Vulcanibacillus modesticaldus gen. nov., sp. nov., a strictly anaerobic, nitrate-reducing bacterium from deep-sea hydrothermal vents

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A novel anaerobic, moderately thermophilic, spore-forming bacterium, designated strain BR^T, was isolated from deep-sea hydrothermal core samples collected at the Rainbow vent field on the Mid-Atlantic Ridge (36° 14′ N 33° 54′ W). The cells were found to be rod-shaped, non-motile, Gram-positive and spore-forming. The organism grew in the temperature range 37–60 °C, with an optimum at 55 °C, and at pH values in the range 6–8·5, with an optimum around pH 7. NaCl concentrations for growth were in the range 10–40 g l^{-1}, with an optimum at 20–30 g l^{-1}. Strain BR^T grew chemo-organoheterotrophically with carbohydrates, proteinaceous substrates and organic acids with nitrate as electron acceptor. The novel isolate was not able to ferment. The G+C content of the genomic DNA was 34·5 mol%. Phylogenetic analysis of the 16S rRNA gene sequence placed strain BR^T in the *Bacillaceae* within the class '*Bacilli*'. On the basis of the phenotypic and phylogenetic data, this isolate should be described as a member of a novel genus, for which the name *Vulcanibacillus* gen. nov. is proposed. The type species is *Vulcanibacillus modesticaldus* sp. nov., with the type strain BR^T (=DSM 14931^T=JCM 12998^T).

Deep-sea hydrothermal vents encompass diverse microbial habitats inhabited by unique microbial communities. Traditional culture-based approaches have led to the characterization of numerous microbes that include mesophiles, thermophiles and hyperthermophiles from diverse trophic groups (Jeanthon, 2000). Of the thermal classes isolated from deep-sea vents, less attention has been paid to mesophiles and moderate thermophiles in comparison with hyperthermophiles. In particular, mesophilic and moderately thermophilic micro-organisms belonging to the *Epsilonproteobacteria*, a prevalent group in culture-independent molecular analyses, have only recently been obtained in culture (Miroshnichenko *et al.*, 2002, 2004; Takai *et al.*, 2003).

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain BR^T is AM050346.

Electron micrographs and details of the polar lipid and cellular fatty acid compositions of strain BR^T are available as supplementary material in IJSEM Online.

Of the thermophiles isolated from these environments, only a few belong to the Gram-positive bacteria. To date, thermophilic Gram-positive isolates from deep-sea hydrothermal vents comprise aerobic bacilli (Marteinsson *et al.*, 1996) and strict anaerobes of the orders '*Thermoanaerobacteriales*' (Sokolova *et al.*, 2001; Fardeau *et al.*, 2004) and *Clostridiales*. The latter include recently described species of the genera *Caloranaerobacter* (Wery *et al.*, 2001), *Clostridium* (Brisbarre *et al.*, 2003) and *Tepidibacter* (Slobodkin *et al.*, 2003; Urios *et al.*, 2004).

In this paper, we describe a strictly anaerobic, moderately thermophilic, Gram-positive, spore-forming bacterium that was isolated from a deep-sea hydrothermal vent sediment sample; this novel organism has phenotypic, chemotaxonomic and phylogenetic characteristics that allow its assignment to a novel genus within the family *Bacillaceae*.

Chimney and core samples were collected at the Rainbow hydrothermal vent field on the Mid-Atlantic Ridge (36° 16′ N 33° 54′ W; 2300 m depth) by the remotely operated vehicle *Victor* during the Iris Cruise. A brief description of

the hydrothermal site and the sampling procedures has been reported elsewhere (Nercessian *et al.*, 2005). Once on board, the samples were aseptically transferred in 50 ml glass vials under N_2. The vials were closed tightly with butyl rubber stoppers (Bellco), pressurized with N_2 (100 KPa), reduced with sodium sulfide, if required, and stored at 4 °C until needed.

The enrichment medium (EM) contained the following (g l^{-1} unless indicated otherwise): NH_4Cl, 0·33; KCl, 0·33; KH_2PO_4, 0·33; $CaCl_2.2H_2O$, 0·33; $MgCl_2.6H_2O$, 0·33; NaCl, 25·0; $NaNO_3$, 3·0, sodium acetate, 2·0; yeast extract, 0·1; trace element solution (Balch *et al.*, 1979), 10 ml l^{-1}; vitamin solution (Wolin *et al.*, 1963), 10 ml l^{-1}. The medium was prepared anaerobically (Balch *et al.*, 1979) and dispensed in 15 ml Hungate tubes; the headspace (5 ml) was filled with N_2 (atmospheric pressure). No reducing agents were added to the medium. The pH of the medium was 6·5. Single colonies were isolated using EM in which acetate had been replaced by tryptone (2 g l^{-1}) and which had been solidified with 1·5 % agar (Difco), using a serial 10-fold dilution technique in agar shake tubes. During routine cultivation of strain BR^T in the laboratory, it was shown that the addition of $FeSO_4.7H_2O$ (0·1 g l^{-1}) and yeast extract (0·5 g l^{-1}) to the medium significantly improved the stability and reproducibility of cell growth. An Olympus BH-2 microscope equipped with an Olympus OM-2 camera was used routinely to observe and count the bacteria. Gram staining was carried out as described by Murray *et al.* (1994). Staining of intracellular inclusions was done with Sudan black B, according to the protocol of Murray *et al.* (1994), and with Nile blue A, as described by Spring *et al.* (2005). Cells of *Malikia granosa* DSM 15619^T that accumulate polyhydroxyalkanoates served as the control. The ultrastructure of whole cells and thin-section preparations was studied using a model JEM-100 electron microscope. Cells were prepared as described previously (Bonch-Osmolovskaya *et al.*, 1990).

The influence of pH on growth was assessed using 10 mM acetate/acetic acid buffer, MES, PIPES, HEPES and Tris between pH 4 and 8·5 and without buffer above pH 8·5. The pH of the medium was adjusted after sterilization of the medium. The effect of NaCl on growth was determined on the same medium containing increasing NaCl concentrations. The effects of pH and of NaCl concentrations were determined at the optimal temperature for growth. Physiological tests were performed in EM from which sodium acetate and sodium nitrate had been omitted. Proteinaceous potential growth substrates were added at a concentration of 1 g l^{-1} to the EM medium without acetate; carbohydrates, sodium salts of organic acids and alcohols were tested at a concentration of 3 g l^{-1}. When molecular hydrogen served as substrate, the headspace (10 ml) was filled with a H_2/CO_2 mixture (4 : 1, v/v). Possible electron acceptors, such as elemental sulfur (5 g l^{-1}), thiosulfate (10 mM), sulfate (10 mM) and nitrite (10 mM), were tested. The ability to reduce amorphous Fe(III) oxide (90 mM) or Fe(III) citrate (20 mM) was tested as described previously (Slobodkin *et al.*, 1999). The ability to grow aerobically was determined in 100 ml flasks containing the same medium (10 ml). To check for microaerophilic growth, air was added to the headspace (10 ml) of tubes filled with EM (5 ml) lacking sodium nitrate. The final oxygen concentration varied from 0·5 to 9 %.

Growth was determined by measuring changes in turbidity at 600 nm by inserting culture tubes directly in a Spectronic 20D spectrophotometer (Bioblock). All growth experiments were performed in duplicate. NO, N_2O and N_2 were tested by GC with a Porapak-Q column at 70 °C and at flow rates of 4 ml min^{-1} (the carrier gas was argon). For quantitative nitrite analysis, 0·1 ml culture medium was added to a mixture containing 0·9 ml deionized water, 0·5 ml 0·6 % solution of sulfanilic acid in 20 % HCl and 0·5 ml (60 mg per 50 ml) solution of *N*-naphthylethylenediamine. The absorbance at 548 nm was measured after 15 min (the time necessary for colour development). For quantitative nitrate analysis, the method of Cataldo *et al.* (1975) was used. Oxidase activity was assayed using discs impregnated with dimethyl-*p*-phenylenediamine (bioMérieux). Catalase activity was assayed by mixing a pellet of fresh culture with a drop of H_2O_2 (10 %, v/v).

EM medium was inoculated with different samples collected at the Rainbow hydrothermal site. After incubation at 55 °C, long thin rods were observed in enrichments from one chimney sample and two core samples. The cultures were diluted and transferred into EM agar. After 5 days incubation at 60 °C, single cream-coloured colonies about 2 mm in diameter were visible in the agar shake tubes. The pure culture obtained after regrowth in liquid EM medium was designated strain BR^T. Cells of strain BR^T consisted of long thin rods, 4–5 μm in length and 0·2–0·3 μm in diameter (see Supplementary Fig. S1 in IJSEM Online). Cells generally occurred singly and stained Gram-positive. In the early phase of growth, inclusions of reserve polymers were frequently detected, and caused the cells to swell. The reserve compound was highly refractile under phase-contrast microscopy and stained black with the lipophilic stain Sudan black B. In contrast, the affinity for the stain Nile blue A, which is known to be highly specific for polyhydroxyalkanoates, was quite low, such that no red fluorescence could be detected by epifluorescence microscopy. Hence, we presume that the reserve compound might represent an unknown storage lipid. Flagella were not seen on negatively stained electron preparations, and no swimming motility was observed. Spore-forming cells could be observed in the early stage of propagation of the strain after its isolation. Cells lysed rapidly at the beginning of the stationary phase of growth. Colonies showed a negative reaction for oxidase and catalase.

Strain BR^T grew at temperatures between 37 and 60 °C, with an optimum at 55 °C; no growth was observed at 30 or 65 °C. The pH range for growth was between 6 and 8·5, with an optimum at pH 7; no growth was observed at

pH 5·5 or 9. Strain BR^T grew with NaCl concentrations ranging from 10 to 40 g l^{-1}, with an optimum at 20–30 g l^{-1}; no growth was observed without NaCl or in the presence of 50 g NaCl l^{-1}.

Strain BR^T was strictly anaerobic: it was unable to grow under aerobic or microaerophilic conditions. Nitrate was essential for growth and was reduced to nitrite. Nitrate was not further reduced to ammonia or N_2. Elemental sulfur, thiosulfate, sulfate, nitrite, amorphous Fe(III) oxide (90 mM) and Fe(III) citrate (20 mM) could not be used as alternative electron acceptors. The substrates used for growth in the presence of nitrate as electron acceptor were acetate, yeast extract, tryptone, Bio-Trypticase, Bactopeptone, sucrose, glucose, fructose, starch, cellobiose, ethanol and pyruvate. Malate, succinate, methanol, formate, lactate, galactose, arabinose, lactose, butyrate and propionate were not used. No fermentative growth was observed.

Cell-wall preparations were obtained by boiling cells in 10 % (w/v) aqueous trichloroacetic acid solution for 20 min, washing them with water and subsequently treating them with trypsin at 37 °C, as described by Schleifer & Kandler (1972). Diaminopimelic acid isomers were detected in cell-wall hydrolysates (4 M HCl, 100 °C, 16 h) by TLC on cellulose sheets (Merck), using the solvent system of Rhuland *et al.* (1955).

Respiratory lipoquinones and polar lipids were extracted from 100 mg freeze-dried cell material using the two-stage method described by Tindall (1990a, b). Fatty acids were analysed as the methyl ester derivatives prepared from 10 mg dry cell material. Cells were subjected to differential hydrolysis in order to detect ester-linked and non-ester-linked (amide-bound) fatty acids (B. J. Tindall, unpublished). Fatty acid methyl esters were analysed by GC using a 0·2 μm × 25 m non-polar capillary column and flame-ionization detection. The run conditions were as follows: injection and detector port temperature, 300 °C; inlet pressure, 60 kPa, split ratio, 50 : 1; injection volume, 1 μl; temperature program, 130–310 °C at a rate of 4 °C min^{-1}.

The peptidoglycan of strain BR^T contained *meso*-diaminopimelic acid as the diagnostic diamino acid, which is consistent with the occurrence of peptidoglycan type A1γ according to Schleifer & Kandler (1972). The predominant menaquinone was MK-7. The fatty acids comprised a mixture of straight-chain and branched (iso- and anteiso-) fatty acids (see Supplementary Table S1); i-15 : 0 predominates among the fatty acids (> 69 %), but ai-15 : 0, 16 : 0, i-17 : 0 (4·8–8·1 %) and 14 : 0, 15 : 0, ai-16 : 0, ai-17 : 0, 18 : 1ω9 and 18 : 0 (1·0–2·6 %) are also present. The polar lipids comprised a comparatively simple pattern of phospholipids, including phospholipids and aminophospholipids. The major polar lipids comprised phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, two unidentified phospholipids and an unidentified aminophospholipid (see Supplementary Fig. S2).

The presence of predominantly iso- and/or anteiso-fatty acids, the absence of hydroxy fatty acids and the presence of menaquinones as the major respiratory quinones are features of organisms associated with '*Bacillus* and relatives'. Although most of the members of this group produce MK-7, there is evidence that shorter (MK-6) or longer (MK-8 or MK-9) menaquinones may also predominate, providing a limited degree of differentiation within the group. With regard to the fatty acids, i-/ai-15 : 0 or i-/ai-17 : 0 usually predominate, although the relative proportions of the fatty acids, together with the presence/absence of other fatty acids, may indicate that there are larger groupings within the bacilli. The resolution of such data, however, are not usually sufficient to allow clear definition of groupings that might correlate with genera (Kämpfer, 1994, 2002). With regard to the polar lipids, the chemical diversity of various *Bacillus* species is well documented (Minnikin & Minnikin, 1981; O'Leary & Wilkinson, 1988), although there have been no serious attempts to use such data, together with physiological, morphological and genetic data, to define groupings within the bacilli that might be regarded as genera.

In describing a novel taxon that, through 16S rRNA gene sequence analysis, shows a degree of similarity to the type species of the genus *Bacillus* (*Bacillus subtilis*), it is important not only to develop a concept of what constitutes the novel genus, but also to consider how the genus *Bacillus* should be defined in the future. Although great emphasis has been placed on the use of 16S rRNA gene sequences (with the definition of RNA groups), this has still not led to a new definition of the genus *Bacillus*. Given the well-documented chemical diversity of various species previously or currently assigned to the genus *Bacillus*, it would appear opportune to consider the differences between the chemical composition of the type species of the genus (*B. subtilis*), as the nomenclatural type of the genus, and the new taxon. Although both strain BR^T and *B. subtilis* DSM 10^T share MK-7 and iso-/anteiso-branched fatty acids, there are some differences in the fatty acid patterns (Supplementary Table S1), as well as in the polar lipid profiles. In particular, *B. subtilis* DSM 10^T produces phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, a glycolipid and an aminophospholipid with an R_F value different from that found in strain BR^T (Supplementary Fig. S2). The similarities between the two organisms are clearly indicative of the fact that they both belong to the bacilli, but the differences indicate that strain BR^T should not be included in the genus *Bacillus*.

DNA was isolated after disruption of cells using a French pressure cell (Thermo Spectronic) and purified by hydroxyapatite chromatography (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 DNA G+C content was determined by using the HPLC method described by Tamaoka & Komagata (1984).

Genomic DNA extraction, PCR-mediated amplification of the 16S rRNA gene and sequencing of PCR products were

carried out as described by Rainey *et al.* (1996). The sequence reaction mixtures were electrophoresed using a model 373A automated DNA sequencer (Applied Biosystems).

The 16S rRNA gene sequence of strain BR^T was aligned with published sequences obtained from the EMBL nucleotide sequence database and the Ribosomal Database Project using the ae2 editor (Maidak *et al.*, 2001) and the ARB program (Ludwig *et al.*, 2004). Evolutionary distance calculations were based on the algorithms of Jukes & Cantor (1969), DeSoete (1983) and Felsenstein (1993), using neighbour-joining, maximum-likelihood and parsimony methods of tree reconstruction.

The G+C content of the DNA of strain BR^T was shown to be 34·5 mol%. The 16S rRNA gene sequence of strain BR^T had only similarity values below 93 % with those of its closest relatives. Its phylogenetic position differed slightly according to the algorithm used and the selection of sequences included in the analysis. In no case, however, did strain BR^T cluster specifically with one of the established taxa (Fig. 1).

Of the thermophilic, endospore-forming rods belonging to genera of the family *Bacillaceae* (Garrity *et al.*, 2003), only a few are strict anaerobes. *Bacillus infernus*, *Bacillus macyae* and *Bacillus arseniciselenatis* are the only three strictly anaerobic representatives of the genus *Bacillus* (Boone *et al.*, 1995; Switzer Blum *et al.*, 1998; Santini *et al.*, 2004). The species *Bacillus selenitireducens* and *Anoxybacillus pushchinoensis* are facultatively aerobic, but grow better under anaerobic conditions (Switzer Blum *et al.*, 1998; Pikuta *et al.* 2000). Other significant phenotypic differences (e.g. in DNA G+C contents, the presence of catalase and the cellular fatty acid composition) were found between these organisms (Table 1). Therefore, on the basis of physiological and genomic characteristics, fatty acid composition and 16S rRNA gene sequence analysis, we propose the creation of a novel genus, *Vulcanibacillus* gen. nov., with *Vulcanibacillus modesticaldus* sp. nov. as the type species.

Description of *Vulcanibacillus* gen. nov.

Vulcanibacillus (Vul.ca.ni.ba.cil′lus. L. n. *Vulcanus* the Roman god of fire; L. dim. n. *bacillus* a small rod; N.L. masc. *Vulcanibacillus* a bacillus living in the vicinity of volcanic areas).

Cells are rod-shaped and produce spores. Cells occur mostly singly and are not motile. The cell-wall structure is Gram-positive. Moderately thermophilic, neutrophilic and adapted to the salinity of sea water. Strictly anaerobic. Chemo-organotrophic, using nitrate as the sole electron acceptor, this being reduced to nitrite. Nitrate is not further reduced to ammonia or N_2. No fermentation occurs. Oxidase- and catalase-negative. Produces MK-7 as the major respiratory lipoquinone. The predominant fatty acid is i-15 : 0, but 14 : 0, ai-15 : 0, 15 : 0, ai-16 : 0, 16 : 0, i-17 : 0, ai-17 : 0, 18 : 1ω9 and 18 : 0 are also present. The polar lipids comprise phosphatidylglycerol, diphosphatidylglycerol and phosphatidylethanolamine, as well two unidentified phospholipids (PL1 and PL2) and a single unidentified aminophospholipid. The DNA G+C content is 34·5 mol%. 16S rRNA gene sequence analysis places *Vulcanibacillus* in the class '*Bacilli*'. The type species of the genus is *Vulcanibacillus modesticaldus*.

Description of *Vulcanibacillus modesticaldus* sp. nov.

Vulcanibacillus modesticaldus (mo.des′ti.cal.dus. L. adj. *modestus* moderate; L. adj. *caldus* warm, hot; N.L. masc. adj. *modesticaldus* moderately hot).

In addition to having the characteristics given in the genus description (above), cells are rods, 4–5 μm in length and 0·2–0·3 μm in diameter. Colonies of strain BR^T are lens-shaped and cream in colour. The temperature range for growth is 37–60 °C, with an optimum at 55 °C. Neutrophilic. The optimum NaCl concentration for growth is 2–3 %. Substrates used for growth in the presence of nitrate as electron acceptor include acetate, yeast extract, tryptone, Bio-Trypticase, Bacto-peptone, sucrose, glucose, fructose, starch, cellobiose, ethanol and pyruvate. Elemental sulfur, thiosulfate, sulfate, nitrite, amorphous Fe(III) oxide (90 mM) and Fe(III) citrate (20 mM) cannot be used as alternative electron acceptors. Malate, succinate, methanol, formate, lactate, galactose, arabinose, lactose, butyrate and

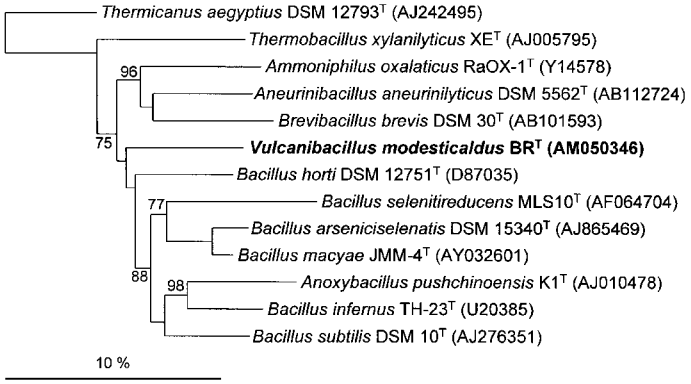

Fig. 1. Phylogenetic tree of strain BR^T and selected members of *Bacillus* and related taxa based on neighbour-joining analysis. Numbers (%) at branch points are bootstrap values greater than 70 %. Bar, 10 % sequence divergence.

Table 1. Salient characteristics of genera of the family *Bacillaceae* (Garrity *et al.*, 2003) that contain thermophilic, endospore-forming rods

Data were taken from Nystrand (1984), Nazina *et al.* (2001) and Fortina *et al.* (2001a, b). V, Variable; ND, not determined.

Characteristic	*Vulcanibacillus*	*Geobacillus*	*Bacillus*	*Brevibacillus*	*Aneurinibacillus*	*Thermobacillus*	*Saccharococcus*	*Ureibacillus*
Anaerobic growth	+	+/−	+/−	+/−	−	−	−	−
Catalase	−	+	+	+	+	+	+	ND
Main isoprenoid quinone	MK-7	MK-7	MK-7	MK-7	MK-7	MK-7	ND	V
Main cellular fatty acids	i-15:0, ai-15:0, 16:0, i-17:0	ai-15:0, i-16:0, i-17:0	V	ai-15:0 and i-15:0 or i-15:0	i-15:0, 16:0, i-16:0	i-16:0, 16:0, ai-17:0	i-15:0, 16:0, i-17:0	i-16:0, i-15:0, i-17:0
DNA G+C content (mol%)	34·5	49–58	32–69	43–57	41–43	57·5	48	36–41·5

propionate are not used. The chemical composition of the species is identical to that given for the genus.

The type strain, BR^T (=DSM 14931^T=JCM 12998^T), was isolated from a core sample collected at the Rainbow vent field on the Mid-Atlantic Ridge (36° 14′ N).

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References

Balch, W. E., Fox, G. E., Magrum, G. E., Woese, G. E. & Wolfe, R. S. (1979). Methanogens: reevaluation of a unique biological group. *Microbiol Rev* **43**, 260–296.

Bonch-Osmolovskaya, E. A., Sokolova, T. G., Kostrikina, N. A. & Zavarzin, G. A. (1990). *Desulfurella acetivorans* gen. nov. and sp. nov. – a new thermophilic sulfur-reducing eubacterium. *Arch Microbiol* **153**, 151–155.

Boone, D. R., Liu, Y., Zhao, Z. J., Balkwill, D. L., Drake, G. R., Stevens, T. O. & Aldrich, H. C. (1995). *Bacillus infernus* sp. nov., an Fe(III)- and Mn(IV)-reducing anaerobe from the deep terrestrial subsurface. *Int J Syst Bacteriol* **45**, 441–448.

Brisbarre, N., Fardeau, M.-L., Cueff, V. & 7 other authors (2003). *Clostridium caminithermale* sp. nov., a slightly halophilic and moderately thermophilic bacterium isolated from an Atlantic deep-sea hydrothermal chimney. *Int J Syst Evol Microbiol* **53**, 1043–1049.

Cashion, P., Holder-Franklin, M. A., McCully, J. & Franklin, M. (1977). A rapid method for the base ratio determination of bacterial DNA. *Anal Biochem* **81**, 461–466.

Cataldo, D. A., Haroon, M., Schrader, L. E. & Youngs, V. L. (1975). Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. *Commun Soil Sci Plant Anal* **6**, 71–80.

DeSoete, G. (1983). A least squares algorithm for fitting additive trees to proximity data. *Psychometrika* **48**, 621–626.

Fardeau, M.-L., Bonilla Salinas, M., L'Haridon, S., Jeanthon, C., Verhé, F., Cayol, J.-L., Patel, B. K. C., Garcia, J.-L. & Ollivier, B. (2004). 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. *Int J Syst Evol Microbiol* **54**, 467–474.

Felsenstein, J. (1993). PHYLIP (phylogeny inference package), version 3.5c. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle, USA.

Fortina, M. G., Pukall, R., Schumann, P., Mora, D., Parini, C., Manachini, P. L. & Stackebrandt, E. (2001a). *Ureibacillus* gen. nov., a new genus to accommodate *Bacillus thermosphaericus* (Andersson *et al.* 1995), emendation of *Ureibacillus thermosphaericus* and description of *Ureibacillus terrenus* sp. nov. *Int J Syst Evol Microbiol* **51**, 447–455.

Fortina, M. G., Mora, D., Schumann, P., Parini, C., Manachini, P. L. & Stackebrandt, E. (2001b). Reclassification of *Saccharococcus caldoxylosilyticus* as *Geobacillus caldoxylosilyticus* (Ahmad *et al.* 2000) comb. nov. *Int J Syst Evol Microbiol* **51**, 2063–2071.

Garrity, G. M., Bell, J. A. & Lilburn, T. G. (2003). Taxonomic outline of the prokaryotes. In *Bergey's Manual of Systematic Bacteriology*, release 4.0. http://141.150.157.80/bergeysoutline/main.htm

Jeanthon, C. (2000). Molecular ecology of hydrothermal vent microbial communities. *Antonie van Leeuwenhoek* **77**, 117–133.

Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.

Kämpfer, P. (1994). Limits and possibilities of total fatty acid analysis for classification and identification of *Bacillus* species. *Syst Appl Microbiol* **17**, 86–96.

Kämpfer, P. (2002). Whole-cell fatty acid analysis in the systematics of *Bacillus* and related genera. In *Applications and Systematics of Bacillus and Relatives*, pp. 271–299. Edited by R. Berkeley, M. Heyndrickx, N. Logan & P. De Vos. Oxford: Blackwell.

Ludwig, W., Strunk, O., Westram, R. & 29 other authors (2004). ARB: a software environment for sequence data. *Nucleic Acids Res* **32**, 1363–1371.

Maidak, B. L., Cole, J. R., Lilburn, T. G. & 7 other authors (2001). The RDP-II (Ribosomal Database Project). *Nucleic Acids Res* **29**, 173–174.

Marteinsson, V. T., Birrien, J. L., Jeanthon, C. & Prieur, D. (1996). Numerical taxonomic study of thermophilic *Bacillus* isolated from three geographically separated deep-sea hydrothermal vents. *FEMS Microbiol Ecol* **21**, 255–266.

Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.

Minnikin, D. E. & Minnikin, M. (1981). Lipids in the classification of *Bacillus* and related taxa. In *The Aerobic Endospore-Forming Bacteria* (Special Publications of the Society for General Microbiology no. 4), pp. 59–90. Edited by R. C. W. Berkeley & M. Goodfellow. London: Academic Press.

Miroshnichenko, M. L., Kostrikina, N. A., L'Haridon, S., Jeanthon, C., Hippe, H., Stackebrandt, E. & Bonch-Osmolovskaya, E. A. (2002). *Nautilia lithotrophica* gen. nov., sp. nov., a thermophilic sulphur-reducing ε-proteobacterium isolated from a deep-sea hydrothermal vent. *Int J Syst Evol Microbiol* **52**, 1299–1304.

Miroshnichenko, M. L., L'Haridon, S., Schumann, P., Spring, S., Bonch-Osmolovskaya, E. A., Jeanthon, C. & Stackebrandt, E. (2004). *Caminibacter profundus* sp. nov., a novel thermophile of *Nautiliales* ord. nov. within the class '*Epsilonproteobacteria*', isolated from a deep-sea hydrothermal vent. *Int J Syst Evol Microbiol* **54**, 41–45.

Murray, R. G. E., Doetsch, R. N. & Robinow, C. F. (1994). Determinative and cytological light microscopy. In *Methods for General and Molecular Bacteriology*, pp. 21–41. Edited by P. Gerhardt, R. G. E. Murray, W. A. Wood & N. R. Krieg. Washington, DC: American Society for Microbiology.

Nazina, T. N., Tourova, T. P., Poltaraus, A. B. & 8 other authors (2001). Taxonomic study of aerobic thermophilic bacilli: descriptions of *Geobacillus subterraneus* gen. nov., sp. nov. and *Geobacillus uzenensis* sp. nov. from petroleum reservoirs and transfer of *Bacillus stearothermophilus*, *Bacillus thermocatenulatus*, *Bacillus thermoleovorans*, *Bacillus kaustophilus*, *Bacillus thermoglucosidasius* and *Bacillus thermodenitrificans* to *Geobacillus* as the new combinations *G. stearothermophilus*, *G. thermocatenulatus*, *G. thermoleovorans*, *G. kaustophilus*, *G. thermoglucosidasius* and *G. thermodenitrificans*. *Int J Syst Evol Microbiol* **51**, 433–446.

Nercessian, O., Fouquet, Y., Pierre, C., Prieur, D. & Jeanthon, C. (2005). Diversity of *Bacteria* and *Archaea* associated with a carbonate-rich metalliferous sediment sample from the Rainbow vent field on the Mid-Atlantic Ridge. *Environ Microbiol* **7**, 698–714.

Nystrand, R. (1984). *Saccharococcus thermophilus* gen. nov., sp. nov., isolated from beet sugar extraction. *Syst Appl Microbiol* **5**, 204–219.

O'Leary, W. M. & Wilkinson, S. G. (1988). Gram-positive bacteria. In *Microbial Lipids*, pp. 117–201. Edited by C. Ratledge & S. G. Wilkinson. London: Academic Press.

Pikuta, E., Lysenko, A., Chuvilskaya, N., Mendrock, U., Hippe, H., Suzina, N., Nikitin, D., Osipov, G. & Laurinavichius, K. (2000). *Anoxybacillus pushchinensis* gen. nov., sp. nov., a novel anaerobic alkaliphilic, moderately thermophilic bacterium from manure, and description of *Anoxybacillus flavithermus* comb. nov. *Int J Syst Evol Microbiol* **50**, 2109–2117.

Rainey, F. A., Ward-Rainey, N., Kroppenstedt, R. M. & Stackebrandt, E. (1996). The genus *Nocardiopsis* represents a phylogenetically coherent taxon and a distinct actinomycete lineage: proposal of *Nocardiopsaceae* fam. nov. *Int J Syst Bacteriol* **46**, 1088–1092.

Rhuland, L. E., Work, E., Denman, R. F. & Hoare, D. S. (1955). The behaviour of the isomers of α,ε-diaminopimelic acid on paper chromatograms. *J Am Chem Soc* **77**, 4844–4846.

Santini, J. M., Streimann, I. C. A. & vanden Hoven, R. N. (2004). *Bacillus macyae* sp. nov., an arsenate-respiring bacterium isolated from an Australian gold mine. *Int J Syst Evol Microbiol* **54**, 2241–2244.

Schleifer, K. H. & Kandler, O. (1972). Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol Rev* **36**, 407–477.

Slobodkin, A. I., Tourova, T. P., Kuznetsov, B. B., Kostrikina, N. A., Chernyh, N. A. & Bonch-Osmolovskaya, E. A. (1999). *Thermoanaerobacter siderophilus* sp. nov., a novel dissimilatory Fe(III)-reducing anaerobic thermophilic bacterium. *Int J Syst Bacteriol* **49**, 1471–1478.

Slobodkin, A. I., Tourova, T. P., Kostrikina, N. A., Chernyh, N. A., Bonch-Osmolovskaya, E. A., Jeanthon, C. & Jones, B. E. (2003). *Tepidibacter thalassicus* gen. nov., sp. nov., a novel moderately thermophilic, anaerobic, fermentative bacterium from a deep-sea hydrothermal vent. *Int J Syst Evol Microbiol* **53**, 1131–1134.

Sokolova, T. G., Gonzalez, J. M., Kostrikina, N. A., Chernyh, N. A., Tourova, T. P., Kato, C., Bonch-Osmolovskaya, E. A. & Robb, F. T. (2001). *Carboxydobrachium pacificum* gen. nov., sp. nov., a new anaerobic, thermophilic, CO-utilizing marine bacterium from Okinawa Trough. *Int J Syst Evol Microbiol* **51**, 141–149.

Spring, S., Wagner, M., Schumann, P. & Kämpfer, P. (2005). *Malikia granosa* gen. nov., sp. nov., a novel polyhydroxyalkonate- and polyphosphate-accumulating bacterium isolated from activated sludge and reclassification of *Pseudomonas spinosa* as *Malikia spinosa* comb. nov. *Int J Syst Evol Microbiol* **55**, 621–629.

Switzer Blum, J., Burns Bindi, A., Buzzelli, J., Stolz, J. F. & Oremland, R. S. (1998). *Bacillus arsenicoselenatis*, sp. nov., and *Bacillus selenitireducens*, sp. nov.: two haloalkaliphiles from Mono Lake, California that respire oxyanions of selenium and arsenic. *Arch Microbiol* **171**, 19–30.

Takai, K., Inagaki, F., Nakagawa, S., Hirayama, H., Nunoura, T., Sako, Y., Nealson, K. H. & Horikoshi, K. (2003). Isolation and phylogenetic diversity of members of previously uncultivated epsilon-*Proteobacteria* in deep-sea hydrothermal fields. *FEMS Microbiol Lett* **218**, 167–174.

Tamaoka, J. & Komagata, K. (1984). Determination of DNA base composition by reversed-phase high-performance liquid chromatography. *FEMS Microbiol Lett* **25**, 125–128.

Tindall, B. J. (1990a). A comparative study of the lipid composition of *Halobacterium saccharovorum* from various sources. *Syst Appl Microbiol* **13**, 128–130.

Tindall, B. J. (1990b). Lipid composition of *Halobacterium lacusprofundi*. *FEMS Microbiol Lett* **66**, 199–202.

Urios, L., Cueff, V., Pignet, P. & Barbier, G. (2004). *Tepidibacter formicigenes* sp. nov., a novel spore-forming bacterium isolated from a Mid-Atlantic Ridge hydrothermal vent. *Int J Syst Evol Microbiol* **54**, 439–443.

Wery, N., Moricet, J.-M., Cueff, V., Jean, J., Pignet, P., Lesongeur, F., Cambon-Bonavita, M.-A. & Barbier, G. (2001). *Caloranaerobacter azorensis* gen. nov., sp. nov., an anaerobic thermophilic bacterium isolated from a deep-sea hydrothermal vent. *Int J Syst Evol Microbiol* **51**, 1789–1796.

Wolin, E. A., Wolin, M. J. & Wolfe, R. S. (1963). Formation of methane by bacterial extracts. *J Biol Chem* **238**, 2882–2888.