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Isolation from oil reservoirs of novel thermophilic anaerobes phylogenetically related to *Thermoanaerobacter subterraneus*: reassignment of *T. subterraneus*, *Thermoanaerobacter yonseiensis*, *Thermoanaerobacter tengcongensis* and *Carboxydibrachium pacificum* to *Caldanaerobacter subterraneus* gen. nov., sp. nov., comb. nov. as four novel subspecies

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Novel thermophilic, anaerobic, Gram-positive, rod-shaped bacteria, strains SL9 and OCA1, were isolated from oilfields in France and Australia, respectively. Both strains, together with *Thermoanaerobacter yonseiensis* KB-1^T (=DSM 13777^T), *Thermoanaerobacter tengcongensis* MB4^T (=DSM 15242^T) and *Carboxydibrachium pacificum* JM^T (=DSM 12653^T), possessed genomic (DNA–DNA hybridization studies) and phylogenetic similarities with *Thermoanaerobacter subterraneus* SEBR 7858^T (=DSM 13054^T), which was isolated recently from an oilfield reservoir in south-west France. Marked phenotypic differences exist between the three oilfield isolates (*T. subterraneus*, strain OCA1 and strain SL9): they include temperature range for growth and substrates used. Differences were also observed in the DNA G+C contents of all organisms. Similarly to *T. subterraneus*, strains SL9 and OCA1, and also *T. yonseiensis*, *T. tengcongensis* and *Carboxydibrachium pacificum*, produced acetate and L-alanine as major end products of glucose metabolism [0·8–1·0 mol L-alanine produced (mol glucose consumed)$^{-1}$] and reduced thiosulfate, but not sulfate, to sulfide. Because of these significant metabolic and phylogenetic differences between the oilfield isolates (*T. subterraneus*, strain OCA1 and strain SL9), *T. yonseiensis*, *T. tengcongensis* and *Carboxydibrachium pacificum* and other *Thermoanaerobacter* species, it is proposed to reassign them as a novel genus and species, *Caldanaerobacter subterraneus* gen. nov., sp. nov., comb. nov., with the creation of four novel subspecies, *Caldanaerobacter subterraneus* subsp. *subterraneus* subsp. nov., comb. nov., *Caldanaerobacter subterraneus* subsp. *yonseiensis* subsp. nov., comb. nov., *Caldanaerobacter subterraneus* subsp. *tengcongensis* subsp. nov., comb. nov. and *Caldanaerobacter subterraneus* subsp. *pacificus* subsp. nov., comb. nov.

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Abbreviation: RDP, Ribosomal Database Project.

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INTRODUCTION

Petroleum reservoirs constitute a group of unique terrestrial sites, as they present an unusual combination of extreme environmental conditions, including temperature, pressure and salinity. It is also known that petroleum composition varies widely between reservoirs, which may

have an impact on the microbial biodiversity of such environments. Attention has recently been paid to the microbial ecology of petroleum reservoirs, where anaerobes have always been considered to be the dominant micro-organisms. They include fermentative and sulfate-reducing bacteria and methanogenic *Archaea* (Magot *et al.*, 2000). Among the fermentative anaerobes, thermophiles have been studied the most, probably because most oil reservoirs occur at a depth where *in situ* temperatures exceed 100 °C (Stetter *et al.*, 1993). Many of them belong to the *Bacteria* (Jeanthon *et al.*, 1995; Ravot *et al.*, 1995a; Grassia *et al.*, 1996; Fardeau *et al.*, 1997; Rees *et al.*, 1997). They include members of the family '*Thermoanaerobiaceae*' (genera *Thermoanaerobacter* and *Thermoanaerobacterium*) that were isolated from low-saline reservoirs (Grassia *et al.*, 1996). Most oilfield isolates of the genus *Thermoanaerobacter* have not been characterized formally, except for two isolates from French hot continental oil reservoirs, with one being identical to *Thermoanaerobacter thermohydrosulfuricus* (L'Haridon *et al.*, 1995) and the second being a novel species of *Thermoanaerobacter*: *Thermoanaerobacter subterraneus* (Fardeau *et al.*, 2000). Several other strains that were isolated from French and African oilfields were recognized as a novel subspecies of *Thermoanaerobacter brockii*, *T. brockii* subsp. *lactiethylicus* (Cayol *et al.*, 1995). Members of the order *Thermotogales* have also been isolated from oil reservoirs (Stetter *et al.*, 1993). Within this order, novel species of the genera *Thermotoga* (Jeanthon *et al.*, 1995; Ravot *et al.*, 1995a; Fardeau *et al.*, 1997), *Petrotoga* and *Geotoga* (Davey *et al.*, 1993; Lien *et al.*, 1998) have been characterized. Most of these oilfield thermoanaerobes, which belong to the family '*Thermoanaerobiaceae*' (Fardeau *et al.*, 1993, 1996) or to the order *Thermotogales* (Ravot *et al.*, 1995b; Lien *et al.*, 1998), reduced thiosulfate mostly to sulfide. All produced acetate as a major end product of sugar metabolism. L-Alanine production from sugar catabolism by *T. subterraneus* (Fardeau *et al.*, 2000) and members of the order *Thermotogales* was of particular interest; this metabolic trait has been interpreted as a possible remnant of an ancestral metabolism (Ravot *et al.*, 1996).

Here, we describe novel thermophilic anaerobes, strains SL9 and OCA1, that were isolated from oil reservoirs in France and Australia and that reduce thiosulfate to sulfide and produce L-alanine from sugar metabolism. Similarly to *Carboxydibrachium pacificum* (Sokolova *et al.*, 2001), *Thermoanaerobacter yonseiensis* (Kim *et al.*, 2001) and *Thermoanaerobacter tengcongensis* (Xue *et al.*, 2001), they share genotypic and phylogenetic similarities with *T. subterraneus* (Fardeau *et al.*, 2000), which was isolated recently from an oil reservoir in the Paris Basin, France. These six thermoanaerobes are proposed as members of a novel genus of the family '*Thermoanaerobiaceae*', *Caldanaerobacter* gen. nov., comb. nov.

METHODS

Sample and strain source. Oil–water mixture samples were collected in sterile 250 ml glass bottles from production well-heads in France and Australia through a tapping that was fitted on the production line. Samples were stored at 4 °C before being used as inocula. *Carboxydibrachium pacificum* JM^T (= DSM 12653^T), *T. yonseiensis* KB-1^T (= DSM 13777^T), *T. brockii* DSM 1457^T and *Thermoanaerobacter ethanolicus* DSM 2246^T were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Braunschweig, Germany. *T. tengcongensis* $MB4^T$ (= DSM 15242^T) was provided by the authors.

Media and cultivation conditions. The technique of Hungate (1969) was used throughout this study. Enrichments with oilfield samples were performed in medium that contained (l distilled water)$^{-1}$: 1 g NH_4Cl, 3·45 g PIPES, 0·3 g K_2HPO_4, 0·3 g KH_2PO_4, 0·2 g $MgCl_2.6H_2O$, 0·1 g $CaCl_2.2H_2O$, 15 g NaCl, 0·1 g KCl, 0·5 g sodium acetate, 1 g glucose, 5 g yeast extract (Difco), 5 g bio-trypticase (bioMérieux) and 0·001 g resazurin. *T. subterraneus*, *T. tengcongensis* and *T. yonseiensis* were cultivated in medium that contained (l distilled water)$^{-1}$: 1 g NH_4Cl, 0·3 g K_2HPO_4, 0·3 g KH_2PO_4, 0·1 g $CaCl_2.2H_2O$, 0·1 g KCl, 0·5 g $MgCl_2.6H_2O$, 1 g yeast extract, 2 g NaCl, 3·6 g glucose, 0·5 g cysteine/HCl and 10 ml trace elements solution (Balch *et al.*, 1979). *Carboxydibrachium pacificum* was cultivated in the latter medium, modified by replacing NaCl by 30 g sea salts and reducing the yeast extract concentration to 0·5 g l^{-1}. Depending on the medium used, the pH was adjusted to 7·0 with either HCl (5 M) or KOH (10 M). After autoclaving, thiosulfate (10 mM) was added. Aliquots of the medium (9 ml) were then dispensed into Hungate tubes. Prior to inoculation, $Na_2S.9H_2O$ was injected from sterile stock solutions, to obtain a final concentration of 0·1 % (w/v).

Isolation. Isolation medium (9 ml) was inoculated with 1 ml oil–water mixture, pressurized with N_2 (100 kPa) and incubated without shaking at the reservoir temperature (65 °C). Positive enrichments were subcultured and purified by streaking onto plates that contained the same medium, solidified with 0·7 % (w/v) Phytagel (Sigma). Plates were incubated in anaerobic jars pressurized with N_2 (100 kPa) at 65 °C for 3 days. Cultures of *T. subterraneus* were also incubated at 65 °C, whereas those of *T. tengcongensis*, *T. yonseiensis* and *Carboxydibrachium pacificum* were incubated at 70 °C.

Characterization. Temperature, pH and NaCl ranges for growth were determined by using the following medium, which contained (l distilled water)$^{-1}$: 1 g NH_4Cl, 0·3 g K_2HPO_4, 0·3 g KH_2PO_4, 0·5 g $MgCl_2.6H_2O$, 0·1 g $CaCl_2.2H_2O$, 1 g NaCl, 0·1 g KCl, 0·5 g cysteine/HCl, 2 g yeast extract, 0·001 g resazurin and 10 ml trace elements solution (Balch *et al.*, 1979). In Hungate tubes, medium was adjusted to different pH values by injecting $NaHCO_3$ or Na_2CO_3 from 10 % (w/v) sterile anaerobic stock solutions. For studies of NaCl requirements, NaCl was weighed directly in tubes prior to dispensing the medium. Substrates were tested at a final concentration of 20 mM in enrichment medium. To test for electron acceptors, sodium thiosulfate, sodium sulfate and elemental sulfur were added to the medium at final concentrations of 20 mM, 20 mM and 2 % (w/v), respectively.

Analytical techniques. Growth was measured by inserting tubes directly into a Cary 50 Scan UV–visible spectrophotometer (Varian) and measuring OD_{580}. Sulfide was determined photometrically as colloidal CuS, by using the method of Cord-Ruwisch (1985). H_2, CO_2, sugars, alcohols and volatile and non-volatile fatty acids were measured as described previously (Fardeau *et al.*, 1996). L-Alanine was measured by HPLC (Moore *et al.*, 1958). Light microscopy was performed as described previously (Fardeau *et al.*, 1997).

Determination of DNA G+C content. DNA G+C content was determined at DSMZ. DNA was isolated and purified by chromatography on hydroxyapatite and its G+C content was determined by using HPLC, as described by Mesbah *et al.* (1989). Non-methylated λ DNA (Sigma) was used as the standard.

DNA–DNA hybridization studies. DNA was isolated by chromatography on hydroxyapatite by the procedure of Cashion *et al.* (1977). DNA–DNA hybridization was performed at DSMZ as described by De Ley *et al.* (1970), with the modification described by Huss *et al.* (1983) and Escara & Hutton (1980), by using a Gilford System model 2600 spectrophotometer equipped with a Gilford model 2527-R thermoprogrammer and plotter. Renaturation rates were computed with the program TRANSFER.BAS (Jahnke, 1992).

16S rRNA gene sequence analysis. The 16S rRNA gene was amplified as described previously (Miranda-Tello *et al.*, 2003). PCR products were cloned by using a pGEM-T Easy cloning kit (Promega), according to the manufacturer's protocols. Clone libraries were screened by direct PCR amplification from a colony by using the vector-specific primers SP6 (5′-ATTTAGGTGACACTATAGAA-3′) and T7 (5′-TAATACGACTCACTATAGGG-3′) and the following reaction conditions: 2 min at 96 °C; 40 cycles of 30 s at 94 °C, 1 min at 55 °C and 3 min at 72 °C; and a final extension of 10 min at 72 °C. Plasmids that contained an insert of the right length were isolated by using the Wizard Plus SV Minipreps DNA purification system (Promega), according to the manufacturer's protocol. Purified plasmids were sent for sequencing to Genome Express (Grenoble, France). Sequence data were imported into the sequence editor BioEdit version 5.0.9 (Hall, 1999), base-calling was examined and a contiguous consensus sequence was obtained for each isolate. The full sequence was aligned by using the Ribosomal Database Project (RDP)'s Sequence Aligner program (Maidak *et al.*, 2001). The consensus sequence was then adjusted manually to conform to the 16S rRNA secondary structure model (Winker & Woese, 1991). A non-redundant BLASTN search (Altschul *et al.*, 1997) of the full sequence through GenBank (Benson *et al.*, 1999) identified its closest relatives. Sequences used in phylogenetic analysis were obtained from the RDP (Maidak *et al.*, 2001) and GenBank (Benson *et al.*, 1999). Positions of sequence and alignment ambiguity were omitted and pairwise evolutionary distances, based on 1152 unambiguous nucleotides, were calculated by using the method of Jukes & Cantor (1969). Dendrograms were constructed by using the neighbour-joining method (Saitou & Nei, 1987). Confidence in the tree topology was determined by using 100 bootstrapped trees (Felsenstein, 1993). GenBank accession numbers of rDNA sequences from reference organisms are included in Fig. 1.

RESULTS AND DISCUSSION

Although microaerophilic micro-organisms have been reported to inhabit oil reservoirs (Voordouw *et al.*, 1996), their activities have never been interpreted as significant, as the oil-bearing subsurface is essentially anaerobic. Anaerobes have therefore been considered to be dominant in this ecosystem (Magot *et al.*, 2000). The importance of micro-organisms that are closely related, morphologically and physiologically, to members of the order *Thermotogales*, namely the genera *Thermotoga*, *Geotoga* and *Petrotoga*, has been established from various hot oil reservoirs throughout the world (Davey *et al.*, 1993; Stetter *et al.*, 1993; Jeanthon *et al.*, 1995; Ravot *et al.*, 1995a; Grassia *et al.*, 1996; Fardeau *et al.*, 1997; Lien *et al.*, 1998; Magot *et al.*, 2000). Members of the family '*Thermoanaerobiaceae*', which includes the genera *Thermoanaerobacter* and *Thermoanaerobacterium*, were also isolated frequently, in particular from low-saline reservoirs (Cayol *et al.*, 1995; L'Haridon *et al.*, 1995; Grassia *et al.*, 1996; Magot *et al.*, 2000). Species of the genus *Thermoanaerobacter* reduced thiosulfate to sulfide, whereas those of the genus *Thermoanaerobacterium* reduced thiosulfate to elemental sulfur. It was reported recently that, similarly to some members of the order *Thermotogales* (Ravot *et al.*, 1996), a novel species of *Thermoanaerobacter*, *T. subterraneus* (Fardeau *et al.*, 2000), also produced L-alanine as a major end product of glucose fermentation. Enrichment cultures that were performed at 65 °C for 3 days with oil–water mixture samples that originated from oil reservoirs in France and Australia led to the isolation of strains SL9 and OCA1, respectively. Both strains were related phylogenetically to *T. subterraneus* (Table 1). Strains SL9 and OCA1 were rod-shaped bacteria, $0{\cdot}5\times2{\cdot}5$–$8{\cdot}0$ μm, that mostly occurred singly or in pairs. They did not grow in oxidized medium. They grew at temperatures that ranged from 45 to 80 °C (no growth was obtained at 85 °C for strain SL9 or at 80 °C for strain OCA1). At pH 7·0, growth was optimum around 75 °C for strain SL9 and around 70 °C for strain OCA1 (data not shown). Growth occurred at initial pH values between 5·7 and 9·2 at 70 °C; the optimum was at pH 7·0 (data not shown). The isolates grew in the presence

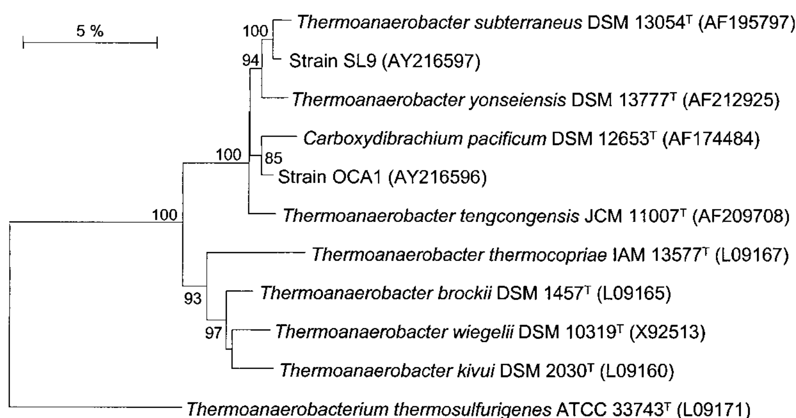

Fig. 1. Dendrogram indicating the position of strains SL9 and OCA1 amongst members of the family '*Thermoanaerobiaceae*'. Sequences were obtained from the RDP (Maidak *et al.*, 2001). Bootstrap values, expressed as percentages of 100 replications, are shown at branching points. Only values >80 % are considered to be significant and reported. Bar, 5 substitutions in 100 nt.

Table 1. Discriminating characteristics of subspecies of *Caldanaerobacter subterraneus* gen. nov., sp. nov., comb. nov.

All subspecies produced L-alanine (determined in this study), acetate, H_2 and CO_2 as diagnostic fermentation products from glucose. +, Positive; −, negative; ND, not determined.

Characteristic	*Caldanaerobacter subterraneus* subspecies			
	*subterraneus**	*tengcongensis*†	*yonseiensis*‡	*pacificus*§
Type strain	DSM 13054^T	JCM 11007^T	DSM 13777^T	DSM 12653^T
Source	Oil well	Hot spring	Geothermal water	Hydrothermal vent, oil well
Temperature for growth (°C):				
Range	40–75	50–80	50–85	50–80
Optimum	65	75	75	70
pH for growth:				
Range	6·0–8·5	5·5–9·0	4·5–9·0	5·8–7·6
Optimum	7·5	7·0–7·5	6·5	6·8–7·2
NaCl concentration for growth (%):				
Range	0–3	0–2·5	0–4	ND
Optimum	0	0·2	0	2–2·5
DNA G+C content (mol%)	41	33	37	33
Use of CO	−	+	+	+
Diagnostic fermentation products from glucose:				
Ethanol	−	+	+	−
Lactate	+	−	+	−

*Data from Fardeau *et al.* (2000).
†Data from Xue *et al.* (2001).
‡Data from Kim *et al.* (2001).
§Data from Sokolova *et al.* (2001).

of NaCl concentrations that ranged from 0 to 2 %. Growth was optimum in the absence of NaCl at pH 7·0 (data not shown). Yeast extract was required for growth on carbohydrates and could not be replaced by vitamins. Strains SL9 and OCA1 grew on the following substrates (at a concentration of 20 mM unless indicated otherwise) in the presence of thiosulfate as the electron acceptor: D-fructose, D-galactose, D-glucose, DL-maltose, D-mannose, D-ribose, starch and glycerol. In contrast to strain OCA1, strain SL9 used cellobiose, lactose, D-xylose and pyruvate and did not use CO. Mannitol, melibiose and xylan were not used by either strain. In the presence of thiosulfate, the glucose metabolic profile of both strains changed dramatically, with an increase in acetate production and disappearance of L-alanine production. Use of valine, isoleucine and leucine was highly enhanced by the presence of thiosulfate. Valine, isoleucine and leucine were oxidized to isobutyrate, 2-methylbutyrate and isovalerate, respectively. Acetate, L-alanine, H_2 and CO_2 were the major end products of glucose fermentation (in the absence of thiosulfate). Similarly to *T. subterraneus*, strains SL9 and OCA1 produced around 1 mol L-alanine (mol glucose consumed)$^{-1}$. Small amounts of lactate were produced, but no ethanol was produced from glucose fermentation by either strain. Serine was fermented to acetate. These bacteria reduce thiosulfate to sulfide and have a DNA G+C content of 38·4 (strain SL9) and 39·4 (strain OCA1) mol%.

As well as phylogenetic and phenotypic similarities, strains SL9 and OCA1 share genotypic similarities with *T. subterraneus* (DNA–DNA hybridization values between *T. subterraneus* and strains SL9 and OCA1 were 92·4 and 78·5 %, respectively), suggesting that they all belong to the same species. Surprisingly, the closest phylogenetic relatives of *T. subterraneus*, including *Carboxydibrachium pacificum* (96·8 % sequence similarity), *T. tengcongensis* (97·7 %) and *T. yonseiensis* (98 %), also presented significant DNA–DNA homology (values ⩾70 %) with this species (DNA–DNA hybridization values between *T. subterraneus* and the three species *T. tengcongensis*, *T. yonseiensis* and *Carboxydibrachium pacificum* were 67·9, 70·5 and 91·0 %, respectively; DNA–DNA hybridization values between *T. tengcongensis* and the two species *T. yonseiensis* and *Carboxydibrachium pacificum* were 78·3 and 74·4 %, respectively; DNA–DNA hybridization between *T. yonseiensis* and *Carboxydibrachium pacificum* was 90·4 %), so that they should also be considered as members of *T. subterraneus* (Wayne *et al.*, 1987). Despite phylogenetic and genomic similarities, significant phenotypic differences were found between all these micro-organisms (Table 1), including their DNA G+C contents and their ability to oxidize CO. In particular, *T. subterraneus*, strain OCA1 and strain SL9 have been isolated from oil reservoirs several hundred kilometres apart, thus suggesting that the phenotypical characteristics that distinguish strains SL9 and OCA1 from *T. subterraneus*

might have resulted from *in situ* physicochemical conditions of the oil wells from which they originated. These findings raise questions on the possible indigenous nature of such micro-organisms in the subsurface environment and in oilfield reservoirs, as hypothesized previously by L'Haridon *et al.* (1995) and Ollivier *et al.* (1998).

To elucidate the taxonomic status of *T. subterraneus*, strains SL9 and OCA1, *Carboxydibrachium pacificum*, *T. yonseiensis* and *T. tengcongensis*, we conducted further experiments to determine whether L-alanine is a major end product of glucose fermentation by the type species of the genus *Thermoanaerobacter*, *T. ethanolicus*, and *T. brockii*, the closest phylogenetic relative of the above bacteria (mean sequence similarity of 93 %). Both micro-organisms are known to be acetate/ethanol/lactate producers (Zeikus *et al.*, 1979; Wiegel & Ljungdahl, 1981), but have not been reported to produce L-alanine from glucose metabolism. We demonstrated that besides ethanol, *T. ethanolicus* and *T. brockii* also produced L-alanine during glucose catabolism [values of $<0{\cdot}2$ mol L-alanine produced (mol glucose consumed)$^{-1}$], but only as a minor end product, compared to *T. subterraneus* and its phylogenetic relatives (strains SL9 and OCA1, *Carboxydibrachium pacificum*, *T. yonseiensis* and *T. tengcongensis*). Therefore, the metabolic products of sugar fermentation by *T. subterraneus*, strains SL9 and OCA1, *Carboxydibrachium pacificum*, *T. yonseiensis* and *T. tengcongensis*, which produce significant quantities of alanine [approx. 1 mol L-alanine produced (mol glucose consumed)$^{-1}$], clearly differ from those of *T. ethanolicus* and *T. brockii*. In addition, all these bacteria constitute a distinct phylogenetic lineage in the family '*Thermoanaerobiaceae*' (Fig. 1). Most of them (*T. tengcongensis*, *T. yonseiensis*, *Carboxydibrachium pacificum* and strain SL9) grow at 80 °C and are extreme thermophiles, rather than thermophiles. To our knowledge, among the 18 species and subspecies of the genus *Thermoanaerobacter*, only *T. tengcongensis T. yonseiensis* (both to be reclassified – this report) and *T. brockii* grow at 80 °C (Zeikus *et al.*, 1979; Schmid *et al.*, 1986; Cayol *et al.*, 1995). Due to high DNA homologies (values close to or higher than 70 %) and high 16S rRNA gene sequence similarities, *T. subterraneus*, *T. tengcongensis*, *T. yonseiensis*, *Carboxydibrachium pacificum*, strain OCA1 and strain SL9 should belong to the same species (Wayne *et al.*, 1987). According to Rule 24a (note 3) of the Bacteriological Code (Lapage *et al.*, 1992), *Carboxydibrachium pacificum* (Sokolova *et al.*, 2001), *T. yonseiensis* (Kim *et al.*, 2001) and *T. tengcongensis* (Xue *et al.*, 2001) should be considered as later heterotypic synonyms of *T. subterraneus* (Fardeau *et al.*, 2000), which has the benefit of anteriority over all these micro-organisms. This automatically invalidates the name of the genus *Carboxydibrachium*. Due to significant phylogenetic and metabolic differences between *T. subterraneus* and *Thermoanaerobacter* species, we propose to reassign *T. subterraneus*, strain SL9, strain OCA1, *Carboxydibrachium pacificum*, *T. yonseiensis* and *T. tengcongensis* to a novel genus and species of the family '*Thermoanaerobiaceae*', as *Caldanaerobacter subterraneus* gen. nov., sp. nov., comb. nov. In addition, marked phenotypic differences between these micro-organisms (Table 1) allow clear identification of three novel subspecies of this genus: *Caldanaerobacter subterraneus* subsp. *pacificus* subsp. nov., *Caldanaerobacter subterraneus* subsp. *yonseiensis* subsp. nov. and *Caldanaerobacter subterraneus* subsp. *tengcongensis* subsp. nov. This automatically places the type strain of *T. subterraneus* (DSM 13054^{T}) as the type strain of *Caldanaerobacter subterraneus* subsp. *subterraneus* subsp. nov. According to Fig. 1, strains SL9 and OCA1 are related phylogenetically to *Caldanaerobacter subterraneus* subsp. *subterraneus* and *Caldanaerobacter subterraneus* subsp. *pacificus*, respectively.

Description of *Caldanaerobacter* gen. nov.

Caldanaerobacter (cal.da.nae.ro.bac′ter. L. adj. *caldus* hot; Gr. pref. *an* not; Gr. n. *aer* air; N.L. masc. n. *bacter* equivalent of Gr. neut. n. *bakterion* rod, staff; N.L. masc. n. *Caldanaerobacter* rod that grows in the absence of air at high temperatures).

Cells are straight rods. Gram-reaction is positive or negative. Endospores may be observed. Growth is strictly anaerobic. Thermophilic, fermentative member of the domain *Bacteria*, family '*Thermoanaerobiaceae*'. Carbohydrates serve as fermentable substrates, with acetate and L-alanine as major end products. Approximately 1 mol L-alanine (mol glucose fermented)$^{-1}$ is produced. DNA G+C content is 33–41 mol%. The type species is *Caldanaerobacter subterraneus*.

Description of *Caldanaerobacter subterraneus* sp. nov., comb. nov.

Caldanaerobacter subterraneus (sub.ter.ra′ne.us. L. pref. *sub* less than; L. n. *terra* earth; L. masc. adj. *subterraneus* underground, subterranean, describing its site of isolation).

Basonym: *Thermoanaerobacter subterraneus* Fardeau *et al.* 2000.

Same description as that given for the genus. Acetate, L-alanine, lactate, H_2 and CO_2 are produced during glucose fermentation. Thiosulfate, but not sulfate, is used as an electron acceptor.

The type strain is DSM 13054^{T} (=CNCM I-2383^{T}).

Description of *Caldanaerobacter subterraneus* subsp. *subterraneus* subsp. nov.

Caldanaerobacter subterraneus subsp. *subterraneus* (sub.ter.ra′ne.us. L. pref. *sub* less than; L. n. *terra* earth; L. masc. adj. *subterraneus* underground, subterranean, describing its site of isolation).

Rods ($0{\cdot}5$–$0{\cdot}7\times2$–8 μm) that occur singly or in pairs and possess laterally inserted flagella. Spores are not observed under microscopic examination, but cultures exposed to

120 °C for 45 min can be subcultured, indicating the presence of heat-resistant forms. Electron microscopic examination reveals a Gram-positive cell wall. Round colonies (3 mm in diameter) develop on Phytagel plates or in roll-tubes after 3 days incubation at 70 °C. Chemo-organotrophic and obligately anaerobic member of the domain *Bacteria*, family '*Thermoanaerobiaceae*'. Thermophilic. Optimum temperature for growth is 65–75 °C at pH 7·5; temperature range for growth is 40–80 °C. Optimum pH is 7·0–7·5; growth occurs between pH 5·7 and 9·2. Halotolerant; grows in the presence of up to 3 % NaCl. Yeast extract or bio-trypticase is required for growth on carbohydrates. Growth on sugars is highly enhanced by the presence of both yeast extract and bio-trypticase. Yeast extract cannot be replaced by vitamins. Ferments cellobiose, D-fructose, D-galactose, D-glucose, DL-lactose, DL-maltose, D-mannose, melibiose, D-ribose, starch, D-xylose, glycerol, mannitol, pyruvate and xylan. L-Arabinose, L-rhamnose, sorbose, sucrose, L-xylose and CO are not used. Acetate, L-alanine, lactate, H_2 and CO_2 are produced during glucose fermentation. Elemental sulfur, thiosulfate and sulfite, but not sulfate, are used as electron acceptors. DNA G+C content is 38·4–41 mol% (as determined by HPLC).

The type strain is SEBR 7858^T (=CNCM I-2383^T=DSM 13054^T). Isolated from oilfield waters.

Description of *Caldanaerobacter subterraneus* subsp. *tengcongensis* subsp. nov., comb. nov.

Caldanaerobacter subterraneus subsp. *tengcongensis* (teng.con.gen'sis. N.L. masc. adj. *tengcongensis* pertaining to Tengcong, China).

Basonym: *Thermoanaerobacter tengcongensis* Xue *et al.* 2001.

Description as that given by Xue *et al.* (2001). Approximately 1 mol L-alanine (mol glucose fermented)$^{-1}$ is produced. Oxidizes CO.

The type strain is MB4^T (=JCM 11007^T=DSM 15242^T).

Description of *Caldanaerobacter subterraneus* subsp. *yonseiensis* subsp. nov., comb. nov.

Caldanaerobacter subterraneus subsp. *yonseiensis* (yon.sei.en'sis. N.L. adj. *yonseiensis* pertaining to Yonsei University, Seoul, Korea, in recognition of its support of research into extreme thermophiles and their thermostable enzymes).

Basonym: *Thermoanaerobacter yonseiensis* Kim *et al.* 2001.

Description as that given by Kim *et al.* (2001). Approximately 1 mol L-alanine (mol glucose fermented)$^{-1}$ is produced. Oxidizes CO.

The type strain is KB-1^T (=KFCC 11116^T=DSM 13777^T).

Description of *Caldanaerobacter subterraneus* subsp. *pacificus* subsp. nov., comb. nov.

Caldanaerobacter subterraneus subsp. *pacificus* (pa.ci'fi.cus. L. masc. adj. *pacificus* peaceful; pertaining to the Pacific Ocean, from the western part of which the type strain was isolated).

Basonym: *Carboxydibrachium pacificum* Sokolova *et al.* 2001.

Description as that given by Sokolova *et al.* (2001). Approximately 1 mol L-alanine (mol glucose fermented)$^{-1}$ is produced. Reduces thiosulfate to sulfide. Oxidizes CO.

The type strain is JM^T (=DSM 12653^T). Isolated from a submarine hot vent.

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