Extremophiles (2008) 12:729–734
DOI 10.1007/s00792-008-0180-8

ORIGINAL PAPER

Acinetobacter sp. strain Ths, a novel psychrotolerant and alkalitolerant bacterium that utilizes hydrocarbon

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Received: 7 May 2008 / Accepted: 23 June 2008 / Published online: 16 July 2008

Abstract A novel psychrotolerant, alkalitolerant bacterium, strain Ths, was isolated from a soil sample immersed in hot spring water containing hydrocarbons and grown on a chemically defined medium containing *n*-tetradecane as the sole carbon source. The isolate grew at 0°C but not at temperatures higher than 45°C; its optimum growth temperature was 27°C. It grew in the pH range of 7–9. The strain utilized C_{13}–C_{30} *n*-alkane and fluorene at pH 9 and 4°C. To our knowledge, this is the first report on the bacterium that utilizes a wide range of hydrocarbons at a high pH and a low temperature. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain Ths is closely related to genomic species 6 ATCC 17979 (99.1% similarity), genomic species BJ13/TU14 ATCC 17905 (97.8% similarity), genomic species 9 ATCC 9957 (97.6% similarity) belonging to the genus *Acinetobacter* and to *Acinetobacter calcoaceticus* JCM 6842^T (97.5% similarity). DNA–DNA hybridization revealed that the isolate has 62, 25, 18 and 19% relatedness, respectively, to genomic species 6 ATCC 17979, genomic species BJ13/TU14 ATCC 17905, genomic species 9 ATCC 9957 and *A. calcoaceticus*, respectively.

Keywords *Acinetobacter* · Hydrocarbon utilizing · Psychrotolerant · Alkalitolerant

Communicated by L. Huang.

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Introduction

Oil pollution in particular areas, such as land used as a petroleum stand, is currently a serious environmental problem. Once soil is contaminated by oil, it is difficult for the oil to be decomposed by a natural population of microorganisms. Although there are several methods, such as physical methods, of removing oil from contaminated soil, most of them are costly and it is difficult to treat huge amounts of oil-contaminated soil. Therefore, bioremediation by certain organisms, especially microorganisms, is a promising method for treating oil-contaminated soil. To date, there have been fewer microorganisms suitable for treating oil-contaminated soil in a cold environment than those in a mesophilic environment. Temperature is an important factor in controlling the nature of hydrocarbons and the metabolism of hydrocarbons in microorganisms. In addition, temperature is important in in situ bioremediation because of the difficulty in artificial regulation. At low temperatures, the viscosity of oil increases, preventing oil spread in the water phase, and the volatilization of toxic short-chain *n*-alkanes is prevented and long-chain *n*-alkanes are solidified. Furthermore, metabolism and propagation are inhibited in mesophilic microorganisms. Cold-adapted microorganisms capable of degrading oil hydrocarbons at

low temperatures have been reported (Westlake et al. 1974; Whyte et al. 1996, 1998, 1999; MacCormack and Fraile 1997; Margesin and Schinner 1997a, 1997b, 1997c, 1999, 2001; Foght et al. 1999; Baranieck et al. 2002; Yumoto et al. 2002; Margesin et al. 2003). However, there are few reports on the isolation of cold-adapted microorganisms utilizing a wide range of hydrocarbons in a wide range of pH.

In this study, considering the variety of oil-contaminated environmental conditions, a psychrotolerant, alkalitolerant bacterium, strain Ths, that can decompose *n*-alkanes was isolated from a soil sample immersed in hot spring water containing hydrocarbons from Toyotomi Hot Spring located in the northern part of Hokkaido, Japan. Phenotypic and chemotaxonomic characteristics, phylogenetic analysis based on 16S rRNA gene sequences and DNA–DNA hybridization showed that the isolate can be identified as a novel strain of the genus *Acinetobacter*. The isolate will be useful in a wide range of application for environmental oil-contaminated water or soil.

Materials and methods

Bacterial strain and cultivation

In June 2002, a soil sample immersed in hot spring water (32.5°C, pH 7.4) containing hydrocarbons was obtained from Toyotomi, in Hokkaido, Japan. A small amount of the obtained soil sample was inoculated using a synthetic medium (AT medium) consisting of KNO_3, 5 g; KH_2PO_4, 0.5 g; $MgSO_4{\cdot}7H_2O$, 0.5 g; $FeSO_4{\cdot}7H_2O$, 0.01 g; $CaCl_2{\cdot}2H_2O$, 0.02 g; $MnSO_4{\cdot}nH_2O$; and 0.001 g; $ZnSO_4{\cdot}7H_2O$, 0.0005 g in 1 l of 100 mM $NaHCO_3$–Na_2CO_3 buffer (pH 9) in deionized water supplemented with 3% *n*-tetradecane as the sole carbon source and incubated at 27°C for 9 days. Part of the obtained cultured medium was spread on the AT medium containing 1.5% agar supplemented with *n*-tetradecane as the sole carbon source. *n*-Tetradecane was vaporized onto the surface of the agar plate by soaking filtration paper in it, which was then placed under the AT agar medium on a plate and incubated at 27°C for 1 week. After the incubation, a colony that had appeared was transferred to Luria–Bertani (LB) agar (pH 7.2) consisting of tryptone (Difco), 10 g; yeast extract (Difco), 5 g; NaCl, 10 g; and agar, 20 g in 1 l of distilled water, reisolated five times and maintained in the same medium. *Acinetobacter junii* ATCC 17908^T, *Acinetobacter johnsonii* ATCC 17909^T, *Acinetobacter haemolyricus* ATCC 17906^T, *Acinetobacter radioresistens* IAM 13186^T, *Acinetobacter ursingii* NIPH 137^T, *Acinetobacter schindleri* NIPH 1034^T, *Acinetobacter parvus* NIPH 384^T, *Acinetobacter bouvetii* DSM 14964^T, *Acinetobacter grimotii* DSM 14968^T, *Acinetobacter baumanii* JCM 6840^T, *Acinetobacter calcoaceticus* JCM 6842^T, *Acinetobacter lowffii* JCM 6840^T, genomic species BJ13/TU14 (ATCC 17905), genomic species 6 (ATCC 17979, LUH 4717 and LUH 286), genomic species 9 (ATCC 9957), genomic species 3 (ATCC 19004), and '*Acinetobacter ventianus*' (ATCC 31012) were used as reference strains for DNA–DNA hybridization. They were cultured in LB broth (with the same constituents as LB medium, without the agar) until the late exponential growth phase at 27°C.

Phenotypic characterization

For phenotypic characterization of the isolate and genomic species 6 ATCC 17979, LB broth or LB agar was used as the basal medium. The culture was incubated at 27°C for 2 weeks, and experiments were performed three times to confirm the reproducibility of results. Acid production from carbohydrates was determined by the method of Hugh and Leifson (1953). Determination of substrate utilization as the sole carbon and energy source was performed US medium containing substrate, 0.2%; NH_4Cl, 2 g; Na_2HPO_4, 2 g; KH_2PO_4, 1 g; $MgSO_4{\cdot}7H_2O$, 0.1 g; $CaCl_2{\cdot}2H_2O$, 0.05 g; and 1 ml metal mixture in 1 l of distilled water. The metal mixture included (per 100 ml distilled water) EDTA·2Na, 1.8 g; $ZnSO_4{\cdot}7H_2O$, 5.0 g; $FeSO_4{\cdot}7H_2O$, 5.0 g; $MnSO_4{\cdot}4H_2O$, 1.5 g; $CuSO_4{\cdot}5H_2O$, 0.4 g; $Co(NO_3)_2{\cdot}6H_2O$, 0.25 g; and H_2BO_3, 0.1 g. Other physiological and biochemical characteristics were examined according to methods described in Cowan and Steel's Manual (Barrow and Feltham 1993). For comparison with previously reported *Acinetobacter* strains, physiological and biochemical characteristics were examined according to methods described by Bouvet and Grimont (1987). The optimum growth temperature and pH of strain Ths were determined using a temperature gradient incubator (model TN-2612; Advantec, Tokyo, Japan). L-shaped tubes, that each contained 10 ml of LB broth, were used. The temperature gradient range used was 5–50°C. The growth at a temperature below 5°C was examined using a conventional incubator. Growth experiments at pH 7–10 were performed using LB broth containing 100 mM NaH_2PO_4/Na_2HPO_4 buffer (pH 7–8) and 100 mM $NaHCO_3/Na_2CO_3$ buffer (pH 9–10). Growth depending on the pH (pH range of pH 7–10) was estimated at 27°C. Hydrocarbon utilization was tested using AT medium containing 1% (v/v) hydrocarbon at pH 9 (100 mM $NaHCO_3/Na_2CO_3$ buffer) at 4 and 10°C.

Electron microscopy

To observe negatively stained cells by transmission electron microscopy (TEM) (model H-800, Hitachi), cells were grown on LB agar medium. TEM preparation and observation were performed as described previously (Yumoto et al. 2001).

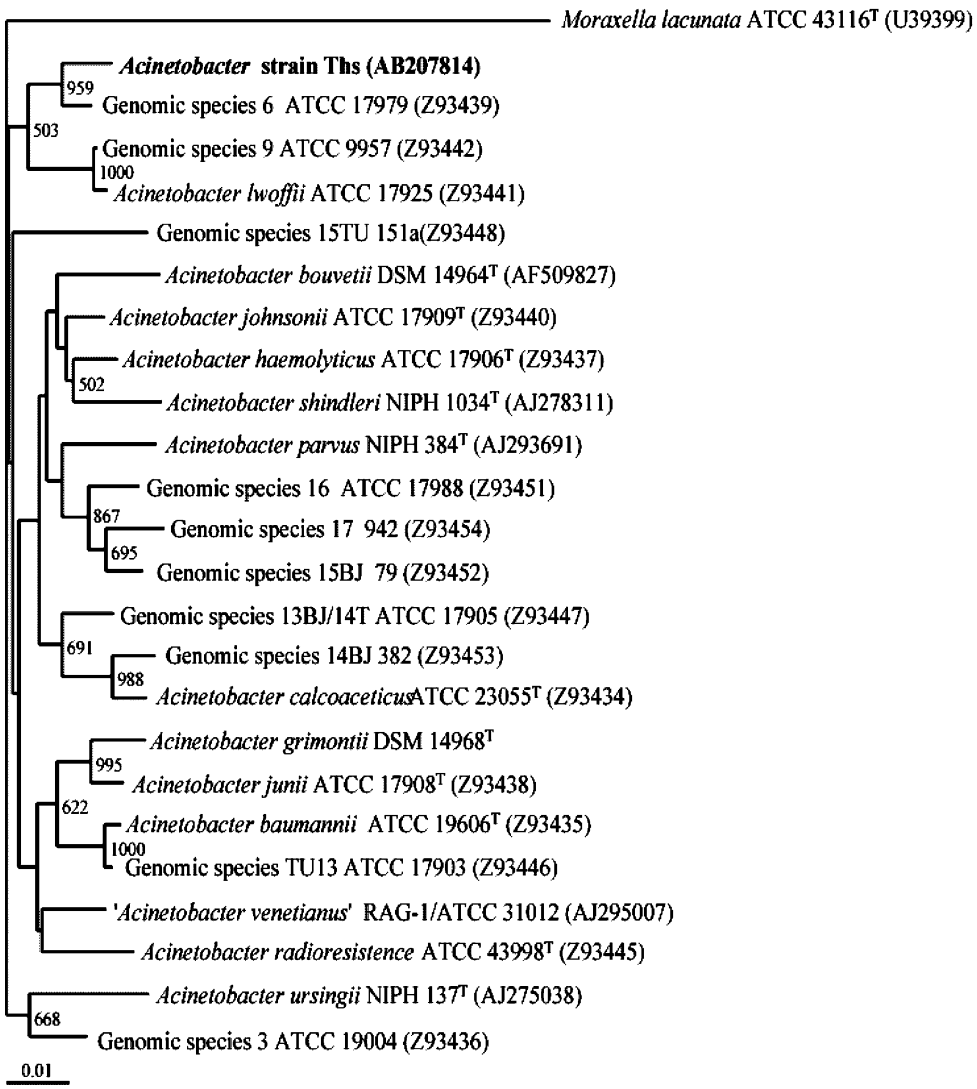

Fig. 1 Phylogenetic tree derived from 16S rRNA gene sequence data of *Acinetobacter* sp. strain Ths and other related organisms was contracted by the neighbor-joining method. *Numbers* indicate bootstrap values (from 1,000 replications) greater than 500. *Bar* 0.01 K_{nuc}

Chemotaxonomic characterization

Whole-cell fatty acids and isoprenoid quinones were performed as described previously (Yumoto et al. 2001). Bacterial DNA was prepared according to the method of Marmur (1961). DNA base composition was determined by the method of Tamaoka and Komagata (1984).

16S rRNA sequencing

The 16S rRNA gene sequence of strain Ths was analyzed to determine the phylogenetic position. The 16S rRNA gene was amplified using the PCR method with the primers 9F (GAGTTTGATCCTGGCTCAG) and 1541R (AAGG AGGTGATCCAGCC). The PCR product of approximately 1.5 kb was sequenced directly by the dideoxynucleotide chain-termination method using a DNA sequencer (ABI PRISM 3100) with a BigDye™ Termination RR mix, version 3.1 (Applied Biosystems), according to the manufacturer's instructions. Primers 9F, 515F, 907F and 357R were used in gene sequencing reaction. Multiple alignments of sequences were performed, and nucleotide substitution rate (K_{nuc} value) was calculated. A phylogenetic tree was constructed by the neighbor-joining method (Kimura 1980; Saitou and Nei 1987) using the CLUSTAL W program (Thompson et al. 1994). Sequence similarity was calculated using the GENETYX computer program (Software Development, Tokyo, Japan). The nucleotide sequence of the 16S rRNA gene of strain Ths has been deposited in GenBank/EMBL/DDBJ under the accession number AB207814.

DNA–DNA hybridization

The level of DNA–DNA relatedness was determined fluorometrically by the method of Ezaki et al. (1989) using photobiotin-labeled DNA probes and black microplates (Nage Nunc International). Photobiotin-labeled DNA was

prepared using photoprobebiotin (SP-1000, Vector Laboratories Inc., CA, USA). The fluorescence was detected by a microplate reader (type MTP-32, Corona electric, Tokyo, Japan).

Results and discussion

The isolate grew between −1 and 44°C with optimum growth at 27°C. The isolate grew at almost the same growth rate and intensity in the pH range of 7–9. Cells were rod-shaped (0.4–0.5 × 0.4–0.8 μm), Gram-negative and without a flagellum. Colonies were circular and colorless, with an entire margin. The species was positive for catalase and negative for oxidase. The isolate was positive for Simmons' citrate medium and H_2S production but negative for methyl red in the Voges-Proskauer test, the ONPG test, urease test and reduction of NO_3^- to NO_2^-. It hydrolyzed casein and Tweens 20, 40, 60 and 80, but not starch, DNA, lipid or alginic acid. No acid was produced in either aerobic or anaerobic conditions from D-fructose, D-maltose, D-mannose, sucrose, D-xylose, raffinose, *myo*-inositole, mannitol, sorbitol, D-galactose, L-rhamnose or trehalose. The isolate utilized L-alanine, L-histidine, L-glutamate, L-asparagine, L-proline, citrate, aspartate, DL-4-aminobutyrate, L-histidine and D-malate as substrates but not L-arabinose, D-fructose, glycerol, lactose, D-maltose, D-mannose, raffinose, sucrose, D-xylose, sorbitol, L-rhamnose, D-cellobiose, inuline, L-tryptophan, L-treonine, L-valine, L-lysine, L-glycine, DL-lactate, malonate or L-phenylalanine.

The cellular fatty acid compositions of strain Ths were as follows (% of total fatty acids): $C_{10:0}$ 1.0, $C_{12:0}$ 3.7, $C_{14:0}$ 0.3, $C_{15:0}$ 0.3, $C_{16:0}$ 27.7, $C_{16:1(9t)}$ 0.6, $C_{16:1(7c)}$ 0.4, $C_{16:1(9c)}$ 17.8, $C_{17:0}$ 1.8, 2-OH $C_{12:0}$ 1.3, $C_{17:1}$ 1.6, $C_{18:0}$ 0.9, $C_{18:1(9t)}$ 0.8, $C_{18:1(9c}$ 39.9 and 3-OH $C_{12:0}$ 2.1. The major isoprenoid quinone was ubiquinone-9 (Q-9). The DNA G + C content was 45.5%.

The sequence of 1,490 bases of the 16S rRNA gene of strain Ths was compared with those of previously reported strains. A phylogenetic tree constructed using the obtained data (Fig. 1) showed that strain Ths^T formed a monophyletic group with genomic species 6 and *A. lwoffii* ATCC 17925. The highest similarities to strain Ths was observed with the genomic species 6 ATCC 17979 (99.1%), followed by genomic species BJ13/TU14 ATCC 17905 (97.8%), genomic species 9 ATCC 9957 (97.6%) and *A. calcoaceticus* JCM 6840^T (97.5%).

According to the results of the 16S rRNA gene sequence analysis of strain Ths, 17 related strains exhibiting more than 95% similarity were chosen as reference strains for DNA–DNA hybridization. DNA–DNA hybridization results indicated that strain Ths was distinct from all of the strains examined except genomic species 6 ATCC 17979 (62% similarity) (Table 1). When reversed reaction was performed using genomic species 6 ATCC 17979 as a probe with strain Ths in the hybridization, similarity 58% value was obtained. DNA–DNA hybridization revealed that the isolate has 25, 18 and 19% relatedness, respectively, to genomic species BJ13/TU14 ATCC 17905, genomic species 9 ATCC 9957 and *A. calcoaceticus* JCM 6840^T, respectively. As shown in Table 1, 17 related strains based on 16S rRNA gene sequence exhibited low DNA–DNA reassociation value (below 63%).

Although strain Ths and genomic species 6 ATCC 17979 have similar phenotypic characteristics, strain Ths was different from genomic species 6 ATCC 17979 in growth at −1 and 40°C, growth in 3% NaCl, production of acid from D-glucose, melibiose or D-galactose, and utilization of D-glucose, DL-4-aminobutyrate, or L-histidine as the sole carbon source (data not shown). Strain Ths also differed from other relatively closely related strains in growth temperature range, acid production from D-glucose, gelatin hydrolysis and utilization of substrates (Table 2).

On the basis of the16S rRNA sequence, the low DNA–DNA reassociation and the specific combination of phenotypic characteristics, it has been shown that strain Ths is not affiliated with any known species of genus *Acinetobacter*. Although the isolate is thought to be a new species, it is considered that data on more than two strains is necessary to confirm that it is indeed a new species belonging to the genus *Acinetobacter*.

The isolate utilized C_{13}–C_{30} *n*-alkane and fluorene at pH 9 under 4°C. Furthermore, the isolate utilized C_{12}–C_{30}

Table 1 DNA–DNA relatedness (%) between strain Ths and other strains of *Acinetobacter* species

Strain	Ths
Genomic species 6 ATCC 17979	62
A. parvus NIPH 384^T	41
A. haemolyticus ATCC 17906^T	34
A. ventianus RAG-1 ATCC 31012	30
A. junii ATCC 17908^T	27
A. johnsonii ATCC 17909^T	26
Genomic species BJ13/TU14 ATCC 17905	25
A. baumanii JCM 6840^T	24
A. grimontii DSM 14968^T	23
A. ursingii NIPH 137^T	23
A. schindleri NIPH 1034^T	22
A. lowffii JCM 6840^T	20
A. bouvetii DSM 14964^T	19
A. calcoaceticus JCM 6842^T	19
Genomic species 9 ATCC 9957	18
Genomic species 3 ATCC 19004	17
A. radioresistens IAM 13186^T	5

Table 2 Characteristics of *Acinetobacter* sp. strain Ths and related species

	1	2	3	4
Growth at				
44°C	–	–	–	–
41°C	–	–	+	+
37°C	+	+	+	+
Acid from D-glucose	–	d	–	+
Gelatin hydrolysis	+	+	–	–
Utilization of				
DL-Lactate	–	–	+	+
DL-4-Aminobutyrate	+	–	d	+
Citrate	+	+	d	+
Aspartate	+	d	d	+
L-Histidine	+	–	+	+
D-Malate	+	+	+	+
Malonate	–	–	–	d
L-Phenylalanine	–	–	–	d

Data for genomic species 6, *A. junii*, and genomic species 3 were taken from Bouvet and Grimont (1987). The numbers are percentages of positive strains

1 Strain Ths, *2* genomic species 6, *3 A. junii*, *4* genomic species 3, + all strains positive, − all strains negative, *d* 11–89% positive

Table 3 Utilization of hydrocarbon by *Acinetobacter* sp. strain Ths at pH 9

Hydrocarbons	4°C	10°C
n-Dodecane	–	+
n-Tridecane	+	+
n-Tetradecane	+	+
n-Pentadecane	+	+
n-Hexadecane	+	+
n-Eicosane	+	+
n-Tetracosane	+	+
n-Octacosane	+	+
n-Dotriacontane	+	+
Cyclododecane	–	–
Fluorene	+	+
Anthracene	w	+
Pyrene	–	–

+ Positive, − negative, *w* weakly positive

n-alkane, fluorene and anthracene at pH 9 and 10°C (Table 3). To our knowledge, this is the first report on the bacterium that utilizes a wide range of hydrocarbon at high pH and a low temperature. Although there are several reports on hydrocarbon utilizing *Acinetobacter* spp. (Gallagher 1971; Ishige et al. 2000) or psychrotolerant *Acinetobacter* spp. (Breuil et al. 1975; Pratuangdejkul and Dharmsthiti 2000), there is no report on hydrocarbon-utilizing and alkaline- and low-temperature-tolerant *Acinetobacter* spp. To our knowledge, strain Ths is the first example of a strain belonging to the genus *Acinetobacter* that utilizes hydrocarbons at a low temperature with high pH tolerance.

Acknowledgments The authors thank Dr. A. Nemec for providing *A. parvus* NIPH 384^T and *A. ursingii* NIPH 137^T.

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