Extremophiles (2009) 13:505–510
DOI 10.1007/s00792-009-0235-5

ORIGINAL PAPER

Thermovorax subterraneus, gen. nov., sp. nov., a thermophilic hydrogen-producing bacterium isolated from geothermally active underground mine

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Received: 4 December 2008 / Accepted: 26 February 2009 / Published online: 21 March 2009

Abstract A thermophilic, rod-shaped, motile, Gram-positive, spore-forming bacterium strain 70B^T was isolated from a geothermally active underground mine in Japan. The temperature and pH range for growth was 50–81°C (optimum 71°C) and 6.2–9.8 (optimum pH 7–7.5), respectively. Growth occurred in the presence 0–2% NaCl (optimum 1% NaCl). Strain 70B^T could utilize glucose, fructose, mannose, mannitol, pyruvate, cellobiose and tryptone as substrates. Thiosulfate was used as electron acceptor. Major whole-cell fatty acids were iso-$C_{15:0}$, $C_{16:0}$ DMA (dimethyl acetal), $C_{16:0}$ and anteiso-$C_{15:0}$. The G+C mol% of the DNA was 44.2%. Phylogenetic analysis based on the 16S rRNA gene sequence revealed that the closest relatives of strain 70B^T were *Thermosediminibacter oceani* DSM 16646^T (94% similarity) and *Thermosediminibacter litoriperuensis* DSM 16647 (93% similarity). The phenotypic, chemotaxonomic and phylogenetic properties suggest that strain 70B^T represents a novel species in a new genus, for which the name *Thermovorax subterraneus* gen. nov., sp. nov. is proposed. The type strain of *Thermovorax subterraneus* is 70B^T (=DSM 21563 = JCM 15541).

Keywords *Thermovorax subterraneus* · Gen. nov · Sp. nov. · Thermophilic · Hydrogen-producing bacterium

Communicated by L. Huang.

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Introduction

Thermophilic microorganisms have increasingly attracted attention because of their great potential in biotechnological applications such as fermentative hydrogen production using hot industrial effluents as substrate. Thermophilic microbiological hydrogen production trough dark fermentation may have many advantages as compared to mesophilic fermentation but has remained less studied. At elevated temperatures reaction rates and thermodynamic favorability of hydrogen production reactions will increase, solubility of hydrogen will decrease and less side products are formed (Hawkes et al. 2002; van Groenestijn et al. 2002; van Niel et al. 2003). High temperature also decreases the risk of contamination of bioprocesses by H_2-consuming organisms and pathogens (van Groenestijn et al. 2002; Kotsopoulos et al. 2006). Thus, there is a need to isolate and characterize novel thermophilic hydrogen fermenting bacteria to further the progress in this promising area.

So far, thermophilic bacteria used in hydrogen dark fermentation studies have been isolated, for example, from hot spring environments (Jannasch et al. 1989; Rainey et al. 1994; Orlygsson and Baldursson 2007), effluent of anaerobic reactors (Ueno et al. 2001; O-Thong et al. 2008) and oil-producing well (Ravot et al. 1995). Also geothermal underground environments, such as volcanic waters, soils and sediment, provide excellent habitat for thermophilic anaerobic bacteria where they usually form microbial mats utilizing metabolites of other bacteria for growth (Lowe et al. 1993). This paper describes one hydrogen-producing strain 70B^T isolated from geothermal underground sediment. On the basis of phylogenetic analysis, the closest relatives of this isolate are *Thermosediminibacter oceani* and *Thermosediminibacter litoriperuensis,* both thermophilic fermentative bacteria isolated from deep-sea sediment (Lee et al. 2005).

Materials and methods

Bacterial strain and cultivation

Strain 70B^T was isolated from an underground mine 250 m below ground, from a black sediment layer beneath a thin red crust of ferric iron on a tunnel wall. The temperature at the site was 70–80°C. Sediment samples were collected into sterile 50 mL plastic tubes and tubes were filled with water from the sampling site to replace air from the tubes. First enrichment of strain 70B^T was carried out at 80°C using modified Pfenning medium (Kaksonen et al. 2006). Sediment samples were inoculated on to anaerobic Hungate tubes in anaerobic chamber. Subsequent enrichment and isolation of strain 70B^T were performed at 70°C. The medium used was based on the formulation of Zehnder et al. (1980). Following adjustments described in Karlsson et al. (1999) were made to vitamin solution of the medium: 250 mg L^{-1} pyridoxamine, 100 mg L^{-1} nicotinic acid, 50 mg L^{-1} cyanocobalamin, 100 mg L^{-1} thiamine hydrochloride and 100 mg L^{-1} nicotinamide; and following additions described by Ejlertsson et al. (1996) were made to the medium: 33 μg L^{-1} $Na_2WO_4 \cdot H_2O$, 0.5 g L^{-1} cysteine HCl and 0.2 g L^{-1} yeast extract. The medium contained 27.8 mM glucose as substrate. Isolation was performed in anaerobic roll tubes solidified with 2% agar.

Phenotypic characterization

The isolate was examined under phase-contrast microscopy (Axioskop 2; Zeiss) and photomicrographs were obtained using the agar-coated slides. Flagellum staining was performed according to Heimbrook et al. (1989). Spore formation by the strain was examined by spore staining, microscopically and by testing for growth after heat treatment (95°C for 25 min). Gram staining and the KOH test (Gregersen 1978) were used to determine the Gram's reaction of the cells. The capability of growth with O_2 was determined using 2%O_2, v/v, in the gas phase and reducing agents were omitted from the growth medium. The effect of temperature on growth was determined by measuring the specific growth rate by means of optical density and the effects of pH and NaCl concentration on growth were determined by measuring final optical density (600 nm) (Ultrospec 500 Pro Visible spectrophotometer; Amersham Biosciences). The temperature range for growth was determined using temperature gradient incubator (Test tube incubator; Terratec). To examine the effect of pH on growth the growth medium was adjusted by omitting $NaHCO_3$, the concentrations of KH_2PO_4 and Na_2HPO_4 were standardized to 1 mM and the following pH buffers (each at 10 mM) were used: MES (morpholineethanesulfonic acid; pH 6.0, 6.2 and 6.5); MOPS (morpholinepropanesulfonic acid; pH 7.0 and 7.5); HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; pH 8); Tris [2-amino-2-(hydroxymethyl)-1-3-propanediol; pH 8.5]; CHES [2-(N-cyclohexylamino) ethanesulfonic acid; pH 9.0 and 9.5); and CAPS [3-(cyclohexylamino)-1-propanesulfonic acid; pH 9.7, 9.8 and 9.9]. The ability of the strain to use different substrates was studied using 20 mM substrate concentration. Liquid fermentation products were measured with a high-performance liquid chromatograph (Waters 510) with a Shodex Sugar SH1011 column (Showa denko K·K.) and a Δn –1,000 refraction index detector (WGE Dr. Bures GmbH & Co. KG) in isocratic mode. Gaseous fermentation products were measured with a gas chromatograph (GC-2014, Shimadzu) equipped with a packed (packing, Porapak N 80-100 mesh), stainless steel column, and a thermal conductivity detector. Oven, injector and detector temperatures were 80, 110, and 110°C, respectively, and nitrogen was used as the carrier gas. Electron acceptor utilization was determined using glucose (27.8 mM) as an electron donor. The cultures were incubated for 2 weeks at 70°C. Amorphous iron(III) oxyhydroxide was prepared by neutralizing $FeCl_3$ solution to pH 7 with NaOH. Utilization of different electron acceptors (sulfate, sulfite, thiosulfate, sulfur, nitrate, Fe(III)oxide, MnO_2) was determined by measuring bacterial growth, and also monitored by sulfide, sulfate, thiosulfate, sulfite, nitrate, nitrite, ferrous iron content in the medium or color change (MnO_2). Sulfide production was determined spectrophotometrically (UV-1601 Visible spectrophotometer; Shimadzu) according to Cord-Ruwisch (1985). Concentrations of sulfate, thiosulfate, sulfite, nitrate and nitrite was determined using ion chromatograph (Dionex DX-120). Ferrous iron formation was determined colorimetrically (UV-1601 Visible spectrophotometer; Shimadzu) with 1,10-*ortho*-phenanthroline according to 3500-Fe (Anonymous 1992).

Chemotaxonomic characterization

The composition of fatty acid methyl esters of cellular fatty acids was analyzed at the Identification Service of the DSMZ according to method of Sasser (1990). Polar lipid analyses and analysis of respiratory quinones were carried out by the Identification Service of the DSMZ and Dr. B. J. Tindall, DSMZ, Braunschweig, Germany. G+C content of genomic DNA was determined using HPLC at the Identification Service of the DSMZ.

Genetic and phylogenetic analysis

Nucleic acids were extracted with a VIOGENE Blood and Tissue Genomic DNA kit (Proteogenix SA). The nearly complete (1,469 bp) 16S rRNA gene of isolate was

amplified using PCR with primer pair 27F (5′-GTTG ATCCTGGCTCAG-3′) and 1492R (5′-ACGGYTACC TTGTTACGACTT-3′). Sequence data were analyzed with Bioedit-software (version 7.0.5.2; Hall 1999) and compared with sequences in GenBank (http://www.ncbi.nlm.nih.gov/blast/). A 16S rRNA sequence similarities were analyzed with Bioedit-software using pairwise alignment. The 16S rRNA gene sequence of isolate was aligned against closest relatives using fast aligner in ARB software (Ludwig et al. 2004). Phylogenetic tree was constructed using distance matrix and neighbor-joining algorithms in ARB software. The robustness of the phylogeny was tested by bootstrap analysis with 1,000 iterations.

Results and discussion

Phenotypic characteristics

Cells of strain 70B^T were straight or slightly curved, sometimes elongated rods, 0.3–0.7 μm in diameter and 1.4–20 μm in length, which occurred singly, in pairs or in chains (Fig. 1). In the late-exponential or stationary growth phase, cells started to swell giving them circular shape. The strain was able to grow after heat treatment at 95°C for 25 min. Gelatin stimulated spore formation. The strain formed spherical spores which were located terminally (Fig. 1). The strain was motile, based on flagella staining and microscopy strain had bipolar lophotrichous flagella of 5–10 μm in length (Fig. 1). The strain 70B^T was Gram-positive as determined by KOH test but stained Gram-negative. The strain was not able to grow in the presence of O_2. The ranges of temperature, pH and NaCl concentration for growth of strain 70B^T are listed in Table 1. The shortest doubling time at optimum temperature (71°C) was 4.9 h. The temperature at the sampling site was 70–80°C which is in the growth range of strain 70B^T. Table 1 lists various substrates and electron acceptors for strain 70B^T. The strain fermented glucose mainly to H_2, CO_2, acetate, ethanol and lactate. H_2 yield based on cumulative H_2 production of strain 70B^T is shown in Fig. 2.

Strain 70B^T has some similar phenotypic characteristics to species of genus *Thermosediminibacter* such as rod shape, sometimes highly elongated and in late exponential growth phase swelling cells. However, it differs from its closest relatives based on spore formation and ability or inability to use certain electron acceptors and substrates (Table 1).

Chemotaxonomic characteristics

The whole-cell fatty acid composition of strain 70B^T is given in Table 2. The major fatty acids were iso-$C_{15:0}$, $C_{16:0}$ DMA (dimethyl acetal), $C_{16:0}$ and anteiso-$C_{15:0}$ (Table 2). Rf values and staining properties of detected polar lipids were not consistent with any known phospholipids. This differentiates strain 70B^T clearly from its closest relatives in genus *Thermosediminibacter* which have known phospholipid fatty acid profiles (Lee et al. 2005). Respiratory quinones were not detected in strain 70B^T. The G+C content of the total DNA of the strain 70^T (44.2 mol%) is lower than that of *T. oceani* (50 mol%) and *T. litoriperuensis* (50 mol%) (Lee et al. 2005).

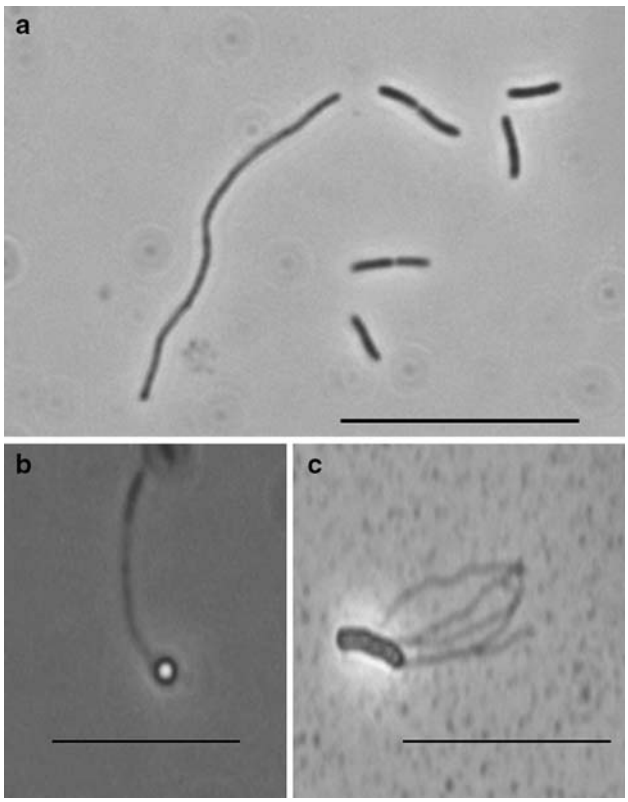

Fig. 1 Phase-contrast micrographs of strain 70B^T. Cells from mid-exponential growth phase (**a**), sporulating cell (**b**), flagellum-stained cell (**c**). *Bars* 10 μm

Genetic and phylogenetic characteristics

Phylogenetic analysis (Fig. 3) showed that the closest relatives of strain 70B^T are *Thermosediminibacter oceani* DSM 16646^T (94% 16S rRNA gene sequence similarity) and *Thermosediminibacter litoriperuensis* DSM 16647^T (93% 16S rRNA gene sequence similarity), both isolated from a deep-sea sediment (Lee et al. 2005). These phenotypic, chemotaxonomic and phylogenetic differences discussed above suggest that the strain 70B^T represents a novel species of a new genus, for which the name *Thermovorax subterraneus* gen. nov., sp. nov. is proposed.

Table 1 Characteristics of strain 70B^T in comparison of those of its closest phylogenetic relatives

Characteristic	Strain		
	1	2	3
Temperature range (°C)	50–81	52–76	43–76
Optimal temperature (°C)	71	68	64
pH range	6.2–9.8	6.3–9.3	5–9.5
Optimal pH	7.0–7.5	7.5	7.9–8.4
NaCl range (%)	0–2	0–6	0–4.5
Optimal salinity (NaCl %)	1	1	0.5–2
GC content (mol%)	42.2	50	50
Substrates			
Mannitol (20 mM)	+	−	+
Pyruvate (20 mM)	+	−	NR
Cellobiose (20 mM)	+	−	NR
Tryptone (0.2% w/v)	+	−	+
Lactate (20 mM)	−	−	+
Xylose (20 mM)	−	+	+
Myo-inositol (20 mM)	−	−	+
Raffinose (20 mM)	−	−	+
Sucrose (20 mM)	−	+	+
Galactose (20 mM)	−	NR	+
Cellulose (0.2% w/v)	−	NR	NR
Starch (0.2% w/v)	−	NR	NR
Gelatin (0.2% w/v)	−	NR	NR
Xylan (0.2% w/v)	−	NR	NR
Electron acceptors			
Sulfur (20 mM)	−	+	+
MnO_2 (10 mM)	−	+	+

All taxa shared the following characteristics: motile, negative for Gram staining, positive for Gram reaction (results from this study), ability to use glucose (20 mM), fructose (20 mM), mannose (20 mM) as substrates, thiosulfate (20 mM) reduction and inability to use sulfate (20 mM), sulfite (2 mM), nitrate (20 mM) and Fe(III)oxide (90 mM) as electron acceptors. The concentrations apply only to this study

Taxa: 1, 70B^T (This study); 2, *Thermosediminibacter oceani* (Lee et al. 2005); 3, *T. litoriperunsis* (Lee et al. 2005)

+ Positive, − negative, *NR* not reported

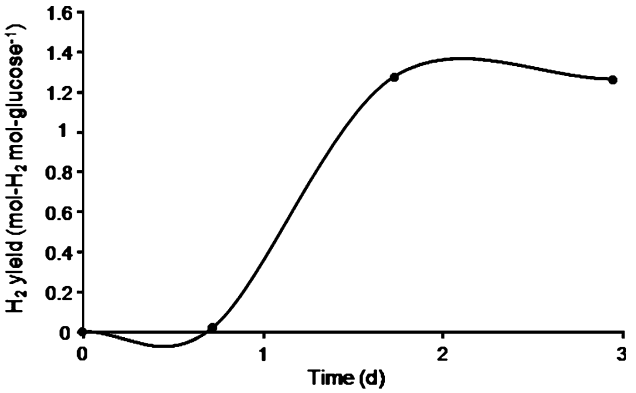

Fig. 2 Hydrogen yield based on the cumulative hydrogen production showing the hydrogen production property of strain 70B^T

Table 2 Whole cell fatty acid composition of strain 70B^T

ECL	Fatty acid	Content (%)
11.61	iso-$C_{12:0}$	0.13
12.61	iso-$C_{13:0}$	0.63
12.70	anteiso-$C_{14:0}$	0.12
13.62	iso-$C_{14:0}$	6.41
14	$C_{14:0}$	4.62
14.11	iso 3OH-$C_{13:0}$	0.28
14.47	$C_{14:0}$ DMA	0.66
14.62	iso-$C_{15:0}$	15.43
14.71	anteiso-$C_{15:0}$	9.64
14.95	$C_{16:0}$ aldehyde	2.81
15	$C_{15:0}$	4.92
15.11	iso-$C_{15:0}$ DMA	2.96
15.20	anteiso-$C_{15:0}$ DMA	0.92
15.63	iso-$C_{16:0}$	8.76
16	$C_{16:0}$	10.96
16.11	$C_{16:0i}$ DMA	7.64
16.47	$C_{16:0}$ DMA	12.83
16.63	iso-$C_{17:0}$	1.42
16.72	anteiso-$C_{17:0}$	0.59
17	$C_{17:0}$	0.77
17.10	$C_{17:0i}$ DMA	2.07
17.20	anteiso-$C_{17:0}$ DMA	0.93
17.47	$C_{17:0}$ DMA	1.22
18	$C_{18:0}$	0.47
18.47	$C_{18:0}$ DMA	0.32
	Total unidentified	2.49

ECL equivalent chain length, *DMA* dimethyl acetal

Description of *Thermovorax* gen. nov

Thermovorax (ther.mo.vo'rax. Gr. adj. *thermos* hot; L. n. *vorax* devourer; N. L. masc. n. *thermovorax* eating at elevated temperature).

Based on the phylogenetic position, the genus *Thermovorax* belongs to the Thermoanaerobacteraceae family within the low G+C, Gram-type-positive *Bacillus–Clostridium* subphylum. Cells are rod-shaped and swollen in late-exponential growth phase, spore-forming, Gram-positive, anaerobic and thermophilic. Growth occurs mainly fermentatively. Optimal growth occurs at neutral pH. The genomic DNA G+C content of the type species is 44.2 mol%. The type species is *Thermovorax subterraneus*.

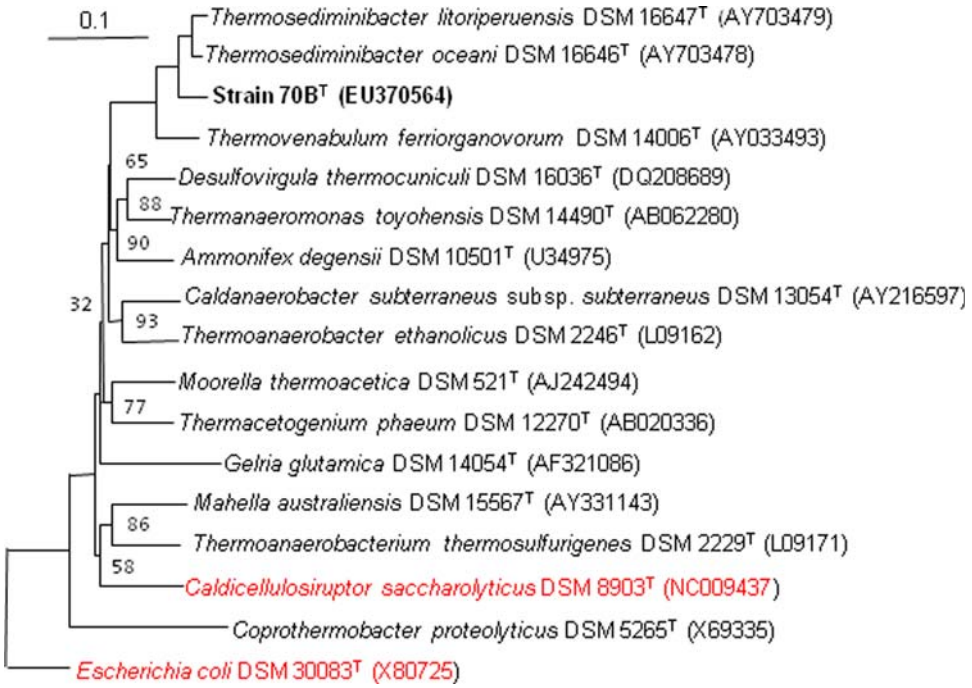

Fig. 3 Phylogenetic tree generated using distance matrix and neighbor-joining methods based on the 16S rRNA gene sequences of strain 70B^T (1,469 bp between *Escherichia* coli positions 28 and 1,490) and related taxa. *E. coli*^{T} (X80725) was used as outgroup. *Numbers* at *nodes* represent bootstrap values based on 1,000 samplings. *Bar* 0.1 nucleotide changes per position

Description of *Thermovorax subterraneus* sp. nov

Thermovorax subterraneus (sub.ter.ra'ne.us. L. masc. adj. *subterraneus* subterranean, underground; *subterraneus* inhabitant in the subsurface). In addition to the properties given in the description of the genus, the following properties are observed. Cells are motile, 0.3–0.7 μm in diameter and 1.4–20 μm in length and occur singly, in pairs or in chains. Growth occurs at 50–81°C (optimum 71°C), pH 6.2–9.8 (optimum pH 7–7.5) and NaCl concentrations of 0–2% (optimum 1% NaCl). Thiosulfate is used as electron acceptor and reduced to sulfide but sulfate, sulfite, sulfur, nitrate, Fe(III) or MnO_2 are not used as electron acceptors. Glucose, fructose, mannose, mannitol, pyruvate, cellobiose and tryptone can be used as substrates. Fermentation products from glucose are H_2, CO_2, acetate, ethanol and lactate. The type strain, 70B^T (=DSM 21563 = JCM 15541) was isolated from a geothermally active underground mine. The GenBank 16S rRNA gene sequence accession number is EU370564.

Acknowledgments This research was funded by the Academy of Finland (HYDROGENE project, no. 107425) and Nordic Energy Research (BIOHYDROGEN project, no. 28–02, BioH2 project, no. 06-Hydr-C13). Noora Männistö and Marika Nissilä are acknowledged for the technical assistance.

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