***Thermacetogenium phaeum* gen. nov., sp. nov., a strictly anaerobic, thermophilic, syntrophic acetate-oxidizing bacterium**

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A novel anaerobic, thermophilic, syntrophic acetate-oxidizing bacterium, strain PB^T, was isolated from a thermophilic (55 °C) anaerobic methanogenic reactor which had been treating kraft-pulp waste water. The bacterium oxidized acetate in co-culture with a thermophilic hydrogenotrophic methanogen. Strain PB^T, a Gram-positive, spore-forming, rod-shaped bacterium grew optimally at 58 °C and pH 6.8. The bacterium grew acetogenically on several alcohols, methoxylated aromatics, pyruvate, glycine, cysteine, formate and hydrogen/CO₂. Strain PB^T also oxidized acetate with reduction of sulfate or thiosulfate as the electron acceptor. The bacterium contained MK-7 as the major quinone. The G+C content of the DNA was 53.5 mol%. Comparative 16S rDNA analysis indicated that strain PB^T belongs to the *Bacillus*–*Clostridium* subphylum. However, it was distant from any known genera or micro-organism. The closest known relative was *Thermoterrabacterium ferrireducens* with 87.4% similarity. The name *Thermacetogenium phaeum* gen. nov., sp. nov. is proposed. The type strain is strain PB^T (= DSM 12270^T).

Keywords: *Thermacetogenium phaeum* gen. nov., sp. nov., thermophile, syntrophic acetate oxidation, acetogen, sulfate reduction

INTRODUCTION

Acetate is one of the most important intermediates for methanogenesis in anaerobic mineralization of organic materials. It has been estimated that approximately 70–80% of methane is derived from acetate in anoxic environments (Mountfort & Asher, 1978; Mackie & Bryant, 1981; Lovley & Klug, 1982). Of the number of methanogens described, only the genera *Methanosarcina* and *Methanosaeta* are known to produce methane from acetate. Methanogenesis from acetate in these organisms is catalysed by an acetoclastic reaction in which the methyl group of acetate is reduced to methane (Ferry, 1992). In contrast to these organisms, some syntrophic proton-reducing organisms were found to oxidize acetate to form methane in association with hydrogenotrophic methanogens (Zinder & Koch, 1984; Lee & Zinder, 1988; Schnürer *et al.*, 1994, 1996). Metabolically, this syntrophic association consists of two reactions that were originally proposed by

Barker (1936). In the first reaction, acetate is oxidized to form hydrogen and CO₂, which are, in the second reaction, converted to methane. As with other anaerobic syntrophic fatty acid oxidations, the former reaction is thermodynamically unfavourable under standard conditions, unless it couples with the latter reaction (Stams, 1994; Schink, 1997). Thus, syntrophic acetate degradation is possible only when syntrophic and hydrogen-consuming micro-organisms cooperate. To date, two syntrophic acetate oxidizers in association with hydrogenotrophic methanogens are known. The first description was strain AOR, which was a thermophilic acetate oxidizer isolated from a methanogenic reactor in co-culture with *Methanobacterium* sp. (Zinder & Koch, 1984). The isolate was later found to be a homoacetogen which forms acetate from hydrogen and CO₂ in pure culture, whereas acetate oxidation, i.e. the reverse of acetogenesis, occurs in co-culture with the methanogen (Lee & Zinder, 1988). The second acetate syntroph described was *Clostridium ultunense*, which mesophilically oxidizes acetate in the presence of hydrogenotrophic methanogens (Schnürer *et al.*, 1994, 1996). Although *C. ultunense* is phylo-

The GenBank/EMBL/DBJ accession number for the 16S rDNA sequence of strain PB^T is AB020336.

genetically and chemotaxonomically well characterized, the phylogenetic position of the thermophilic strain AOR is not known. Moreover, the isolate has not been deposited in any culture collection and is no longer available. Considering the other findings that syntrophic oxidation may be involved in methanogenesis from acetate in thermophilic methanogenic reactors (Petersen & Ahring, 1991; Uemura & Harada, 1993), the phylogenetic position and the physiological traits of thermophilic acetate-oxidizing syntrophs are of great interest to elucidate their importance in the environment. Ten years ago, we found methanogenesis from acetate in a thermophilic methanogenic enrichment in which neither *Methanosarcina*- nor *Methanosaeta*-like cells were observed (Kamagata & Mikami, 1989). In this report, we describe the isolation and characterization of a new thermophilic, syntrophic acetate-oxidizing bacterium, strain PB^T, from the enrichment and propose a new name for the isolate.

METHODS

Source of organisms. The syntrophic acetate-oxidizing bacterium, strain PB^T, and a thermophilic hydrogen- and formate-utilizing methanogen, *Methanothermobacter thermoautotrophicus* strain TM (previously *Methanobacterium thermoautotrophicum*) were isolated from a thermophilic (60 °C) anaerobic methanogenic reactor that treated waste water from a kraft-pulp production plant in Japan. *M. thermoautotrophicus* strain ΔH (= DSM 1053) was obtained from the DSMZ (Braunschweig, Germany).

Media and cultivation. The composition of the basal medium used for all experiments was based on DSM 334 medium (DSMZ, 1983). The basal medium contained (per litre): 1.0 g NH₄Cl, 0.3 g KH₂PO₄, 0.6 g NaCl, 0.1 g MgCl₂·2H₂O, 0.08 g CaCl₂·2H₂O, 3.5 g KHCO₃, 1 mg sodium resazurin, 10 ml vitamin solution and 5 ml trace element solution. The vitamin solution was replaced with that of DSM 141 medium (DSMZ, 1983). The composition was as follows (per litre): 2.0 mg biotin, 2.0 mg folic acid, 10.0 mg pyridoxine hydrochloride, 5.0 mg thiamin hydrochloride, 5.0 mg riboflavin, 5.0 mg nicotinic acid, 5.0 mg DL-calcium pantothenate, 0.1 mg vitamin B₁₂, 5.0 mg *p*-aminobenzoate and 5.0 mg lipoic acid. The trace element solution was based on DSM 318 medium (DSMZ, 1983) and contained (per litre): 12.8 g nitrilotriacetic acid, 1.35 g FeCl₃·6H₂O, 0.1 g MnCl₂·4H₂O, 0.024 g CoCl₂·6H₂O, 0.1 g CaCl₂·2H₂O, 0.1 g ZnCl₂, 0.025 g CuCl₂·2H₂O, 0.01 g H₃BO₃, 0.024 g Na₂MoO₄·4H₂O, 1.0 g NaCl, 0.12 g NiCl₂·6H₂O, 4.0 mg Na₂SeO₃·5H₂O, 4.0 mg Na₂WO₄·2H₂O. The trace element solution was adjusted to pH 6.5 with 1 M KOH. For growth of the syntrophic acetate-oxidizing co-culture, the medium contained 80 mM sodium acetate. For the isolation and maintenance of strain PB^T, 80 mM sodium pyruvate was used as the substrate. The medium was anaerobically dispensed into serum vials or bottles under N₂/CO₂ (4:1, v/v) atmosphere. The vials or bottles were sealed with butyl rubber stoppers fitted with caps. The medium was autoclaved for 20 min at 121 °C. The pH of the autoclaved medium was approximately 6.9–7.1. Prior to inoculation, the medium was reduced with sterile stock solutions of Na₂S and cysteine/HCl (final concentration 0.3 g l⁻¹ each). Unless otherwise noted, cultures were incubated at 55 °C in the dark without shaking.

Determination of growth. To determine the growth, a 25 ml

Hungate tube containing 10 ml medium was used. Growth was determined by measuring OD₆₆₀ with a Hitachi spectrophotometer model 100-10. Unless otherwise noted, the growth experiments were performed at least in duplicate.

Temperature, pH and NaCl ranges for growth. Medium containing 20 mM sodium pyruvate was used, and a late-log culture of strain PB^T was inoculated into the fresh basal medium. For determination of the temperature ranges for growth, cultures were incubated at 30–70 °C. For the pH experiment, the pH of the medium was adjusted with sterile stock solutions of 10% K₂CO₃ or 1 M HCl. The pH value was calibrated at 25 °C. The range of pH tested was 5.4–9.1. To determine the effect of salt concentrations on growth, sodium chloride was added to the medium before autoclaving to give final concentrations of 0.05 to 5%.

Determination of acetate-oxidation rate. The acetate-oxidation rate [mmol acetate consumed h⁻¹ (g dry cell wt)⁻¹] was determined by using washed-cell suspension. Strain PB^T was co-cultured with *Methanothermobacter thermoautotrophicus* strain TM on medium containing 80 mM acetate (500 ml) for a month. The cells were harvested anaerobically, suspended in the acetate medium (20 ml) and incubated at 55 °C. Methane and acetate were determined at appropriate intervals.

Utilization of substrates and electron acceptors. Several compounds including organic acids, alcohols, methoxylated aromatics, sugars, amino acids and hydrogen were used for the substrate-utilization tests. Substrates were added from filter-sterilized or autoclaved stock solutions (pH 7.0) to give the desired concentrations. When hydrogen was used as the substrate, the head space (15 ml) of the tube was replaced with hydrogen/CO₂ (4:1, v/v, 151 kPa). For determining electron acceptors, 40 mM sodium acetate was used as the electron donor, and the following organic or inorganic compounds were tested as electron acceptors (mM): sulfate (20), sulfite (2.5), nitrate (20), nitrite (2.5), thiosulfate (20), amorphous Fe(III) (5) and fumarate (10). Two negative controls were incubated simultaneously: (1) medium containing strain PB^T and acetate but without an electron acceptor and (2) the medium containing acetate and electron acceptor but without cells.

Microscopy. Cultures were routinely examined with an Olympus model AX-80 microscope equipped with phase-contrast and epifluorescence apparatus. For transmission electron microscopy, cells were centrifuged and fixed with 5% (v/v) glutaraldehyde and 1% (v/v) osmium tetroxide. The samples were embedded in Spurr resin and the ultrathin sections of the samples were prepared with a Reichert-Nissei ultramicrotome. The samples were stained with 1% uranyl acetate and 0.3% lead citrate. After treatment of vapor deposition with carbon, the samples were observed using a Hitachi model H-7000 electron microscope operating at 75 kV.

Analytical methods. Methane was analysed by a Shimadzu model GC-8AIT gas chromatograph equipped with a molecular sieve 5A 60/80 mesh column and a thermal conductivity detector. Argon was used as the carrier gas at a flow rate of 27 ml min⁻¹. The column and the detector temperatures were kept at 60 °C and 110 °C, respectively. Acetate and fermentation products were determined by using a Shimadzu model LC-6A HPLC equipped with a Shimadzu SCR-101H column and a model SPD-6A UV detector. The column was operated at 40 °C, and 17 mM HClO₄ was used as the eluent at a flow rate of 1 ml min⁻¹. Sulfate, sulfite, nitrate, nitrite and thiosulfate were determined by using a Shimadzu model LC-6A HPLC

equipped with a Shimadzu IC-A3 ion-exchange column and a model CDD-6A electronic conductivity detector. Operating conditions were as follows: column temperature, 40 °C; eluent, 8.0 mM *p*-hydroxybenzoic acid and 3.2 mM bis-tris; flow rate, 1.2 ml min⁻¹. Utilization of amorphous Fe(III) was assessed by measuring changes in the visible colour of the medium (Slobodkin *et al.*, 1997a).

DNA extraction and purification. Genomic DNA was extracted and purified according to the method of Hiraishi (1992). For determination of DNA base composition, the crude DNA was further purified with an equal volume of phenol/chloroform/isoamylalcohol (25:24:1, by vol).

DNA base composition. The G + C content of strain PB^T was determined by enzymic digestion of genomic DNA and HPLC separation using a Yamasa GC kit as described by Kamagata & Mikami (1991). The purified DNA sample was separated by using a Shimadzu model LC-6A HPLC equipped with a Shimadzu CLC-ODS reverse-phase column and a model SPD-6A UV detector at 270 nm. The column was operated at 40 °C, and 10 mM potassium phosphate buffer (pH 3.5) containing 5% methanol was used as the eluent at a flow rate of 1 ml min⁻¹.

Amplification and purification of 16S rDNA. Crude DNA was used as template for PCR amplification of 16S rDNA. PCR was performed by using an AmpliTaq Gold reagent kit (Perkin Elmer) according to the manufacturer's protocol. The PCR primers used for amplification were the forward primer 8F (5'-AGAGTTTGATCCTGGCTCAG-3', *Escherichia coli* positions 8–27) and the reverse primer 1491R (5'-GGTTACCTTGTACGACTT-3', *E. coli* positions 1509–1491) (Weisburg *et al.*, 1991). PCR conditions were as follows: pre-heating at 95 °C for 9 min, 35 cycles of denaturing at 95 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 2 min.

16S rDNA sequence determination and phylogenetic analysis. Purified PCR products of 16S rDNA were sequenced directly with a PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) according to the manufacturer's protocol. Six primers were used for sequencing according to the method of Hiraishi (1992). Both obtained and reference 16S rDNA sequences were aligned with the CLUSTAL W program, version 1.5 (Thompson *et al.*, 1994). The phylogenetic tree was constructed by the neighbour-joining method (Saito & Nei, 1987) with the MEGA program. Bootstrap values were obtained for 100 replicates to estimate the confidence of tree topologies.

Quinone analysis. Quinone was determined according to the method of Tamaoka *et al.* (1983). Approximately 2 g (wet weight) of the late-exponential-phase culture of strain PB^T grown on pyruvate was used for the analysis.

Nucleotide sequence accession numbers. The 16S rDNA sequence of strain PB^T has been deposited in the DDBJ/GenBank/EMBL database under the accession number AB020336. The accession numbers of the reference sequences used in the phylogenetic analysis are as follows: *Thermoterrabacterium ferrireducens* DSM 11225^T, U76364; *Moorella glycerini* DSM 11254^T, U82327; *Moorella thermoacetica* ATCC 39073^T, M59121; *Moorella thermoautotrophica* DSM 1974^T, L09168; *Thermoanaerobacter ethanolicus* DSM 2355^T, L09164; *Thermoanaerobacterium thermosulfurigenes* DSM 2229^T, L09171; *Syntrophomonas wolfei* DSM 2245^T, AF022248; *Syntrophospora bryantii* DSM 3014^T, M26491; *Thermosyntropha lipolytica* DSM

11003^T, X99980; *Desulfotomaculum australicum* DSM 11792, M96665; *Desulfotomaculum thermoacetoxidans* DSM 5813^T, Y11573; *Desulfotomaculum thermobenzoicum* DSM 6193^T, Y11574; *Desulfotomaculum nigrificans* DSM 574^T, X62176; *Desulfotomaculum ruminis* DSM 2154^T, Y11572; *Bacillus subtilis* NCDO 1769, X60646; *Clostridium ultunense* DSM 10521^T, Z69293; *Clostridium butyricum* DSM 552^T, M59085; *Arthrobacter globiformis* DSM 20124^T, X80736.

RESULTS

Enrichment and isolation

Strain PB^T was originally derived from an enrichment culture which converted acetate to methane. For the primary enrichment of a syntrophic acetate-oxidizing co-culture, a sample taken from a thermophilic anaerobic reactor was serially diluted and inoculated into the basal medium containing 80 mM sodium acetate. After 2 months incubation at 55 °C, the cultures receiving 10⁻¹ to 10⁻⁶ dilutions completely degraded acetate and produced nearly equimolar amounts of methane. Under microscopic observation, the cultures receiving 10⁻¹ to 10⁻⁴ dilutions contained several kinds of microbes such as *Methanobacterium*- and *Methanosaeta*-like methanogens. However, neither *Methanosarcina* nor *Methanosaeta*-like cells were observed in the cultures that received the 10⁻⁵ to 10⁻⁶ dilutions. The highest-dilution culture was again serially diluted into fresh acetate medium. After several transfers, the culture was found to be composed of two morphologically distinct rod-shaped bacteria. One organism had a terminal endospore and showed non-autofluorescence. The other, showing autofluorescence indicative of F₄₂₀, was morphologically similar to *Methanobacterium* cells. Neither *Methanosarcina* nor *Methanosaeta* types of methanogen were found. These observations suggested that syntrophic acetate oxidation, as has been previously described by Zinder & Koch (1984) and Schnürer *et al.* (1994) occurred in the culture. However, the culture was not still a defined co-culture, since other types of heterotroph appeared vigorously when the culture was incubated with yeast extract or other carbon sources such as glucose and tryptone. To eliminate those contaminants, we tried to repeat serial dilutions, roll-tube culture or antibiotic-containing culture. However, all attempts were unsuccessful. Therefore, we tried to isolate each member separately from the culture. For isolation of the syntrophic acetate-oxidizing bacterium, several compounds such as ethylene glycol were tested according to the method previously described (Lee & Zinder, 1988; Schnürer *et al.*, 1996). Of the substrates tested, 80 mM sodium pyruvate allowed a spore-forming bacterium to grow after 7 d incubation at 55 °C. The microbe resembled the bacterium which had predominated as well as *Methanobacterium*-like cells in the enrichment. We therefore focused on this bacterium and inoculated the cells into a 2% agar (Agar Noble, Difco) medium containing 80 mM pyruvate as the sole carbon and energy source. After 2 months incubation, the colonies which grew up to 2 mm in

Fig. 1. Syntrophic degradation of acetate to methane by a reconstituted co-culture of strain PB^T and *M. thermoautotrophicus* strain TM. ○, acetate; ■, methane.

diameter were picked with a sterile Pasteur pipette and subcultured in liquid medium containing 80 mM pyruvate and 20 mM BES. Purity of the strain, designated PB^T, was checked by inoculating it into the medium containing 0.1% yeast extract and 1% Bacto Tryptone (Difco), and by microscopy. The thermophilic hydrogen- and formate-utilizing methanogen, *M. thermoautotrophicus* strain TM was also isolated from the primary enrichment culture by using the same procedure, except that pyruvate was replaced with hydrogen/CO₂ (4:1, v/v, 151 kPa) and BES was omitted.

Reconstruction of syntrophic acetate-oxidizing co-culture

To prove strain PB^T to be a syntrophic acetate-oxidizing bacterium, strains PB^T and TM were co-inoculated into basal medium containing 80 mM sodium acetate as the sole carbon and energy source, and incubated at 55 °C. As the control experiments, strains PB^T and TM were separately inoculated under the same conditions. Growth was observed only in the mixed culture after 40 d incubation. Acetate was converted to methane. The co-culture was able to retain methanogenic activity from acetate after further transfer into the fresh medium. Fig. 1 shows that the co-culture completely degraded 40 mM acetate and stoichiometrically produced methane within 24 d. The acetate-oxidation rate using washed-cell suspension was calculated to be 1.29 mmol acetate consumed h⁻¹ (g dry cell wt)⁻¹. Strain PB^T could also be co-cultured with a hydrogen-utilizing methanogen, *M. thermoautotrophicus* strain ΔH.

Colony and cell morphology

Colonies of strain PB^T were disc shaped, 1.0–3.0 mm in diameter, smooth and brownish. Figs 2 and 3 show the morphology and ultrathin sections of the syntrophic

Fig. 2. (a) Phase-contrast micrograph of strain PB^T in pure culture grown on methanol; (b) co-culture of strain PB^T with *M. thermoautotrophicus* strain TM grown on acetate; (c) epifluorescence micrograph of the same field as (b). Bars, 10 µm.

co-culture and the pure culture of strain PB^T. Gram-staining was negative. However, transmission electron microscopy indicated a Gram-positive type of cell wall (Fig. 3). Cells were straight or slightly curved rods with rounded ends, 0.4–0.7 µm wide and 2.0–12.6 µm long. The cells occurred singly, in pairs or in chains depending on the growth phase and growth conditions, i.e. under syntrophic conditions, strain PB^T elongated the cell length at late-exponential phase and occurred singly. In pure culture, the cells often occurred in pairs or in chains at the exponential phase. Terminal endospores were much more frequently observed in the syntrophic co-culture than in the pure culture (Fig. 3).

Fig. 3. Transmission electron micrographs of pure culture (a) and syntrophic co-culture showing a terminal endospore (b). The arrow in (b) indicates the strain PB^T cell. Bars, 1 µm.

Growth properties

The temperature range for growth of strain PB^T was 40–65 °C, with an optimum temperature around 58 °C (Fig. 4a). No growth was observed within 2 months at 35 °C and 70 °C. The pH range for growth was 5.9–8.4 and optimal growth was at pH 6.8 (Fig. 4b). Growth was not observed at pH 5.4 and pH 8.8. The doubling time under the optimum conditions was calculated to be 22.8 h. Strain PB^T was able to grow at NaCl concentrations ranging from 0.05 to 4.5% (w/v).

Substrate utilization

The following substrates were utilized for growth in pure culture (unless otherwise noted, substrates were added at a final concentration of 20 mM): methanol, ethanol, n-propanol, n-butanol, 2,3-butanediol, ethanolamine, pyruvate, 3,4,5-trimethoxybenzoate (10 mM), syringate (10 mM), vanillate (10 mM), glycine, cysteine, formate (40 mM) and hydrogen/CO₂ (4:1, v/v, 151 kPa). Acetate was the sole or major fermentation product from these substrates. In co-culture with hydrogenotrophic methanogens, the bacterium syntrophically oxidized acetate (40 mM) to form methane. No growth was observed on 1,2-propanediol, ethylene glycol, glycerol, citrate,

fumarate, malate, lactate, sarcosine, betaine, meth-ylamine, dimethylamine, trimethylamine, glucose, fructose, galactose, maltose, sucrose, lactose and yeast extract (0.1 %).

Electron acceptors

In the presence of sulfate, strain PB^T consumed 12 mM acetate (initial concentration, 40 mM) with concomitant reduction of sulfate. Acetate was not degraded further after prolonged incubation. Thiosulfate was also utilized as an electron acceptor. Fe(III), sulfite, nitrate, nitrite and fumarate were not utilized.

Chemotaxonomic characteristics

The genomic DNA G+C content of strain PB^T was 53.5 mol % as determined by HPLC. Quinone analysis revealed that strain PB^T contained MK-7 as the major quinone component.

Phylogenetic analysis

Nearly the full length (1462 bp) of the 16S rDNA sequence of strain PB^T was determined. The sequence was aligned and compared with 18 representative

Fig. 4. Effect of temperature (a) and pH (b) on the growth of strain PB^T.

sequences of other bacteria. On the basis of the phylogenetic analysis (Fig. 5), strain PB^T was found to be a member of the *Bacillus*–*Clostridium* subphylum of the Gram-positive bacteria, and the closest relative was *Thermoterrabacterium ferrireducens* (87.4% similarity).

DISCUSSION

In this report, we have described a novel strictly anaerobic, thermophilic acetate-oxidizing syntroph. In pure culture, this strain grew acetogenically on several alcohols, organic acids, methoxylated aromatics and some amino acids. The strain could also grow autotrophically on hydrogen/CO₂ to produce acetate as the major product.

With regard to the other syntrophic acetate-oxidizing bacteria, two syntrophs have been previously isolated and characterized: a thermophilic syntroph, strain AOR (Lee & Zinder, 1988), and a mesophilic syntroph, *Clostridium ultunense* (Schnürer *et al.*, 1996). Table 1 shows the properties of these two isolates and our strain. All the isolates show acetogenic growth in pure culture.

One of the very interesting features that distinguish strain PB^T from the other two strains was that the strain can oxidize acetate in pure culture by using sulfate as the electron acceptor. This trait is very similar to several sulfate-reducing bacteria belonging to the genus *Desulfotomaculum*. *Desulfotomaculum acetoxidans* and *D. thermoacetoxidans* are able to oxidize acetate completely with sulfate reduction (Widdel & Pfennig, 1977; Min & Zinder, 1990). Strain PB^T is phylogenetically affiliated with the Gram-positive low G + C group, which also includes *Desulfotomaculum* species (Fig. 5). Moreover, strain PB^T, as well as *Desulfotomaculum* species, contains MK-7 as the major quinone component. These indicate that our isolate harbours a sulfate-respiration system. The other feature of such micro-organisms including our isolate is that they are also capable of growing acetogenically on pyruvate or methoxylated aromatics in the absence of sulfate (Min & Zinder, 1990; Tasaki *et al.*, 1992, 1993; Nilsen *et al.*, 1996). Considering the fact that those *Desulfotomaculum* strains harbour the enzymes of the carbon monoxide dehydrogenase pathway that allows the microbes to convert methoxylated compounds to acetate, it is very likely that our isolate has a similar pathway.

Fig. 5. Phylogenetic position of strain PB^T within the *Bacillus*–*Clostridium* subphylum of the Gram-positive bacteria. The tree was based on 16S rDNA sequences and was constructed using the neighbour-joining method. Bar, 0.02 base substitutions per nucleotide position.

Table 1. Characteristics of strain PB^T and other syntrophic acetate-oxidizing bacteria

ND, Not determined.

Characteristic	Strain PB ^T *	Strain AOR†	<i>C. ultunense</i> ‡
Cell size (µm)	0.4–0.7 × 2–12.6	0.4–0.6 × 2–3	0.5–0.7 × 0.5–7
Spore formation	+	–	+
Gram stain	+	+	+
Temperature range (°C)	40–65	50–65	15–50
Optimum temperature (°C)	58	60	37
pH range	5.9–8.4	ND	5–10
Optimum pH	6.8	ND	7
DNA G + C content (mol %)	53.5	47	32
Major menaquinone	MK-7	ND	ND
Supplement required	None	Yeast extract	Yeast extract
Utilization of substrate:			
In pure culture			
Hydrogen/CO ₂	+	+	–
Formate	+	+	+
Ethylene glycol	–	+	+
Methanol	+	–	–
Ethanol	+	–	–
n-Propanol	+	–	ND
n-Butanol	+	–	–
1,2-Propanediol	–	+	–
2,3-Butanediol	+	–	ND
Ethanolamine	+	–	–
Glycerol	–	–	–
Pyruvate	+	+	+
3,4,5-Trimethoxybenzoate	+	–	–
Syringate	+	ND	–
Vanillate	+	ND	–
Glycine	+	–	–
Betaine	–	+	+
Methylamine	–	–	–
Cysteine	+	ND	+
Glucose	–	–	+
Acetate	–	–	–
Acetate plus sulfate	+	–	–
In co-culture with methanogen			
Acetate	+	+	+

* This study.

† Data from Lee & Zinder (1988).

‡ Data from Schnürer *et al.* (1996).

Phylogenetic analysis based on 16S rDNA sequence showed that strain PB^T was most closely related to an iron-reducing, non-spore forming bacterium, *Thermoterrabacterium ferrireducens* (Slobodkin *et al.*, 1997a) (Fig. 5). However, the sequence similarity was very low (87.4 %). Moreover, strain PB^T differed from *Thermoterrabacterium ferrireducens* in several features: (1) strain PB^T is not capable of utilizing Fe(III) as an electron acceptor; (2) it is a spore former; (3) it has a much higher DNA G + C content. Our strain is rather related to the genus *Moorella* than to *Thermo-*

terrabacterium in several features such as cell morphology, substrate utilization, physiological properties, quinone composition and the G + C content of DNA (Fontaine *et al.*, 1942; Wiegel *et al.*, 1981; Hippe *et al.*, 1992; Collins *et al.*, 1994; Slobodkin *et al.*, 1997b). However, *Moorella* spp. do not have the ability to reduce sulfate. These special features are clearly enough to distinguish our isolate from the other genera. On the basis of phenotypic and phylogenetic distinctiveness of the isolate, we concluded that the organism characterized here represents a novel and

distinct taxon for which we propose the name *Thermacetogenium phaeum* gen. nov., sp. nov.

Description of *Thermacetogenium* gen. nov.

Thermacetogenium (therm.a.ce.to.ge'ni.um. Gr. adj. *thermos* warm; L. n. *acetum* vinegar; Gr. v. suff. *genium* producing; M.L. neut. n. *Thermacetogenium* thermophilic vinegar producer).

Cells are rod shaped. Strictly anaerobic and thermophilic. Chemoautotrophic and chemo-organotrophic. Gram reaction is negative, but shows a Gram-positive cell-wall structure. Round terminal endospores are formed. Colonies are disc shaped. Cells are able to oxidize acetate in co-culture with hydrogenotrophic micro-organisms. Acetate can be utilized also by sulfate reduction in pure culture. MK-7 is the major quinone. Grows acetogenically on several alcohols, methoxylated aromatics, organic acids, amino acids and hydrogen/CO₂. Sugars are not utilized. Additional supplements are not required.

Description of *Thermacetogenium phaeum* sp. nov.

Thermacetogenium phaeum (phae'um. Gr. adj. *phaios* brown, referring to the colour of the colonies; M.L. neut. adj. *phaeum* brown).

Cells are straight or slightly curved and rod shaped, 0.4–0.7 µm wide and 2.0–12.6 µm long. Occur singly, in pairs or in chains. Round terminal endospores are observed. Colonies are disc shaped, 1–3 mm in diameter, smooth and brownish. Methanol, ethanol, n-propanol, n-butanol, 2,3-butanediol, ethanolamine, pyruvate, 3,4,5-trimethoxybenzoate, syringate, vanillate, glycine, cysteine, formate, and hydrogen/CO₂ are utilized in pure culture. Acetate is the primary fermentation product. Sugars are not utilized. Organic nutrients such as yeast extract are not required for growth. Acetate is oxidized to CO₂ in co-culture with hydrogen-utilizing methanogens. Acetate is also oxidized to CO₂ in pure culture with reduction of sulfate or thiosulfate as the electron acceptor. Sulfite, nitrate, nitrite, Fe(III) and fumarate are not utilized when acetate is used as the electron donor. Temperature range for growth is 40–65 °C, with an optimum at 58 °C. Growth is observed at a pH range from 5.9 to 8.4, and the optimum is pH 6.8. Growth occurs in the presence of NaCl at 0.05–4.5% (w/v). The G+C content of DNA is 53.5 mol%. The major quinone is MK-7. The type strain is *Thermacetogenium phaeum* strain PB^T (= DSM 12270^T).

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REFERENCES

- Barker, H. A. (1936). On the biochemistry of methane fermentation. *Arch Microbiol* **7**, 404–419.
- Collins, M. D., Lawson, P. A., Willems, A., Cordoba, J. J., Fernandez-Garayzabal, J., Garcia, P., Cai, J., Hippe, H. & Farrow, J. A. E. (1994). The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. *Int J Syst Bacteriol* **44**, 812–826.
- DSMZ (1993). Catalogue of strains, 5th edn. Braunschweig: Gesellschaft für Biotechnologische Forschung.
- Ferry, J. G. (1992). Methane from acetate. *J Bacteriol* **174**, 5489–5495.
- Fontaine, F. E., Peterson, W. H., McCoy, E., Johnson, M. J. & Ritter, G. J. (1942). A new type of glucose fermentation by *Clostridium thermoaceticum* n. sp. *J Bacteriol* **43**, 701–715.
- Hippe, H., Andreesen, J. R. & Gottschalk, G. (1992). The genus *Clostridium*—nonmedical. In *The Prokaryotes*, p. 1800–1866. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. New York: Springer.
- Hiraishi, A. (1992). Direct automated sequencing of 16S rDNA amplified by polymerase chain reaction from bacterial cultures without DNA purification. *Lett Appl Microbiol* **15**, 210–213.
- Kamagata, Y. & Mikami, E. (1989). Diversity of acetotrophic methanogens in anaerobic digestion. In *Recent Advances in Microbial Ecology*, pp. 459–464. Edited by T. Hattori, Y. Ishida, Y. Maruyama, R. Y. Morita & A. Uchida. Tokyo: Japan Scientific Society.
- Kamagata, Y. & Mikami, E. (1991). Isolation and characterization of a novel thermophilic *Methanosaeta* strain. *Int J Syst Bacteriol* **41**, 191–196.
- Lee, M. J. & Zinder, S. H. (1988). Isolation and characterization of a thermophilic bacterium which oxidizes acetate in syntrophic association with a methanogen and which grows acetogenically on H₂/CO₂. *Appl Environ Microbiol* **54**, 124–129.
- Lovley, D. R. & Klug, M. J. (1982). Intermediary metabolism of organic matter in the sediments of a eutrophic lake. *Appl Environ Microbiol* **43**, 552–560.
- Mackie, R. I. & Bryant, M. P. (1981). Metabolic activity of fatty acid-oxidizing bacteria and the contribution of acetate, propionate, butyrate, and CO₂ to methanogenesis in cattle waste at 40 and 60 °C. *Appl Environ Microbiol* **41**, 1363–1373.
- Min, H. & Zinder, S. H. (1990). Isolation and characterization of a thermophilic sulfate-reducing bacterium *Desulfotomaculum thermoacetoxidans* sp. nov. *Arch Microbiol* **153**, 399–404.
- Mountfort, D. O. & Asher, R. A. (1978). Changes in proportions of acetate and carbon dioxide used as methane precursors during the anaerobic digestion of bovine waste. *Appl Environ Microbiol* **35**, 648–654.
- Nilsen, R. K., Torsvik, T. & Lien, T. (1996). *Desulfotomaculum thermocisternum* sp. nov., a sulfate reducer isolated from a hot north sea oil reservoir. *Int J Syst Bacteriol* **46**, 397–402.
- Petersen, S. P. & Ahring, B. K. (1991). Acetate oxidation in a thermophilic anaerobic sewage-sludge digester: the importance of non-aceticlastic methanogenesis from acetate. *FEMS Microbiol Ecol* **86**, 149–158.
- Saito, N. & Nei, M. (1987). The neighbor-joining method: a new method for constructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

- Schink, B. (1997). Energetics of syntrophic cooperation in methanogenic degradation. *Microbiol Mol Biol Rev* **61**, 262–280.
- Schnürer, A., Houwen, F. P. & Svensson, B. H. (1994). Mesophilic syntrophic acetate oxidation during methane formation by a triculture at high ammonium concentration. *Arch Microbiol* **162**, 70–74.
- Schnürer, A., Schink, B. & Svensson, B. H. (1996). *Clostridium ultunense* sp. nov., a mesophilic bacterium oxidizing acetate in syntrophic association with a hydrogenotrophic methanogenic bacterium. *Int J Syst Bacteriol* **46**, 1145–1152.
- Slobodkin, A., Reysenbach, A. L., Strutz, N., Dreier, M. & Wiegel, J. (1997a). *Thermoterrabacterium ferrireducens* gen. nov., sp. nov., a thermophilic anaerobic dissimilatory Fe(III)-reducing bacterium from a continental hot spring. *Int J Syst Bacteriol* **47**, 541–547.
- Slobodkin, A., Reysenbach, A. L., Mayer, F. & Wiegel, J. (1997b). Isolation and characterization of the homoacetogenic thermophilic bacterium *Moorella glycerini* sp. nov. *Int J Syst Bacteriol* **47**, 969–974.
- Stams, A. J. M. (1994). Metabolic interactions between anaerobic bacteria in methanogenic environments. *Antonie Leeuwenhoek* **66**, 271–294.
- Tamaoka, J. & Komagata, K. (1984). Determination of DNA base composition by reversed-phase high-performance liquid chromatography. *FEMS Microbiol Lett* **25**, 125–128.
- Tamaoka, J., Katayama-Fujimura, Y. & Kuraishi, H. (1983). Analysis of bacterial menaquinone mixtures by high performance liquid chromatography. *J Appl Bacteriol* **54**, 31–36.
- Tasaki, M., Kamagata, Y., Nakamura, K. & Mikami, E. (1992). Utilization of methoxylated benzoates and formation of intermediates by *Desulfotomaculum thermobenzoicum* in the presence or absence of sulfate. *Arch Microbiol* **157**, 209–212.
- Tasaki, M., Kamagata, Y., Nakamura, K., Okamura, K. & Minami, K. (1993). Acetogenesis from pyruvate by *Desulfotomaculum thermobenzoicum* and differences in pyruvate metabolism among three sulfate-reducing bacteria in the absence of sulfate. *FEMS Microbiol Lett* **106**, 259–264.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
- Uemura, S. & Harada, H. (1993). Microbial characteristics of methanogenic sludge consortia developed in thermophilic UASB reactors. *Appl Microbiol Biotechnol* **39**, 654–660.
- Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**, 697–703.
- Widdel, F. & Pfennig, N. (1977). A new anaerobic, sporing, acetate-oxidizing, sulfate-reducing bacterium, *Desulfotomaculum* (emend.) *acetoxidans*. *Arch Microbiol* **112**, 119–122.
- Wiegel, J., Braun, M. & Gottschalk, G. (1981). *Clostridium thermoautotrophicum* species novum, a thermophile producing acetate from molecular hydrogen and carbon dioxide. *Curr Microbiol* **5**, 255–260.
- Zinder, S. H. & Koch, M. (1984). Non-aceticlastic methanogenesis from acetate: acetate oxidation by a thermophilic syntrophic coculture. *Arch Microbiol* **138**, 263–272.