***Thermoanaerobacter yonseiensis* sp. nov., a novel extremely thermophilic, xylose-utilizing bacterium that grows at up to 85 °C**

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A novel strictly anaerobic, extremely thermophilic, spore-forming and xylose-utilizing bacterium, designated strain KB-1^{TP} (type and patent strain), was isolated from a geothermal hot stream at Sileri on Java island, Indonesia. The cells were rod-shaped, motile and had terminal spores. The newly isolated strain stained Gram-positive and the cells occurred singly or in pairs during the exponential growth phase. The temperature optimum for growth was 75 °C and growth occurred in the range 50–85 °C. The pH range for growth was 4.5–9.0, with an optimum at pH 6.5. Strain KB-1^{TP} grew chemo-organotrophically by fermenting a wide range of substrates such as glucose, fructose, D-xylose, lactose, maltose, sucrose, mannose, galactose, cellobiose, pullulan and soluble starch. Arabinose, xylan, cellulose, olive oil and Tween 80 were not fermented. The predominant fermentation end products after growth on glucose were lactate, acetate, ethanol, CO₂ and small amounts of isovaleric acid, butyric acid, propionic acid, 1-pentanol and 2-propanol. Thiosulfate was reduced to H₂S. Strain KB-1^{TP} was sensitive to tetracycline, chloramphenicol, penicillin G, neomycin, kanamycin, vancomycin and rifampicin at concentrations of 100 µg ml⁻¹. No effect was observed with chloramphenicol and neomycin at concentrations of 10 µg ml⁻¹. This indicates that strain KB-1^{TP} belongs to the bacterial domain. The G+C content of the DNA was 37 mol%. The comparison of the 16S rDNA sequence to that of closely related strains revealed that strain KB-1^{TP} belonged to clostridial cluster V, showing highest sequence identities (92.7%) to members of the genus *Thermoanaerobacter*. Taking into account the physiological and molecular properties of the new isolate, it is proposed that strain KB-1^{TP} should be classified as a new species of the genus *Thermoanaerobacter*, designated *Thermoanaerobacter yonseiensis*. The type strain, KB-1^{TP}, has been deposited in the Korean Federation of Culture Collections (KFCC 11116^P) as a patent strain and in the Deutsche Sammlung von Mikroorganismen und Zellkulturen as a type strain (= DSM 13777^T).

Keywords: *Thermoanaerobacter yonseiensis*, thermophiles

INTRODUCTION

In the last two decades, our understanding of the biology of thermophilic and other extremophilic micro-organisms has been greatly advanced. Thermophiles, which are found in both the bacterial and the archaeal domains, have been detected in association

with all types of thermal habitats, including hot springs, volcanoes, solfataric fields and deep-sea hydrothermal vents (Stetter, 1996). Due to the biotechnological potential of anaerobic microbes, many research groups have focused on the thermophilic anaerobes that are able to utilize monomeric and polymeric carbohydrates. Many thermophilic, anaerobic and saccharolytic bacteria were first described as members of *Clostridium* and *Desulfotomaculum*, but were later transferred to the genera *Thermo-*

The GenBank accession number for the 16S rDNA sequence of *Thermoanaerobacter yonseiensis* strain KB-1^{TP} is AF212925.

anaerobacterium and *Thermoanaerobacter* (Lee *et al.*, 1993a). As molecular biological techniques such as 16S rDNA sequencing and DNA–DNA hybridization have improved, the taxonomic and phylogenetic relationships of these thermophilic genera have been further defined by Lee *et al.* (1993a) and Rainey *et al.* (1993). The genus *Thermoanaerobacter* falls into cluster A of thermophiles as defined by Rainey *et al.* (1993) and currently comprises 12 species, namely *Thermoanaerobacter brockii* (formerly *Thermoanaerobium brockii*; now including the three subspecies, *brockii*, *finnii* and *lactiethylicus*; Zeikus *et al.*, 1979; Cayol *et al.*, 1995), *Thermoanaerobacter mathranii* (Larsen *et al.*, 1997), *Thermoanaerobacter italicus* (Kozianowski *et al.*, 1997), *Thermoanaerobacter wiegelii* (Cook *et al.*, 1996), *Thermoanaerobacter acetoethylicus* (formerly *Thermobacteroides acetoethylicus*; Ben-Bassat & Zeikus, 1981; Rainey & Stackebrandt, 1993; Collins *et al.*, 1994), *Thermoanaerobacter ethanolicus* (Wiegel & Ljungdahl, 1981), *Thermoanaerobacter thermocopriæ* (formerly *Clostridium thermocopriæ*; Jin *et al.*, 1988), *Thermoanaerobacter sulfurophilus* (Bonch-Osmolovskaya *et al.*, 1997), *Thermoanaerobacter thermohydrosulfuricus* (formerly *Clostridium thermohydrosulfuricum*; Wiegel *et al.*, 1979; Lee *et al.*, 1993a), *Thermoanaerobacter kivui* (formerly *Acetogenium kivui*; Leigh *et al.*, 1981; Collins *et al.*, 1994), *Thermoanaerobacter siderophilus* (Slobodkin *et al.*, 1999) and *Thermoanaerobacter subterraneus* (Fardeau *et al.*, 2000). In general, members of the genus *Thermoanaerobacter* are thermophilic and rod-shaped anaerobes that grow chemo-organotrophically on a variety of saccharides as energy and carbon source, forming mainly ethanol, acetate and lactate as fermentation end products (Lee *et al.*, 1993a). *Thermoanaerobacter kivui* is the only homoacetogen in this genus, but it clearly does not belong to other genera that contain thermophilic homoacetogenic organisms, e.g. *Moorella thermoacetica* (formerly *Clostridium thermoaceticum*) and *Moorella thermoautotrophica* (formerly *Clostridium thermoautotrophicum*) (Collins *et al.*, 1994). The majority of *Thermoanaerobacter* species were found to be sporulating organisms. However, lack of spore formation may only be the result of the inability of the taxonomist to provide conditions to induce sporulation, as was found in the case of *Thermoanaerobacter brockii* (Cook *et al.*, 1991). The Gram-reaction is negative, positive or variable. In contrast to the report of Lee *et al.* (1993a), some species of the genus *Thermoanaerobacter* also accumulate elemental sulfur at the same time as they reduce thiosulfate to H_2S (Kozianowski *et al.*, 1997).

Much attention has been paid to xylose-utilizing extreme thermophiles or hyperthermophiles since these micro-organisms are a promising source of thermostable and thermoactive D-xylose isomerases. These enzymes are of great interest for the production of high-fructose corn syrup (HFCS) in the starch processing industry (Brown *et al.*, 1993; Dekker *et al.*, 1991a, b; Liu *et al.*, 1996). Only a limited number of

extreme thermophilic micro-organisms are able to utilize xylose. Thermostable and thermoactive D-xylose (D-glucose) isomerases have been characterized from members of the genera *Thermus* (Dekker *et al.*, 1991a; Park *et al.*, 1997), *Thermotoga* (Brown *et al.*, 1993; Vielle *et al.*, 1995), *Thermoanaerobacterium* (Lee *et al.*, 1993b; Meaden *et al.*, 1994; Liu *et al.*, 1996) and *Thermoanaerobacter* (Erbezniak *et al.*, 1998; Dekker *et al.*, 1991b). Except for some *Thermus* and *Thermotoga* strains, the majority of these bacteria is unable to grow above 80 °C with xylose as sole carbon source.

In this paper we report on the isolation and characterization of the xylose-utilizing strain KB-1^{TP} that grows up to 85 °C. Based on morphological, physiological and phylogenetic studies, it is concluded that this extremely thermophilic isolate is a new species of the genus *Thermoanaerobacter* for which the name *Thermoanaerobacter yonseiensis* is proposed.

METHODS

Reference strains. *Thermococcus siculi* DSM 12349^T, *Thermotoga maritima* DSM 3109^T, *Thermoanaerobacter brockii* subsp. *brockii* DSM 1457^T and *Thermoanaerobacter ethanolicus* DSM 2246^T were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany.

Origin of the isolate. Samples of hot water, mud and soil were collected from hot streams at Sileri (Java island) associated with volcanic activity in Indonesia. The samples were transported in sterile plastic bottles at ambient temperature. Each collection bottle was completely filled and supplemented with $Na_2S \cdot 9H_2O$ as reducing agent at a final concentration of 0.5 g l⁻¹. The *in situ* temperature of the streams was between 60 and 80 °C, and the collected samples had a pH range of 4.0–7.0.

Culture conditions. An enrichment culture medium (EM-KB1 medium) for strictly anaerobic thermophiles was designed and contained the following components (per litre distilled water): 2.0 g NaCl, 0.5 g KH_2PO_4 , 0.1 g K_2HPO_4 , 1.0 g $(NH_4)_2SO_4$, 0.5 g $NaHCO_3$, 0.1 g KBr, 0.03 g H_3BO_3 , 0.1 g KCl, 0.5 g $MgCl_2 \cdot 6H_2O$, 0.8 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.03 g $SrCl_2 \cdot 6H_2O$, 0.033 mg Na_2WO_4 , 0.026 mg Na_2SeO_3 , 10 ml trace element solution (Balch *et al.*, 1979), 10 ml vitamin solution (Wolin *et al.*, 1964), 1.0 g yeast extract, 10 g elemental sulfur, 1 mg resazurin and 3 ml $Na_2S \cdot 9H_2O$ (25% solution, w/v, pH 8.0). To this medium, filter-sterilized solutions of xylose, tryptone, starch or pyruvate (final concentration 5.0 g l⁻¹) were added. Growth medium GM-KB1 contained (per litre distilled water): 2.0 g NaCl, 0.1 g KCl, 0.2 g $NaHCO_3$, 0.5 g KH_2PO_4 , 0.5 g $(NH_4)_2SO_4$, 0.1 g KBr, 0.03 g H_3BO_3 , 0.5 g $MgCl_2 \cdot 6H_2O$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g $CaCl_2 \cdot 2H_2O$, 0.03 g $SrCl_2 \cdot 6H_2O$, 0.033 mg Na_2WO_4 , 0.026 mg Na_2SeO_3 , 10 ml trace element solution (Balch *et al.*, 1979), 10 ml vitamin solution (Wolin *et al.*, 1964), 5.0 g carbon source (separately sterilized), 1.0 g yeast extract, 2.0 g $Na_2S_2O_3 \cdot 5H_2O$, 1 mg resazurin and 3 ml $Na_2S \cdot 9H_2O$ (25% solution, w/v, pH 8.0). Both media were prepared without Na_2S , boiled for 20 min and cooled down on ice under a N_2 atmosphere. The media were finally reduced by the addition of 3 ml $Na_2S \cdot 9H_2O$ solution (25% solution, w/v, pH 8.0). Sodium thiosulfate was filter-sterilized and added separately after autoclaving. Unless otherwise stated, the media were adjusted to pH 6.5 with

6 M HCl and dispensed into appropriate glass containers sealed with butyl-rubber stoppers under a N₂ atmosphere. Sterilization of the media was performed by autoclaving at 121 °C for 20 min or at 100 °C for 1 h if the medium contained elemental sulfur. Unless otherwise indicated, cultures were incubated under atmospheric pressure at 75 °C. All chemicals were purchased from Merck, except yeast extract and tryptone, which were obtained from Difco Laboratories.

Enrichment and isolation. Enrichment cultures (10% inoculum, v/v) were grown on EM-KB1 medium at temperatures between 70 and 100 °C and at pH values between 5.0 and 7.0 for several days. Isolates were obtained by successively growing end point dilutions at 80 °C and pH 6.5 in medium EM-KB1, followed by spreading on 2.0% (w/v) agar-solidified EM-KB1 medium in Hungate tubes. Purified isolates were stored in liquid culture at 4 °C and could be used as inocula for at least 12 months. For long-term storage pure cultures were stored under anaerobic conditions in the presence of 50% (w/v) glycerol at -20 °C.

Determination of growth parameters. Microbial growth was either monitored by direct cell counting using a Petroff-Hausser chamber (depth 0.02 mm) and phase-contrast microscopy at a magnification of $\times 400$, or by measuring the optical density in a spectrophotometer at 600 nm. To determine the optimum temperature, cells were grown in serum bottles containing 50 ml GM-KB1 medium supplemented with soluble starch (5.0 g l⁻¹) at pH 7.0. Determination of the optimum pH was performed at 75 °C. KH₂PO₄ and NaHCO₃ were omitted from the GM-KB1 medium and CaCl₂ was reduced to a final concentration of 0.05 g l⁻¹ to prevent precipitation. Additionally, the following filter-sterilized components (at a final concentration of 10 mM, except glucose) were added to GM-KB1 medium: glucose (5.0 g l⁻¹); trisodium citrate, pH range 3.0–5.5; MES sodium salt, pH range 5.5–6.5; Tris (trizma base), pH range 6.5–7.5; and Na₂CO₃, pH range 7.5–9.5. Elemental sulfur was used as electron acceptor instead of sodium thiosulfate in the acidic medium which contained trisodium citrate (pH 4.0–5.5) to prevent precipitation. To monitor growth, samples (in duplicate) were withdrawn every hour. Utilization of various substrates as energy and carbon source was tested in a modified GM-KB1 medium with a reduced amount of yeast extract (0.1 g l⁻¹). Unless otherwise indicated, the carbon sources were tested at a concentration of 5.0 g l⁻¹. Cultures containing no additional carbon source served as control. The ability of the isolate to grow in the presence of various electron acceptors was determined in thiosulfate-free GM-KB1 medium supplemented with elemental sulfur (10.0 g l⁻¹), L-cystine (2.0 g l⁻¹), sodium thiosulfate (2.0 g l⁻¹), sodium nitrate (2.0 g l⁻¹) or sodium sulfite (2.0 g l⁻¹). To determine the requirement of sodium thiosulfate as electron acceptor, microbial growth was monitored by direct cell counting using a Petroff-Hausser chamber after full growth (70 °C, 18 h) of the examined strains (1% inoculum, v/v) in both GM-KB1 medium and thiosulfate-free GM-KB1 medium. Unless otherwise noted, the growth experiments were performed at least in duplicate.

Physiological tests and determination of fermentation products. Hucker's method (Brock & Madigan, 1988) was applied for Gram-staining. The reduction of thiosulfate to H₂S was tested by the addition of 0.2 g FeSO₄ · 7H₂O l⁻¹ to late exponential-phase cultures grown on medium GM-KB1 supplemented with 5.0 g glucose l⁻¹ and 2.0 g sodium thiosulfate l⁻¹. Blackening of the medium indicated the formation of H₂S (Jander & Blasius, 1995). Uninoculated

medium treated identically served as control. The formation of CO₂ was monitored by barium carbonate precipitation as described by Jander & Blasius (1995). Susceptibility of the new isolate to antibiotics was tested under standard growth conditions at 70 °C. The bacterium *Thermotoga maritima* DSM 3109^T and the archaeon *Thermococcus siculi* DSM 12349^T were used as controls to ensure the effectiveness of the antibiotics at elevated temperatures. Inhibition was defined by cell densities of less than 1×10^7 cells ml⁻¹ in cultures containing antibiotics. Volatile fatty acids and alcohols were analysed by flame-ionization detection (FID) using a model Sigma 2000 gas chromatograph (Perkin Elmer). Lactate was determined enzymologically in a YSI model 2700 SELECT Biochemistry analyser as recommended by the manufacturer.

Electron microscopy. Cells were harvested by centrifugation at 5000 g for 10 min to prepare specimens for transmission electron microscopy and then fixed overnight in a solution containing 2.5% (w/v) glutaraldehyde in 0.1 M Na₂HPO₄/KH₂PO₄ (pH 7.2) buffer at 4 °C, and post-fixed with 1% osmium tetroxide (OsO₄). Samples were then dehydrated with ethanol and embedded in Spurr. The sections were stained with 1% (w/v) uranyl acetate and 1% (w/v) citrate and examined with a Philips model CM-10 electron microscope at an accelerating voltage of 80 kV. For scanning electron microscopy, cells were collected on a 0.4 µm pore size Millipore HA filter and fixed with 2.5% (v/v) glutaraldehyde in 50 mM Tris/HCl (pH 7.5) containing 3% (w/v) NaCl at 75 °C for 30 min. After dehydration in a graded ethanol series, the specimen was immersed in *t*-butyl alcohol and dried using a freeze drying method, and finally sputter-coated with platinum under vacuum (E-1010 Ion sputter; Hitachi). The specimen was observed using a Hitachi S-800 scanning electron microscope at an accelerating voltage of 20 kV.

Analysis of lipid components. Respiratory lipoquinone, fatty acid and polar lipid analyses were carried out by Dr B. J. Tindall at the DSMZ, Braunschweig, Germany. Respiratory lipoquinones and polar lipids were extracted from 100 mg of freeze-dried cell material using the two-stage method described by Tindall (1990a, b). Respiratory quinones were extracted using methanol/hexane and the polar lipids were extracted by adjusting the remaining methanol/0.3% aqueous NaCl phase (containing the cell debris) to give a chloroform/methanol/0.3% aqueous NaCl mixture (1:2:0.8, by vol.). The extraction solvent was stirred overnight and the cell debris was pelleted by centrifugation. Polar lipids were recovered into the chloroform phase by adjusting the chloroform/methanol/0.3% aqueous NaCl mixture to a ratio of 1:1:0.9 (by vol.). Respiratory lipoquinones were separated into their different classes by TLC on silica gel (Macherey-Nagel), using hexane/*t*-butylmethylether (9:1, v/v) as solvent. UV absorbing bands corresponding to menaquinones or ubiquinones were removed from the plate and further analysed by HPLC (LDC Analytical; Thermo Separation Products) fitted with a reverse phase column (Macherey-Nagel) using methanol as the eluant. Respiratory lipoquinones were detected at 269 nm. Polar lipids were separated by two-dimensional silica gel TLC. The first direction was developed in chloroform/methanol/water (65:25:4, by vol.) and the second in chloroform/methanol/acetic acid/water (80:12:15:4, by vol.). Total lipid material and specific functional groups were detected using dodecamolybdo-phosphoric acid (total lipids), Zinzadze reagent (phosphate), ninhydrin (free amino groups), periodate-Schiff (α -glycols),

Dragendorff reagent (quaternary nitrogen) and anisaldehyde/sulphuric acid (glycolipids). Fatty acids were analysed as the methyl ester derivatives prepared from 10 mg dry cell materials. Cells were subjected to differential hydrolysis to detect ester-linked and non-ester-linked (amide-bound) fatty acids. Fatty acid methyl esters were analysed by GC using a non-polar capillary column and flame ionization detection.

DNA base composition. Isolation of DNA was performed according to the method described by Visuvanathan *et al.* (1989). The DNA base composition was determined at the DSMZ by HPLC (Mesbah *et al.*, 1989; Tamaoka & Komagata 1984).

16S rDNA sequence analysis. DNA was extracted from overnight cultures following the method described by Charbonnier & Forterre (Robb & Place, 1995). The 16S rDNA gene was amplified by PCR using primers designed by DeLong (1992) and sequenced on an ABI 373S automated sequencer (Perkin Elmer/Applied Biosystems). The almost complete 16S rDNA sequence of strain KB-1^{TP}, corresponding to positions 21–1526 (*Escherichia coli* numbering), was determined at the DSMZ and aligned with reference sequences by using the CLUSTAL W program, version 1.81 (Thompson *et al.*, 1994). Pairwise evolutionary distances were computed by using the correction of Jukes & Cantor (1969) and a distance matrix tree was constructed by the neighbour-joining method (Saitou & Nei, 1987) employing the program PHYLIP (Felsenstein *et al.*, 1993). The first and the last 80 bases were excluded from the phylogenetic analysis because of incomplete sequences reported for some strains and due to alignment uncertainties. A total of 1517 nt (corresponding to positions 101–1452, *E. coli* numbering) were used in the analysis and the phylogenetic trees were displayed using version 1.6 of the TREEVIEW program of Page (1996). The root position of the tree was estimated using the 16S rRNA sequence of *Caldicellulosiruptor saccharolyticus* (DSM 8903^T) as outgroup.

Nucleotide sequence accession numbers. Accession numbers for the sequences used as references are as follows: *Thermoanaerobacter brockii* subsp. *brockii* DSM 1457^T, L09165; *Thermoanaerobacter brockii* subsp. *finii* DSM 3389^T, L09166; *Thermoanaerobacter brockii* subsp. *lactiethylicus* DSM 9801^T, U14330; *Thermoanaerobacter mathranii* A3^T (= DSM 11426^T), Y11279; *Thermoanaerobacter italicus* Ab9^T (= DSM 9252^T), AJ250846; *Thermoanaerobacter wiggelii* Rt8.B1^T (= DSM 10319^T), X92513; *Thermoanaerobacter acetoethylicus* DSM 2359^T, L09163; *Thermoanaerobacter ethanolicus* 39E (= DSM 2355), L09164; *Thermoanaerobacter thermocopriae* JT-3^T (= IAM 13577^T), L09167; *Thermoanaerobacter sulfurophilus* L-64^T (= DSM 11584^T), Y16940; *Thermoanaerobacter thermohydrosulfuricus* DSM 567^T, L09161; *Thermoanaerobacter kivui* DSM 2030^T, L09160; *Thermoanaerobacter siderophilus* SR4^T (= DSM 12299^T), AF120479; *Moorella thermoautotrophica* DSM 1974^T, X77849; *Moorella thermoacetica* DSM 12797, AJ242494; *Thermoanaerobacterium saccharolyticum* DSM 7060^T, L09169; *Thermoanaerobacterium thermosaccharolyticum* D120-70, AF247003; *Caldicellulosiruptor saccharolyticus* DSM 8903^T, AF130258.

RESULTS

Enrichment and isolation

Six samples from hot streams at Sileri in the Dieng volcanic area on the island of Java, Indonesia, were

used for the enrichment of extremely thermophilic micro-organisms. To mimic the *in situ* temperatures and pH profile, enrichment cultures on EM-KB1 medium containing various carbon sources (5.0 g l⁻¹) were incubated at temperatures between 70 and 100 °C and pH 4.0–7.0. Primary enrichment cultures were prepared by adding 5.0 ml sample water to 50 ml EM-KB1 medium under a N₂ gas atmosphere. After 2 or 3 d of incubation at 80 °C, growth of rod-shaped micro-organisms was observed. Positive enrichment cultures were repeatedly transferred to fresh EM-KB1 medium supplemented with xylose (5.0 g l⁻¹) and yeast extract (0.2 g l⁻¹) and incubated at 80 °C for 24 h. Pure cultures were successfully obtained by the dilution-to-extinction technique. Each of the diluted cultures was incubated for 3 d at 80 °C and the serial dilutions (1:10) were repeated at least 10 times. The culture showing growth on xylose at the highest dilution was designated strain KB-1^{TP} and was chosen for further investigation. Purity of the strain was confirmed by microscopic examinations and formation of homogeneous colonies on agar-solidified EM-KB1 medium in Hungate tubes. Liquid cultures of strain KB-1^{TP} could be used as inoculum for at least 12 months when stored at 4 °C.

Colony and cell morphology

Colonies of isolate KB-1^{TP} formed on solid EM-KB1 medium after 48 h of incubation at 70 °C appeared uniformly round, non-pigmented and flat with a diameter of 1–2 mm. Straight, rod-shaped single cells were observed in the phase-contrast microscope after growth in liquid culture incubated at 70 and 80 °C for 12 h (in mid- to late exponential growth phase). Vegetative cells measured 0.4–0.8 × 1.0–3.0 µm (Fig. 1a, b). When incubated at lower temperatures (60 °C for 12 h) cells of strain KB-1^{TP} were significantly longer (about 4.0–5.0 µm) than those incubated at 70 or 80 °C (Fig. 1c). An electron micrograph of an ultrathin section of strain KB-1^{TP} is shown in Fig. 2a. Observation at higher magnification revealed a two-layer structure of the cell wall (Fig. 2b), resembling those of *Thermoanaerobacter ethanolicus* JW200^T (Wiegel & Ljungdahl, 1981) and *Thermoanaerobacter wiggelii* Rt8.B1^T (Cook *et al.*, 1996). Sporulating cells could be observed when the new isolate was incubated under suboptimal conditions, such as overnight at 85 °C (≥ 10% inoculum, v/v). Strain KB-1^{TP} formed a spherical, refractile endospore in terminally swollen sporangia. Phase-bright sulfur accumulated in the medium and on the cells. The Gram reaction was positive.

Physiological characterization of growth

The newly isolated bacterium grew strictly anaerobically under N₂ or N₂/CO₂ (80:20, v/v) using Na₂S as a reducing agent. In oxidized medium, as indicated by the pink colour of the redox indicator resazurin, no growth occurred. Isolate KB-1^{TP} grew over a tem-

Fig. 1. Scanning electron micrographs of isolate KB-1^{TP}. Cells were cultured at (a) 80, (b) 70 and (c) 60 °C for 12 h. Bars, 3 µm.

perature range of about 50–85 °C, showing optimal growth at 75 °C (Fig. 3a). No growth was observed at 45 and 86 °C even after prolonged incubation times (> 1 week). Growth of the new isolate at 75 °C occurred between pH 4.5 and 9.0, with an optimum at pH 6.5 (Fig. 3b). No growth was observed at pH 4.0 or below and pH 9.5 or above. Under optimal cultivation conditions (5.0 g soluble starch l⁻¹, 1.0 g yeast extract l⁻¹, 2.0 g thiosulfate l⁻¹) the generation time for the isolate was 60 min. The maximum cell density was 5.5×10^8 cells ml⁻¹. Strain KB-1^{TP} grew well in GM-KB1 medium without the addition of NaCl (final cell yield 5×10^8 cells ml⁻¹). Growth was observed at NaCl concentrations up to 40 g l⁻¹ and no growth was observed at an NaCl concentration of 50 g l⁻¹. Growth

was inhibited by the addition of 100 µg ml⁻¹ each of tetracycline, chloramphenicol, penicillin G, neomycin, kanamycin, vancomycin or rifampicin. At concentrations of 10 µg ml⁻¹, however, the isolate grew in the presence of chloramphenicol and neomycin. These results indicate that strain KB-1^{TP} belongs to the bacterial domain (Böck & Kandler, 1985). In GM-KB1 medium containing sodium thiosulfate (2 g l⁻¹), the final cell concentrations of the strains were as follows: *Thermoanaerobacter brockii* subsp. *brockii* DSM 1457^T, 5.9×10^8 cells ml⁻¹; *Thermoanaerobacter ethanolicus* DSM 2246^T, 2.1×10^8 cells ml⁻¹; strain KB-1^{TP}, 5.5×10^8 cells ml⁻¹. However, in thiosulfate-free GM-KB1 medium, the final cell concentrations were: *Thermoanaerobacter brockii* subsp. *brockii*,

Fig. 2. Ultrastructure of isolate KB-1^{TP}. (a) Transmission electron micrograph. (b) Cell wall structure and cell membrane shown in detail. O, Outer cell wall; D, dense layer; CM, cytoplasmic membrane. Bars, 500 nm.

3.0×10^8 cells ml⁻¹; *Thermoanaerobacter ethanolicus*, 4.5×10^7 cells ml⁻¹; strain KB-1^{TP}, 2.5×10^7 cells ml⁻¹. In contrast to *Thermoanaerobacter brockii* and *Thermoanaerobacter ethanolicus*, the growth of strain KB-1^{TP} was significantly lower without thiosulfate.

Nutritional requirements and fermentation products

Isolate KB-1^{TP} is a chemo-organotrophic bacterium able to utilize a wide variety of sugars, polysaccharides and proteins. Glucose, fructose, D-xylose, D-galactose, lactose, maltose, mannose, D-cellobiose, sucrose, soluble starch, pullulan, yeast extract, tryptone and peptone were fermented. No growth, however, was

Fig. 3. (a) Effect of temperature on growth of strain KB-1^{TP}. (b) Effect of pH on growth of strain KB-1^{TP}. ■, Trisodium citrate buffer; □, MES buffer; ●, Tris buffer; ○, sodium carbonate buffer. The pH values were the initial values adjusted at room temperature.

detected when arabinose, D-sorbitol, xylan, carboxymethylcellulose, olive oil, Tween 80, collagen and gelatin were used as carbon and energy source. Growth of strain KB-1^{TP} was dependent on yeast extract with a concentration of at least 0.1 g l⁻¹. Sodium thiosulfate, sulfur, L-cystine and sodium nitrate served as final electron acceptors and only poor growth occurred if none of these electron acceptors were present in medium GM-KB1 (final cell yield 2.5×10^7 cells ml⁻¹). The newly isolated micro-organism reduced thiosulfate to sulfur and H₂S. Sulfur was formed in the early phase of fermentation as indicated by phase-bright sulfur aggregates in the light microscope (data not shown). In the late exponential growth phase GM-KB1 medium turned black after the addition of a FeSO₄ solution (2.0 g l⁻¹), which precipitated as FeS, indicating the presence of H₂S. The major fermentation products after 15 h of growth on medium GM-KB1 supplemented with glucose (5.0 g l⁻¹) were lactate, acetic acid, ethanol, CO₂ and H₂S. Propionic

Table 1. Characteristics of strain KB-1^{TP} in comparison with other species of the genus *Thermoanaerobacter*

1, *Thermoanaerobacter brockii* subsp. *brockii* DSM 1457^T (Cayol *et al.*, 1995); 2, *Thermoanaerobacter brockii* subsp. *finnii* DSM 3389^T (Cayol *et al.*, 1995); 3, *Thermoanaerobacter brockii* subsp. *lactiethylicus* DSM 9801^T (Cayol *et al.*, 1995); 4, *Thermoanaerobacter mathranii* DSM 11426^T (Larsen *et al.*, 1997); 5, *Thermoanaerobacter italicus* DSM 9252^T (Kozianowski *et al.*, 1997); 6, *Thermoanaerobacter wiegelii* DSM 10319^T (Cook *et al.*, 1996); 7, *Thermoanaerobacter acetoethylicus* DSM 2359^T (Ben-Bassat & Zeikus, 1981; Cayol *et al.*, 1995); 8, *Thermoanaerobacter ethanolicus* DSM 2246^T (Wiegel & Ljungdahl, 1981); 9, *Thermoanaerobacter thermocopriae* IAM 13577^T (Jin *et al.*, 1988); 10, *Thermoanaerobacter sulfurophilus* DSM 11584^T (Bonch-Osmolovskaya *et al.*, 1997); 11, *Thermoanaerobacter thermohydrosulfuricus* DSM 567^T (Lee *et al.*, 1993); 12, *Thermoanaerobacter kivui* DSM 2030^T (Leigh *et al.*, 1981); 13, *Thermoanaerobacter siderophilus* DSM 12299^T (Slobodkin *et al.*, 1999); 14, Strain KB-1^{TP} (data from this study). +, Positive; –, negative; v, variable; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14
16S rDNA similarity to strain KB-1 ^{TP} (%)	92.7	92.7	91.9	91.4	91.8	92.2	90.6	90.7	91.4	92.0	91.8	92.2	91.1	100
G + C content (mol%)	30–31	32	35	37	34–35	35–36	31	32	37–38	30–31	29–32	38	32	37
Growth temperature (°C)														
Maximum	< 85	75	75	< 78	78	78	80	78	74	75	78	75	78	85
Optimum	65–75	65	55–60	70–75	70	65–68	65	69	60	55–60	67–69	66	69–71	75
Spore formation	+	+	+	+	+	+	–	–	+	–	+	–	+	+
Gram staining	+	v	+	v	–	–	–	v	–	+	v	–	ND	+
Utilization of:														
Starch	+	ND	ND	+	+	+	+	+	+	+	+	–	+	+
Xylan	ND	ND	ND	+	+	ND	–	+	ND	+	+	ND	–	–
Xylose	–	+	+	+	+	+	–	+	+	+	+	ND	+	+

Fig. 4. The phylogenetic tree based on 16S rDNA sequence comparisons of strain KB-1^{TP} and selected bacteria was constructed by the neighbour-joining method and rooted with *Caldicellulosiruptor saccharolyticus* DSM 8903^T. The numbers indicate the bootstrap scores of 1000 trials. The bar equals 1 base substitution per 100 nt positions.

acid, butyric acid, isovaleric acid, 2-propanol and 1-pentanol could be detected in low concentrations (< 0.2 mM).

Analysis of lipid components

As shown by TLC, strain KB-1^{TP} possesses glycolipids. Due to the weak staining of the glycolipids, they did not appear to be identical to known glycolipids that contain glucose, mannose or galactose. Some of these unusual glycolipids could not be assigned to any known structure since they did not stain for phosphate or amino groups, nor did they show a strong reaction with the reagents used for sugar detection. Respiratory lipoquinones, such as menaquinones and ubiquinones, were not found. The major cellular fatty acids of the new isolate grown on medium GM-KB1 at 75 °C were: iso-C_{15:0} (42%), iso-C_{17:0} (11%), C_{16:0} (7%),

C_{18:1} ω7c (6%), 3-OH iso-C_{14:0} (6%), anteiso-C_{15:0} (5%) and anteiso-C_{17:0} (5%).

Phylogenetic analysis

The almost complete sequence (1501 bp) of the 16S rRNA gene from strain KB-1^{TP} was determined and aligned with available sequences of members of the *Bacillus/Clostridium* subphylum of the Gram-positive bacteria. Based on the sequence analysis, the 16S rDNA sequence had less than 92.7% similarity with any other known sequences. The most similar rDNA sequences were those of *Thermoanaerobacter brockii* subsp. *brockii*, *Thermoanaerobacter brockii* subsp. *finnii* and *Thermoanaerobacter brockii* subsp. *lactiethylicus* with 91.9–92.7% similarity (Table 1). Pairwise evolutionary distances were computed as described in Methods and employed in the construc-

tion of a phylogenetic dendrogram. The phylogenetic position of isolate KB-1^{TP} within the radiation of cluster V of the clostridia (Collins *et al.*, 1994) is shown in Fig. 4. The G+C content was 37 mol% and the G+C content of the 16S rDNA was 59.6 mol%.

DISCUSSION

According to current classification rules, the new isolate, strain KB-1^{TP}, can be considered to be a species of the genus *Thermoanaerobacter*. This assumption is supported by the examination of the almost complete 16S rDNA sequence, which clearly places strain KB-1^{TP} in cluster V of the clostridia as described by Collins *et al.* (1994). In addition, morphological and physiological characteristics of strain KB-1^{TP}, such as H₂S production from thiosulfate, spore formation and sensitivity to antibiotics, are in agreement with those of other described *Thermoanaerobacter* species.

Bacteria belonging to the genus *Thermoanaerobacter* are all thermophilic, anaerobic, rod-shaped bacteria that grow optimally in a temperature range between 60 and 70 °C. Strain KB-1^{TP} displays a temperature optimum of 75 °C and a maximum growth temperature of 85 °C, which is significantly higher than that of any other *Thermoanaerobacter* species. Among *Thermoanaerobacter* species, only two species are reported to grow at 80 °C, namely *Thermoanaerobacter acetoethylicus* and *Thermoanaerobacter brockii* subsp. *brockii* (Cayol *et al.*, 1995). However, these species do not utilize xylose as sole carbon source (Cayol *et al.*, 1995). Interestingly, unlike *Thermoanaerobacter acetoethylicus* and *Thermoanaerobacter brockii* subsp. *brockii*, the newly isolated strain utilizes D-xylose as sole carbon source (Table 1) and produces a thermostable D-xylose isomerase (data not shown). When grown on glucose and in the presence of thiosulfate the newly isolated micro-organism forms fermentation products which are typical for members of the genus *Thermoanaerobacter*. But in contrast to other validly described *Thermoanaerobacter* species, strain KB-1^{TP} additionally produces propionate and 1-pentanol from glucose. The fact that lactate was found to be the major fermentation product on glucose/thiosulfate is an uncommon finding among *Thermoanaerobacter* species, since members of this genus mostly form ethanol or acetate as major fermentation products. Growth of strain KB-1^{TP} is dependent on the presence of yeast extract which is – except in the case of *Thermoanaerobacter wiegelii* – a common growth factor for *Thermoanaerobacter* species (Cook *et al.*, 1996). Thiosulfate reduction to sulfide is a common trait of sulfate-reducing bacteria such as *Thermoanaerobacterium* and *Thermoanaerobacter* (Lee *et al.*, 1993a). This feature is also observed for the order *Thermotogales* (Ravot *et al.*, 1995). In the case of the genus *Thermoanaerobacter*, a faster growth rate and increased cell yield were obtained in the presence of thiosulfate than in its absence (Fardeau *et al.*, 1996). However, the dependency of thiosulfate as an electron

acceptor is different among *Thermoanaerobacter* species. Unlike *Thermoanaerobacter brockii* subsp. *brockii* DSM 1457^T and *Thermoanaerobacter ethanolicus* DSM 2246^T, strain KB-1^{TP} requires either thiosulfate or sulfur as final electron acceptor and only poor growth was observed when these compounds were not added to the medium. These results suggest that the requirement of electron acceptor for growth is one of the important distinguishing physiologies among *Thermoanaerobacter* species and further analyses for the other *Thermoanaerobacter* species is needed. Analysis of cellular lipids revealed the presence of uncommon glycolipids that could not be assigned to any known bacterial glycolipids. Although the cellular lipid composition is regarded as an important taxonomic factor (Asselineau & Asselineau, 1990; Jenkins, 1981), information in this particular field is not available for other *Thermoanaerobacter* species. Therefore, it is impossible to compare the results of lipid analysis of strain KB-1^{TP} with those of other members of the genus *Thermoanaerobacter*. However, this report on the cellular lipids and respiratory quinones in the genus *Thermoanaerobacter* may encourage other researchers to conduct similar studies of this indicative taxonomic feature.

Beside the physiological differences mentioned above, the new isolate is phylogenetically distant from members of the *Bacillus/Clostridium* subphylum as shown by 16S rDNA sequence analysis. The 16S rDNA sequence of strain KB-1^{TP} showed the highest similarity of 92.7% to those of *Thermoanaerobacter brockii* subsp. *brockii* and subsp. *finnii*. This value is very low and would even justify its classification to a new genus based on the phylogenetic dendrogram (Fig. 4). Strain KB-1^{TP} forms no subcluster with any species of the genus *Thermoanaerobacter* and shows a rather isolated position that branches off very early. This branch received 100% bootstrap support. However, taking into account the morphological and physiological characteristics, strain KB-1^{TP} can be easily accommodated in the genus *Thermoanaerobacter* rather than a new taxonomical group.

Description of *Thermoanaerobacter yonseiensis* sp. nov.

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

Cells are Gram-positive, straight, rod-shaped and motile. Size of vegetative cells in exponential growth phase is 0.4–0.8 × 1.0–3.0 µm. Under suboptimal conditions cells occur in long chains and terminal spores are observed. Optimal conditions for growth are 75 °C (range 50–85 °C) and pH 6.5 (range 4.5–9.0). The doubling time under optimal conditions on medium containing starch/yeast extract/thiosulfate is 60 min. Cells grow chemo-organotrophically under

strict anaerobic conditions on glucose, fructose, lactose, maltose, D-xylose, D-galactose, mannose, sucrose, cellobiose, starch, pullulan, yeast extract, tryptone and peptone. L-Arabinose, D-sorbitol, xylan, carboxymethylcellulose, olive oil, Tween 80, collagen and gelatin are not utilized. Cells require yeast extract, elemental sulfur, L-cystine, sodium nitrate or sodium thiosulfate for growth. Major fermentation products on glucose are lactate, acetate, ethanol, CO₂ and H₂S. Propionate, butyrate, isovalerate, 2-propanol and 1-pentanol are formed in small amounts. Growth on glucose is inhibited by tetracycline, chloramphenicol, penicillin G, neomycin, vancomycin, kanamycin and rifampicin (100 µg ml⁻¹). Growth was observed at NaCl concentrations up to 40 g l⁻¹. Respiratory lipoquinones are absent. Major cellular fatty acids are iso-C_{15:0}, iso-C_{17:0}, C_{16:0}, C_{18:1} ω7c, 3-OH iso-C_{14:0}, anteiso-C_{15:0} and anteiso-C_{17:0}. G + C content of the DNA is 37 mol%. Type strain KB-1^{TP} was isolated from mud samples taken from a hot stream at Sileri in the Dieng volcanic area located on the island of Java, Indonesia, and has been deposited in the Korean Federation of Culture Collections (KFCC 11116^P) as a patent strain and deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen as a type strain (= DSM 13777^T).

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