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

Byoung-Chan Kim,¹ Ralf Grote,² Dong-Woo Lee,¹ Garabed Antranikian² and Yu-Ryang Pyun¹

Author for correspondence: Yu-Ryang Pyun. Tel: +82 2 361 2883. Fax: +82 2 312 6821.
e-mail: yrpyun@yonsei.ac.kr

A novel strictly anaerobic, extremely thermophilic, spore-forming and xylose-utilizing bacterium, designated strain KB-1TP (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-1TP 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-1TP 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-1TP 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-1TP 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-1TP should be classified as a new species of the genus *Thermoanaerobacter*, designated *Thermoanaerobacter yonseiensis*. The type strain, KB-1TP, has been deposited in the Korean Federation of Culture Collections (KFCC 11116P) as a patent strain and in the Deutsche Sammlung von Mikroorganismen und Zellkulturen as a type strain (DSM 13777T).

**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-
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 Thermoanaerobacterium brockii; now including the three subspecies, brockii, fannii and lacteithylicus; Zeikus et al., 1979; Cayol et al., 1995), Thermoanaerobacter mathrani (Larsen et al., 1997), Thermoanaerobacter italicus (Kozianowski et al., 1997), Thermoanaerobacter wiegeli (Cook et al., 1996), Thermoanaerobacter acetothiolyticus (formerly Thermobacteroides acetothiolyticus; Ben-Bassat & Zeikus, 1981; Rainey & Stackebrandt, 1993; Collins et al., 1994), Thermoanaerobacter ethanolicus (Wiegel & Ljungdahl, 1981), Thermoanaerobacter thermophiles (formerly Clostridium thermophiles; Jin et al., 1988), Thermoanaerobacter sulfurophilus (Bonch-Osmolovskaya et al., 1997), Thermoanaerobacter thermohydrodsulfuricus (formerly Clostridium thermohydrodsulfuricum; 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 chem-oorganotrophically 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 thermoautotrophicus (formerly Clostridium thermoautotrophicus) (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$_2$S (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 (Erbezink 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-1TP 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 sicii 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$_2$S•9H$_2$O 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 40–70.

**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$_2$PO$_4$, 0.1 g K$_2$HPO$_4$, 10 g (NH$_4$)$_2$SO$_4$, 0.5 g NaHCO$_3$, 0.1 g KBr, 0.03 g H$_2$BO$_3$, 0.1 g KCl, 0.5 g MgCl$_2$, 6H$_2$O, 0.8 g MgSO$_4$•7H$_2$O, 0.1 g CaCl$_2$•2H$_2$O, 0.03 g SrCl$_2$, 6H$_2$O, 0.033 mg Na$_2$WO$_4$, 0.026 mg Na$_2$SeO$_3$, 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$_2$S•9H$_2$O (25% solution, w/v, pH 8.0). To this medium, filter-sterilized solutions of xylose, tryptone, starch or pyruvate (final concentration 50 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$_2$PO$_4$, 0.5 g (NH$_4$)$_2$SO$_4$, 0.1 g KBr, 0.03 g H$_2$BO$_3$, 0.5 g MgCl$_2$, 6H$_2$O, 0.5 g MgSO$_4$•7H$_2$O, 0.5 g CaCl$_2$, 2H$_2$O, 0.03 g SrCl$_2$, 6H$_2$O, 0.033 mg Na$_2$WO$_4$, 0.026 mg Na$_2$SeO$_3$, 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$_2$S•9H$_2$O, 1.0 mg resazurin and 3 ml Na$_2$S•9H$_2$O (25% solution, w/v, pH 8.0). Both media were prepared without Na$_2$S, 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$_2$S•9H$_2$O 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 anaerobic conditions 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–Hauser chamber (depth 0.02 mm) and phase-contrast microscopy at a magnification of ×400, or by monitoring the optical density at 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 (50 g l−1); trisodium citrate, pH range 3.0–5.0; 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 (2 g l−1), to prevent precipitation of FeS. 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–Hauser 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. Hacker’s method (Brock & Magidan, 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). Unincubated 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 3109T and the archaean Thermococcus sullci DSM 12349T were used as controls to ensure the effectiveness of the antibiotics at elevated temperatures. Inhibition was defined by cell densities of less than 1×106 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 2% (w/v) 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% (w/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 dodecamolybdophosphoric acid (total lipids), Zinzadze reagent (phosphate), ninhydrin (free amino groups), periodate–Schiff (x-glycols),
Dragedorff reagent (quaternary nitrogen) and anil-
sedehyde/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). The DNA base composition was determined according to the method described by Visuvanathan 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, correspond-
ing 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, 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 *Caldivulum sp* var. *saccharolyticus*
(DSM 8903T) as outgroup.

**Nucleotide sequence accession numbers.** Accession
numbers for the sequences used as references are as follows: *Thermo-
aerobacter brockii* subsp. *brockii* DSM 1457T, L09165; *Thermo-
aerobacter brockii* subsp. *finnii* DSM 3389T, L09166; *Thermo-
aerobacter brockii* subsp. *lactiethylicus* DSM 9801T, U14330; *Thermoaqerobacter marxianus* A3T
(= DSM 11426), Y11279; *Thermoanaerobacter italicus* Ab9T
(= DSM 9252T), AJ250846; *Thermoanaerobacter* 
*wiegelii* R18.B1T (= DSM 10319T), X92513; *Thermo-
aerobacter acetethylicus* DSM 2359T, L09163; *Thermo-
aerobacter ethanolicus* 39E (= DSM 2355), L09164; *Thermo-
aerobacter thermocoriae* JT-3T (= IAM 13577T), 
L09167; *Thermoanaerobacter* *sulfurophilus* L-64T (= DSM
11584T), Y16940; *Thermoanaerobacter* *thermoacidophila* 
*sa* DSM 567T, L09161; *Thermoanaerobacter* *kivui* 
DSM 2030T, L09160; *Thermoanaerobacter* *siderophilus* *SR4* (= DSM 12299T), AF120479; *Moorella* 
*thermoautotrophica* DSM 1974T, X77849; *Moorella thermoacida* 
DSM 12797, AJ242494; *Thermaerobacterium* 
*saccharolyticum* DSM 7060T, L09169; *Thermaerobacterium* 
*thermosaccharolyticum* D120-70, AF247003; *Caldivulum* 
*sp* var. *saccharolyticus* DSM 8903T, 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 (50 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 50 ml sample water to 50 ml EM-
KB1 medium under a N2 gas atmosphere. After 2 or 3
day 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 (50 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-
to-extinction technique. Each of the diluted cultures was
incubated for 3 days 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 and was chosen for further
investigation. Purity of the strain was confirmed by
microscopic examinations and formation of homoge-
one colonies on agar-solidified EM-KB1 medium in
Hungate tubes. Liquid cultures of strain KB-1 could
be used as inoculum for at least 12 months when
stored at 4 °C.

**Colony and cell morphology**

Colonies of isolate KB-1 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 were significantly
longer (about 40–50 µm) than those incubated at 70
or 80 °C (Fig. 1c). An electron micrograph of an
ultrathin section of strain KB-1 is shown in Fig. 2a.
Observation at higher magnification revealed a two-
layer structure of the cell wall (Fig. 2b), resembling
those of *Thermaerobacter* *ethanolicus* JW200T
(Wiegel & Ljungdahl, 1981) and *Thermaerobacter* 
*wiegelii* R18.B1T (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 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 anaer-
obically 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 grew over a tem-
Thermoanaerobacter yonseiensis sp. nov.

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

Temperature 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 × 10⁶ cells ml⁻¹. Strain KB-1TP grew well in GM-KB1 medium without the addition of NaCl (final cell yield 5 × 10⁵ 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-1TP 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 1457T, 5.9 × 10⁶ cells ml⁻¹; Thermoanaerobacter ethanolicus DSM 2246T, 2.1 × 10⁶ cells ml⁻¹; strain KB-1TP, 5.5 × 10⁵ cells ml⁻¹. However, in thiosulfate-free GM-KB1 medium, the final cell concentrations were: Thermoanaerobacter brockii subsp. brockii,
3.0 × 10⁸ cells ml⁻¹; *Thermoanaerobacter ethanolicus*, 4.5 × 10⁶ cells ml⁻¹; strain KB-1TP, 2.5 × 10⁶ cells ml⁻¹. In contrast to *Thermoanaerobacter brockii* and *Thermoanaerobacter ethanolicus*, the growth of strain KB-1TP was significantly lower without thiosulfate.

**Nutritional requirements and fermentation products**

Isolate KB-1TP 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 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-1TP 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 × 10⁷ cells ml⁻¹).

Fig. 2. Ultrastructure of isolate KB-1TP. (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.

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

Fig. 3. (a) Effect of temperature on growth of strain KB-1TP. (b) Effect of pH on growth of strain KB-1TP. ■, Trisodium citrate buffer; □, MES buffer; ●, Tris buffer; ○, sodium carbonate buffer. The pH values were the initial values adjusted at room temperature.
Table 1. Characteristics of strain KB-1TP in comparison with other species of the genus Thermoanaerobacter

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rDNA similarity to strain KB-1TP (%)</td>
<td>92.7</td>
<td>92.7</td>
<td>91.9</td>
<td>91.4</td>
<td>91.8</td>
<td>92.2</td>
<td>90.6</td>
<td>90.7</td>
<td>91.4</td>
<td>92.0</td>
<td>91.8</td>
<td>92.2</td>
<td>91.1</td>
<td>100</td>
</tr>
<tr>
<td>Growth temperature (°C)</td>
<td>&lt;85</td>
<td>75</td>
<td>75</td>
<td>&lt;78</td>
<td>78</td>
<td>78</td>
<td>80</td>
<td>78</td>
<td>74</td>
<td>75</td>
<td>78</td>
<td>75</td>
<td>78</td>
<td>85</td>
</tr>
<tr>
<td>Optimum</td>
<td>65–75</td>
<td>65</td>
<td>55–60</td>
<td>70–75</td>
<td>70</td>
<td>65–68</td>
<td>65</td>
<td>69</td>
<td>60</td>
<td>55–60</td>
<td>67–69</td>
<td>66</td>
<td>69–71</td>
<td>75</td>
</tr>
<tr>
<td>Spore formation</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Gram staining</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Utilization of:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Starch</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Xylan</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Xylose</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Xylose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 4. The phylogenetic tree based on 16S rDNA sequence comparisons of strain KB-1TP and selected bacteria was constructed by the neighbour-joining method and rooted with Caldocellulosiruptor saccharolyticus (DSM 8903T). 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-1TP 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-C15:0 (42%), iso-C17:0 (11%), C16:0 (7%), C18:1ω7c (6%), 3-OH iso-C14:0 (6%), anteiso-C15:0 (5%) and anteiso-C17:0 (5%).

Phylogenetic analysis

The almost complete sequence (1501 bp) of the 16S rRNA gene from strain KB-1TP 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-
**DISCUSSION**

According to current classification rules, the new isolate, strain KB-1<sub>TP</sub>, 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<sub>TP</sub> in cluster V of the clostridia as described by Collins *et al.* (1994). In addition, morphological and physiological characteristics of strain KB-1<sub>TP</sub>, such as H<sub>2</sub>S 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<sub>TP</sub> 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 acetoethlyicus* 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 acetoethlyicus* 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<sub>TP</sub> 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<sub>TP</sub> is dependent on the presence of yeast extract which is – except in the case of *Thermoanaerobacter wiegelli* – 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* DSM 1457<sup>T</sup> and *Thermoanaerobacter ethano- licus* DSM 2246<sup>T</sup>, strain KB-1<sub>TP</sub> 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<sub>TP</sub> 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<sub>TP</sub> showed the highest similarity of 92·7% to those of *Thermoanaerobacter brockii* subsp. *brockii* and subsp. *finnti*. 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<sub>TP</sub> 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<sub>TP</sub> 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 x 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-xylene, d-galactose, mannose, sucrose, cellulose, starch, pullulan, yeast extract, trypthone 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 l-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-C₁₅:₀, iso-C₁₇:₀, C₁₆:₀, C₁₈:₁ ω7c, 3-OH iso-C₁₄:₀ and anteiso-C₁₆:₀ and anteiso-C₁₇:₀. G + C content of the DNA is 37 mol%. Type strain KB-1 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 (KFFC 11116₅) as a patent strain and deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen as a type strain (= DSM 13777₅).

ACKNOWLEDGEMENTS

This work was supported by the Korea Science and Engineering Foundation (KOSEF) through the Bioproducts Research Center at Yonsei University (2000-2-0173). We thank Dr B. J. Tindall at the DSMZ for the respiratory lipoquinone, fatty acid and polar lipid analyses, and Professor H. G. Trüper for valuable advice in nomenclature.

REFERENCES


