth in the basal medium was observed without the addition of NaCl, which is equivalent to approximately 0.05 % Na⁺ and 0.1 % Cl⁻. No growth was observed in 1 % (w/v) NaCl. Strain Yu37-1T grew using nitrate (20 mM) as the electron acceptor in the presence of acetate as the electron donor. Ammonium was detected as an end product from the culture of strain Yu37-1T grown using nitrate, as determined by using Ion Selective Pack Test model WAK-NH₄ (Kyoritsu Chemical-Check Lab.); N₂ and sulfide were not detected, determined by GC equipped with Molecular Sieve 5A (Shinwa Chemical Industries) and HPLC equipped with an IC-Pak Anion column (Waters), respectively. Elemental sulfur [1 % (w/v)], sulfate (10 mM), thiosulfate (10 mM), sulfite (1 mM), nitrite (1 mM), iron (III) oxide (10 mM), iron (III) citrate (10 mM), manganese (IV) oxide (10 mM), selenate (1 mM), selenite (1 mM), arsenate (1 mM), arsenite (1 mM), fumarate (10 mM) and oxygen [2 % (v/v) in N₂] were not utilized as alternative electron acceptors in the presence of acetate. Strain Yu37-1T could use acetate (20 mM), pyruvate (20 mM), lactate (20 mM), fumarate (20 mM), succinate (20 mM), malate (20 mM), yeast extract [0.01 % (w/v); Difco], peptone [0.01 % (w/v); Difco] or Casamino acids [0.01 % (w/v); Difco] as electron donors in the presence of nitrate. H₂/CO₂ (4:1 (v/v), about 150 kPa), formate (5 mM), propionate (5 mM), butyrate (5 mM), citrate (5 mM), methanol (5 mM), ethanol (5 mM), glucose (5 mM), fructose (5 mM), lactose (5 mM) and sucrose (5 mM) were not utilized as electron donors in the presence of nitrate. Fermentative growth on pyruvate (20 mM), lactate (20 mM), fumarate (20 mM), succinate (20 mM), malate (20 mM), yeast extract [0.01 % (w/v); Difco], peptone [0.01 % (w/v); Difco] or Casamino acids [0.01 % (w/v); Difco] was not observed in the absence of nitrate as electron acceptor. Strain Yu37-1T was susceptible to ampicillin, chloramphenicol, gentamicin, kanamycin, streptomycin, tetracycline and vancomycin (all at 100 μg ml⁻¹), but was resistant to bacitracin and rifampicin (both at 100 μg ml⁻¹).

The major cellular fatty acids were iso-C₁₄:0 (26.3 %) and anteiso-C₁₃:0 (24.1 %), as determined by using the MIDI microbial identification system (Microbial ID; Agilent Technologies) based on the method described by Sasser (1990). C₁₆:0 (6.2 %), C₁₈:0 (7.2 %), iso-C₁₃:0 (7.7 %), anteiso-C₁₃:0 (5.3 %) and iso-C₁₆:0 (5.7 %) were also detected as minor components. The major quinone was identified as menaquinone MK-8, determined using the HPLC method described by Komagata & Suzuki (1987). The genomic DNA G+C content of strain Yu37-1T was 35.1 mol%, determined by the HPLC method described by Tamaoka & Komagata (1984).

![Fig. 1. Phase-contrast (a) and transmission electron (b) micrographs of cells of strain Yu37-1T. Bars, 5.0 μm (a) and 0.5 μm (b).](image-url)
The 16S rRNA gene of strain Yu37-1T was amplified by PCR and an almost-complete 16S rRNA gene sequence (1487 bases) was determined as described previously (Iino et al., 2007). The 16S rRNA gene sequence of strain Yu37-1T showed similarities of 83.8–86.2% to bacteria belonging to the five genera of the family Deferribacteraceae, Deferribacter, Denitrovibrio, Flexistipes, Geovibrio and Mucispirillum. Similarity to type strains of other bacterial genera was lower than 82%. After alignment with the ARB software (Ludwig et al., 2004), phylogenetic trees were constructed by the neighbour-joining method with the program CLUSTAL_X (Saitou & Nei, 1987; Thompson et al., 1997) and the maximum-likelihood method with the MORPHY software (Adachi & Hasegawa, 1995). Topologies of the trees determined by these two methods were identical and strain Yu37-1T was included in the family Deferribacteraceae, the only family of the order Deferribacterales, the sole order of the class Deferribacteres (Fig. 2).

Morphological, biochemical and physiological properties of strain Yu37-1T are summarized in Table 1, along with those of phylogenetically related organisms. Strain Yu37-1T was isolated from a terrestrial hot spring, whereas most bacteria belonging to the family Deferribacteraceae have been isolated from marine environments such as a North Sea oil reservoir and a deep-sea hydrothermal vent (Fiala et al., 1990; Greene et al., 1997; Janssen et al., 2002; Miroshnichenko et al., 2003; Myhr & Torsvik, 2000; Takai et al., 2003b). The temperature and salinity range for growth of strain Yu37-1T were distinct from those of phylogenetically related organisms, probably because of differences in their habitats. Strain Yu37-1T was moderately thermophilic, whereas members of the genera Deferribacter, Geovibrio and Mucispirillum are mesophilic and members of the genus Flexistipes are slightly thermophilic. In addition, strain Yu37-1T did not grow in 1% (w/v) NaCl, whereas members of the genera Deferribacter, Denitrovibrio and Flexistipes are halophilic.

Some of the morphological and physiological traits of strain Yu37-1T such as vibrio shape, moderate thermophilicity and neutrophilicity were similar to those reported for three species of the genus Deferribacter, namely Deferribacter thermophilus (Greene et al., 1997), Deferribacter abyssii (Miroshnichenko et al., 2003) and Deferribacter desulfuricans (Takai et al., 2003b). However, strain Yu37-1T used only nitrate as an electron acceptor, in contrast to these three Deferribacter species, which are able to use several electron acceptors such as elemental sulfur, nitrate and iron (III) and manganese (IV) salts.

Strain Yu37-1T and Denitrovibrio acetiphilus (Myhr & Torsvik, 2000) were markedly different in the following characteristics: strain Yu37-1T was moderately thermophilic and did not grow fermentatively, whereas Denitrovibrio acetiphilus is mesophilic and can grow fermentatively; the genomic DNA G+C content of strain Yu37-1T was lower than that of Denitrovibrio acetiphilus (42.6 mol%); and strain Yu37-1T showed single polar flagellation, in contrast to bipolar flagellation of Denitrovibrio acetiphilus.

Strain Yu37-1T was significantly different from Flexistipes sinusarabici (Fiala et al., 1990), Geovibrio ferrireducens (Caccavo et al., 1996), Geovibrio thiophilus (Janssen et al., 2002) and Mucispirillum schaedi (Robertson et al., 2005) in its morphological and physiological properties. Cells of strain Yu37-1T were distinctly smaller than cells of F. sinusarabici, G. ferrireducens, G. thiophilus and M. schaedi. Strain Yu37-1T was also different in terms of motility, flagellation and/or the ability to grow by fermentative metabolism. In addition, strain Yu37-1T could be distinguished from F. sinusarabici, G. ferrireducens, G. thiophilus and M. schaedi in the utilization of electron acceptors. The genomic DNA G+C content of strain Yu37-1T was lower than those of members of the genera Flexistipes and Geovibrio.

On the basis of phylogenetic position, morphology and biochemical and physiological properties described above, strain Yu37-1T differed significantly from members of any of the genera of the family Deferribacteraceae. Consequently, a novel species in a new genus in the family Deferribacteraceae, Calditerrivibrio nitroreducens gen. nov., sp. nov., is proposed.

**Description of Calditerrivibrio gen. nov.**

Calditerrivibrio (Cal.di.terri.vib’ri.o. L. adj. caldus hot; L. fem. n. terra the earth; N.L. masc. n. vibrio a vibrio; N.L. masc. n. Calditerrivibrio a vibrio existing in a hot terrestrial environment).

Strictly anaerobic, moderately thermophilic, neutrophilic and chemo-organoheterotrophic bacteria. Stain Gram-negative, non-sporulating and motile by a single polar flagellum. Cells are vibrio-shaped. Catalase is not produced. The major cellular fatty acids are iso-C15:0 and anteiso-C15:0. The major quinone is MK-8. The genus

![Fig. 2. Phylogenetic tree of strain Yu37-1T and related species based on 16S rRNA gene sequences. The tree was based on an alignment of 1209 bp of 16S rRNA gene sequences and constructed by using the neighbour-joining method. Numbers at nodes indicate bootstrap percentages, derived from 1000 bootstrap replications (determined by neighbour-joining analysis/maximum-likelihood method); −, not determined. Bar, 0.02 substitutions per nucleotide position.](http://ijs.sgmjournals.org)
represents a distinct phylogenetic lineage in the family Deferribacteraceae based on 16S rRNA gene sequence analysis. The type species is *Calditerrivibrio nitroreducens*.

**Description of *Calditerrivibrio nitroreducens* sp. nov.**

*Calditerrivibrio nitroreducens* (nit.ro.reduc ens. Gr. n. nitron nitre, nitrate; L. part. adj. reducens drawing backwards, bringing back to a state or condition; N.L. part. adj. nitroreducens nitrate-reducing).

The following properties are given in addition to the genus description. Cells are 0.4–0.5 μm. Growth occurs at 30–65 °C, with the optimum at 55 °C. The pH range for growth is pH 5.5–8.0, with an optimum around pH 7.0–7.5. Growth occurs below 0.5% (w/v) NaCl, with optimum growth in the basal medium without addition of NaCl. Nitrate is the only electron acceptor utilized, with ammonium as the end product. Elemental sulfur, sulfate, sulfite, nitrite, iron (III) oxide, manganese (IV) oxide, selenate, selenite, arsenate, arsenite, fumarate and oxygen are not used as alternative electron acceptors. Acetate, pyruvate, lactate, fumarate, succinate, malate, yeast extract, peptone and Casamino acids are utilized as electron donors with nitrate as the electron acceptor. Fermentative growth is not observed.

The type strain is Yu37-1T (=NBRC 101217T =DSM 19672T), isolated from hot spring water in Yumata, Japan. The genomic DNA G+C content of the type strain is 35.1 mol%.

**Table 1.** Morphological, biochemical and physiological properties of strain Yu37-1T (*Calditerrivibrio nitroreducens* gen. nov., sp. nov.) and phylogenetically related genera

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<td>Straight to bent rods</td>
<td>Vibrios</td>
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<td>Optimum growth conditions</td>
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*Values indicate NaCl concentrations added to the basal medium.

References


Itoh, T., Suzuki, K. & Nakase, T. (2002). *Vulcanisaeta distributa* gen. nov., sp. nov., and *Vulcanisaeta souina* sp. nov., novel hyperther-


