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Article *in* International Journal of Systematic and Evolutionary Microbiology · October 2002

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NOTE

Pelotomaculum thermopropionicum gen. nov., sp. nov., an anaerobic, thermophilic, syntrophic propionate-oxidizing bacterium

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An anaerobic, thermophilic, syntrophic propionate-oxidizing bacterium, strain SI^T, isolated previously from granular sludge in a thermophilic upflow anaerobic sludge blanket (UASB) reactor, was characterized. The strain could grow fermentatively on pyruvate and fumarate in pure culture. The strain grew on propionate, ethanol, lactate, 1-butanol, 1-pentanol, 1,3-propanediol, 1-propanol and ethylene glycol in co-culture with the hydrogenotrophic methanogen *Methanothermobacter thermautotrophicus* strain ΔH^T. The optimum temperature for growth was 55 °C and the pH optimum was 7·0. The G+C content of the DNA was 52·8 mol%. Strain SI^T contained MK-7 and MK-7(H$_4$) as the major quinones and contained iso-C15:0 as the major fatty acid. Based on 16S rDNA sequence analysis, strain SI^T formed a novel lineage within the Gram-positive, spore-forming, sulphate-reducing bacterial group *Desulfotomaculum*. However, the strain lacked the ability to conduct dissimilatory sulphate reduction. Instead, it could reduce fumarate to succinate with concomitant growth on several organic substances as electron donor. These phenotypic and genetic properties support the formation of a novel species of a new genus, for which the name *Pelotomaculum thermopropionicum* gen. nov., sp. nov. is proposed. The type strain is strain SI^T (= DSM 13744^T = JCM 10971^T).

Keywords: anaerobe, syntroph, propionate oxidation, thermophile, *Pelotomaculum thermopropionicum* gen. nov., sp. nov

Propionate is one of the important intermediates in the conversion of complex organic matter to methane and carbon dioxide in methanogenic ecosystems. In anaerobic wastewater and solid-waste treatment systems, it is well known that propionate is the precursor of a large fraction of the methane produced (Mah *et al.*, 1990; Gujer & Zehnder, 1983; Kaspar & Wuhrmann, 1978). In addition, propionate is often found to accumulate as one of the major organic compounds in treated wastewater when the operation of anaerobic digestion processes becomes unstable. These findings have, for a long time, encouraged microbiologists to investigate the microbes responsible for methanogenic propionate degradation in order to elucidate their importance in anaerobic processes.

Under methanogenic conditions, propionate degradation is carried out by syntrophic associations of propionate-oxidizing, proton-reducing microbes and hydrogenotrophic microbes (Schink, 1997). Propionate-oxidizing micro-organisms convert propionate into acetate, carbon dioxide and hydrogen only when the hydrogen partial pressure is kept very low by hydrogenotrophic microbes. Hence, the growth of syntrophs is highly dependent on the presence of hydrogen-consuming microbes; this has led to difficulty in isolating these syntrophs. To date, the four mesophilic species of the genera *Syntrophobacter* and

Published online ahead of print on 1 March 2002 as DOI 10.1099/ijs.0.02212-0.

Abbreviations: FAME, fatty acid methyl ester; UASB, upflow anaerobic sludge blanket.

The GenBank/EMBL/DDBJ accession number for the 16S rDNA sequence of strain SI^T is AB035723.

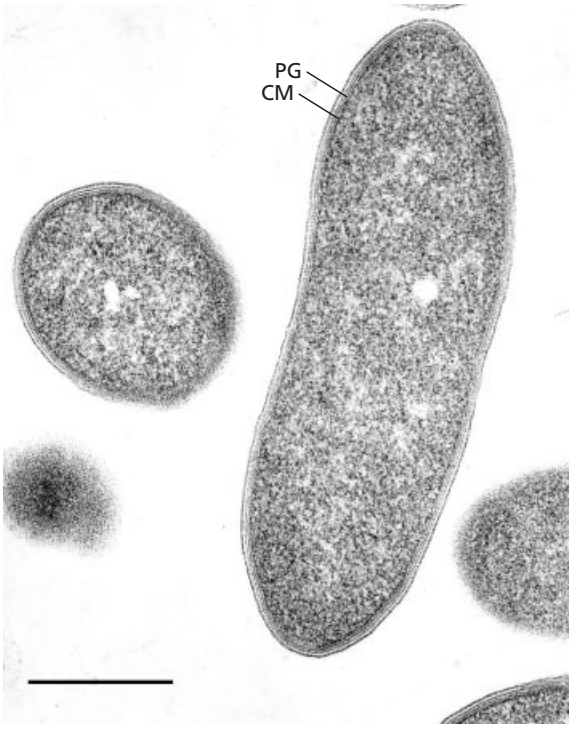


Fig. 1. Transmission electron micrograph of strain SI^T grown on pyruvate (20 mM) medium, showing the peptidoglycan layer (PG) and the cytoplasmic membrane (CM). Bar, 0·5 μm.

Smithella [*Syntrophobacter wolinii* (Boone & Bryant, 1980), *Syntrophobacter pfennigii* (Wallrabenstein *et al*., 1995), *Syntrophobacter fumaroxidans* (Harmsen *et al*., 1998) and *Smithella propionica* (Liu *et al*., 1999)] and two thermophilic members of the genus *Desulfotomaculum* [*Desulfotomaculum thermocisternum* (Nilsen *et al*., 1996) and *Desulfotomaculum thermobenzoicum* subsp. *thermosyntrophicum* (Plugge *et al*., 2002)] represent all of the currently described species that show syntrophic propionate oxidation.

Recently, we have isolated a novel thermophilic anaerobe capable of oxidizing propionate in syntrophic association with hydrogenotrophic methanogens as part of a study on microbial populations responsible for propionate oxidation in the methanogenic granular sludge from a thermophilic (55 °C) upflow anaerobic sludge blanket (UASB) reactor (Imachi *et al*., 2000). The syntroph, designated strain SI^T, was isolated from the granular sludge by conventional techniques combined with *in situ* hybridization detection using an rRNA-targeted fluorescently labelled oligonucleotide probe and was found to be abundant in the sludge (1·1 % of the total cell count) and to be closely associated with *Methanothermobacter*-like cells (Imachi *et al*., 2000). In our previous study, strain SI^T was found to grow on propionate, ethanol and lactate in co-culture with hydrogenotrophic methanogens and to grow on pyruvate in pure culture. On the basis of 16S rDNA analysis, this strain was affiliated with the genus *Desulfotomaculum*, but it was only distantly related to any known species. In this report, we describe the detailed morphological, physiological and chemotaxonomic characteristics of strain SI^T and propose a new genus and species for this strain.

Strain SI^T was isolated in pure culture and in co-culture with methanogens as described in our previous study (Imachi *et al*., 2000). Media for cultivation of strain SI^T were prepared as described previously (Imachi *et al*., 2000). Cells of strain SI^T were sausage-shaped, non-motile, 1·7–2·8 μm long and 0·7–0·8 μm wide and occurred singly or in pairs (Imachi *et al*., 2000). The cells were Gram-reaction negative, but transmission electron microscopy performed as described previously (Hattori *et al*., 2000) showed a Gram-positive bacterial cell-wall structure (Fig. 1). Spores were spherical and central. Spore formation was observed only when strain SI^T was cultivated on propionate medium in tri-culture with *Methanothermobacter thermautotrophicus* strain ΔH^T and *Methanothrix* ('*Methanosaeta*') *thermophila* strain P$_T^T$ (Imachi *et al*., 2000).

Effects of pH, temperature and NaCl concentration on growth were determined by using medium containing 20 mM pyruvate plus 0·01 % yeast extract with exponential-phase cultures of strain SI^T as inoculum. All measurements were performed in triplicate and growth was quantified by measuring the OD_{600}. For determination of the optimum pH for growth, the pH of the pyruvate medium was adjusted to 5·5–8·0 with HCl or NaOH solution under 100 % N_2 gas. Under these conditions, the pH range for growth of strain SI^T was estimated to be 6·5–8·0 with an optimum of pH 7·0 (Fig. 2a). Optimum growth was found at 55 °C (Fig. 2b). No growth occurred below 45 °C or above 65 °C after 2 months of cultivation. Under optimum conditions (pH 7·0, 55 °C), the specific growth rate of the strain on pyruvate in pure culture was $1{\cdot}65 \pm 0{\cdot}17$ day^{-1}. The strain could grow at NaCl concentrations ranging from 0 to 4 g l^{-1}. Growth was inhibited completely by 5 g NaCl l^{-1}. Unstable growth (stagnation of growth and unexpected failure in cultivation) was occasionally observed under all cultivation conditions, particularly in co-culture with methanogens on propionate medium. In addition, cells were readily autolysed in the late growth phase. With pyruvate, autolysis was found to occur within 7 days (data not shown). Autolysed cells were inoculated into fresh medium with 10 % inoculum (v/v), but no growth was recovered.

Strain SI^T grew on pyruvate (20 mM) and fumarate (20 mM) in pure culture. For determination of the fermentation products of strain SI^T, we measured short-chain fatty acids, alcohols and several gases as described previously (Imachi *et al*., 2000) and determined pyruvate, succinate and fumarate by HPLC using a Shim-pack SCR-102H column (Shimadzu;

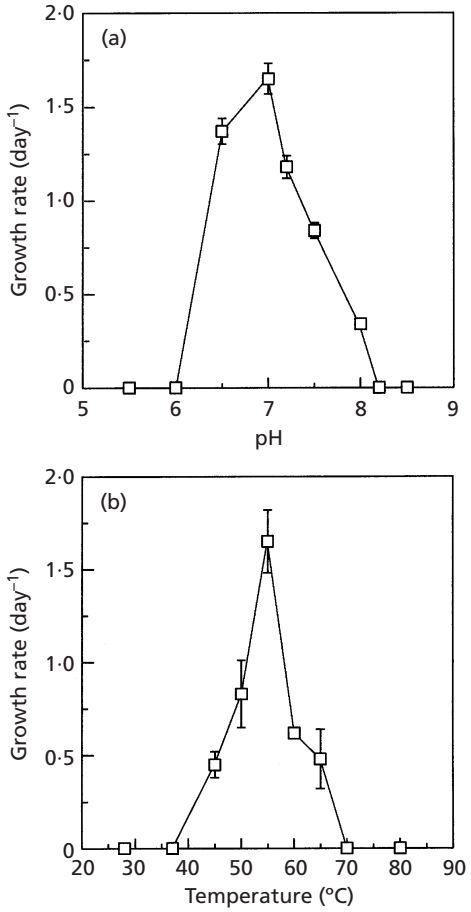


Fig. 2. Effect of pH (at 55 °C) (a) and temperature (b) on the specific growth rate of strain SI^T on pyruvate (20 mM) medium. Error bars indicate standard deviations of triplicate determinations.

mobile phase, 4 mM *p*-toluenesulphonic acid; column temperature, 45 °C). Major fermentation products from pyruvate were acetate and propionate (3:1; molar ratio), whereas the end products from fumarate were succinate, acetate and malate (6·5:1·5:1; molar ratio). Yeast extract (0·01 %) or peptone (0·01 %) were required for growth. The following substrates were not utilized in pure culture (2 months incubation): Casamino acids (0·1 %), yeast extract (0·4 %), tryptone (0·1 %), H_2/CO_2 (80:20, v/v, headspace), betaine (10 mM), glucose (20 mM), ribose (20 mM), xylose (20 mM), lactate (20 mM), glycerol (5 mM), acetate (20 mM), propionate (20 mM), butyrate (20 mM), valerate (5 mM), caproate (5 mM), heptanoate (5 mM), malate (20 mM), succinate (20 mM), ethanol (20 mM), methanol (20 mM), 1-propanol (20 mM), 1-butanol (20 mM), 1-pentanol (10 mM), acetoin (10 mM), acetaldehyde (0·1 %), 1,2-butanediol (10 mM), 2,3-butanediol (10 mM), 1,3-propanediol (10 mM), arabinose (20 mM), fructose (20 mM), galactose (20 mM), mannose (20 mM), raffinose (20 mM), sucrose (20 mM), starch (5 g l^{-1}), formate (40 mM) plus acetate (1 mM), ethylene glycol (10 mM), benzoate (5 mM), hydroquinone (1 mM) and phenol (1 mM).

Anaerobic respiratory growth was observed with fumarate as an electron acceptor. Propionate (20 mM), ethanol (20 mM) and lactate (20 mM) were degraded with the concomitant reduction of fumarate (20 mM) to succinate. The end products from these substrates with fumarate respiration were as follows: acetate and malate from propionate; acetate, malate and propionate from ethanol; and acetate and propionate from lactate. Sulphate (20 mM), sulphite (2·5 mM), thiosulphate (20 mM), elemental sulphur (20 mM), nitrate (20 mM) and ferric ion [Fe(III)-NTA: 5 mM] were not utilized as an electron acceptor with propionate (20 mM), ethanol (20 mM) or lactate (20 mM) as an electron donor. To investigate whether sulphate reduction occurs in the presence of external electron carriers, menadione (500 μg l^{-1}), 1,4-naphthoquinone (500 μg l^{-1}), vitamin K_1 (500 μg l^{-1}) or haemin (50 μg l^{-1}) was added to sulphate medium with propionate, ethanol or lactate (Hippe *et al.*, 1997; Brauman *et al.*, 1998). However, strain SI^T could not reduce sulphate in the presence of these compounds.

Strain SI^T was able to grow on the following substrates in co-culture with *Methanothermobacter themautotrophicus* strain ΔH^T: propionate (20 mM), ethanol (20 mM), lactate (20 mM), 1-butanol (20 mM), ethylene glycol (10 mM), 1-propanol (20 mM), 1-pentanol (10 mM) and 1,3-propanediol (10 mM). Growth was never observed on malate (20 mM), succinate (20 mM), methanol (20 mM), acetoin (10 mM), acetaldehyde (0·1 %), valerate (5 mM), caproate (5 mM), heptanoate (5 mM), 1,2-butanediol (10 mM), 2,3-butanediol (10 mM), benzoate (5 mM), hydroquinone (1 mM) or phenol (1 mM) within 2 months of cultivation. The specific growth rate on 20 mM propionate medium was 0·19 day^{-1} (calculated by measuring methane production) both in co-culture with *Methanothermobacter thermautotrophicus* strain ΔH^T and in tri-culture with *Methanothermobacter thermautotrophicus* strain ΔH^T and *Methanothrix* ('*Methanosaeta*') *thermophila* (Imachi *et al.*, 2000). The specific growth rate of the co-culture with *Methanothermobacter thermautotrophicus* ΔH^T on 10 mM ethanol was 2·4 day^{-1} (Imachi *et al.*, 2000).

Interspecies formate transfer, as well as hydrogen transfer, has been considered important for certain syntrophic cultures (Boone *et al.*, 1989; Thiele & Zeikus, 1988; Hattori *et al.*, 2001). Hattori *et al.* (2001) found that the growth of a thermophilic, acetate-oxidizing syntroph in co-culture with a hydrogen-/formate-utilizing methanogen was much higher than the co-culture with a hydrogen-utilizing methanogen. To investigate whether formate transfer could be involved in the syntrophic growth of strain SI^T on propionate, we replaced the hydrogenotrophic partner with the hydrogen-/formate-utilizing methanogen *Methanothermobacter thermautotrophicus* strain type-

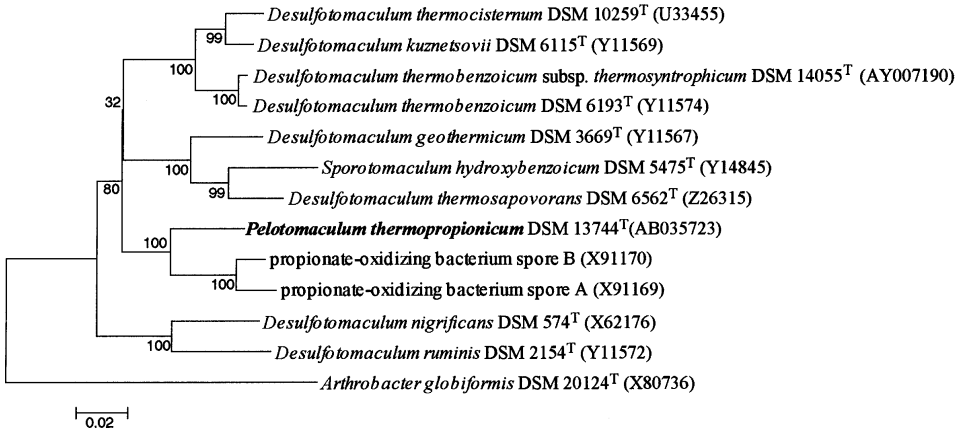


Fig. 3. Phylogenetic tree of strain SI^T (*Pelotomaculum thermopropionicum*) among the genus *Desulfotomaculum* and related organisms in the *Clostridium/Bacillus* subclass. The tree was constructed based on a distance-matrix analysis of 16S rDNA (rRNA gene) sequences (neighbour-joining tree) according to the method of Imachi *et al.* (2000). Bar, 0·02 nucleotide changes per sequence position. Numbers at nodes show bootstrap percentages obtained from 1000 resamplings.

II, which was isolated in our laboratory, and compared the growth rate. However, there was no difference in growth rate between the two co-cultures (data not shown), suggesting that formate transfer is not likely to occur in the co-culture.

On the basis of the phylogenetic analysis described previously, strain SI^T formed a novel lineage in the *Desulfotomaculum* group (Imachi *et al.*, 2000). We reconstructed a phylogenetic tree that included 16S rDNA sequences of *Sporotomaculum hydroxybenzoicum* and *Desulfotomaculum thermobenzoicum* subsp. *thermosyntrophicum* (Fig. 3), both of which were described recently (Brauman *et al.*, 1998; Plugge *et al.*, 2002). The closest relatives of strain SI^T were 16S rDNA clones of propionate-oxidizing bacteria, spore A and spore B (similarity values of 94 and 95%, respectively), which were reported as mesophilic, spore-forming, non-sulphate-reducing, syntrophic propionate-oxidizing bacteria by Harmsen (1996): these microbes have yet to be described either in pure culture or in defined co-culture.

The DNA G+C content of strain SI^T was 52·8 mol%, as determined by the method described previously (Kamagata & Mikami, 1991). For quinone and fatty acid methyl ester (FAME) analyses, cells of strain SI^T were harvested from cultures grown on medium containing 20 mM lactate, 20 mM fumarate and 0·01% yeast extract. The following reference microbes were obtained from the DSMZ and subjected to quinone and FAME analyses for comparison: *Desulfotomaculum thermobenzoicum* DSM 6193^T, *Desulfotomaculum thermosapovorans* DSM 6562^T and *Desulfotomaculum thermocisternum* DSM 10259^T. The FAME analysis (Hanada *et al.*, 2002) showed that strain SI^T contained iso-C15:0 as the major fatty acid, similar to *Desulfotomaculum thermocisternum* and *Desulfotomaculum thermobenzoicum* (Table 1). Quinone analysis (Zhang *et al.*, 2000) revealed that strain SI^T contained MK-7 and MK-7(H_4) as the major quinones,

Table 1. Fatty acid compositions of strain SI^T and thermophilic *Desulfotomaculum* species

Percentages of total acids are shown. Taxa are identified as: 1, *Pelotomaculum thermopropionicum* strain SI^T; 2, *Desulfotomaculum nigrificans* (data from Ueki & Suto, 1979); 3, *Desulfotomaculum thermosapovorans* (Kuever *et al.*, 1999); 4, *Desulfotomaculum thermocisternum*; 5, *Desulfotomaculum thermobenzoicum*.

Fatty acid	1	2	3	4	5
C11:0	–	–	0·5	–	–
iso-C14:0	–	0·8	1·4	–	–
C14:0	3·2	6·1	2·1	6·2	2·0
iso-C15:1	–	–	1·0	–	–
iso-C15:0	76·4	21·1	23·1	63·3	79·4
anteiso-C15:0	–	11·0	–	3·9	–
C15:0	–	1·6	1·4	3·9	–
C16:0 aldehyde	–	–	0·6	–	–
iso-C16:0	–	3·4	2·6	2·4	–
C16:1	–	5·0	5·4	–	–
C16:0	10·7	5·8	15·7	10·4	7·4
iso-C17:1	–	2·0	5·0	–	–
iso-C17:0	2·9	4·1	8·9	–	11·3
branched C17:0	–	–	–	2·4	–
anteiso-C17:0	–	1·4	–	–	–
C17:0	–	0·5	8·9	–	–
iso-C18:0	–	0·6			
C18:1ω9	–	0·7	–	–	–
C18:1ω11	–	0·7	0·9	–	–
C18:0	6·7	1·5	3·2	2·4	–

Table 2. Characteristics of strain SI^T and *Desulfotomaculum* species

Taxa are identified as: 1, *Pelotomaculum thermopropionicum* strain SI^T; 2, *Desulfotomaculum nigrificans* (unless otherwise indicated, data from Campbell & Postgate, 1965; Klemps *et al*., 1985; Widdel, 1992; Liu *et al*., 1997); 3, *Desulfotomaculum thermosapovorans* (Fardeau *et al*., 1995); 4, *Desulfotomaculum thermocisternum* (Nilsen *et al*., 1996); 5, *Desulfotomaculum thermobenzoicum* (Tasaki *et al*., 1991); 6, *Desulfotomaculum thermobenzoicum* subsp. *thermosyntrophicum* (Plugge *et al*., 2002). ND, Not determined; V, variable. All taxa are positive for spore formation and utilization of pyruvate in pure culture.

Characteristic	1	2	3	4	5	6
Cell width (μm)	0·7–0·8	1–1·2	1·5–2	0·7–1·0	1·5–2	1
Cell length (μm)	1·7–2·8	3–6	5–8	2·0–5·2	5–8	3–11
Gram reaction	−	V	ND	ND	+	−
Cell wall structure	+	+	ND	+	+	+
G+C content (mol%)	52·8	48·5–49·9	51·2	57	52·8	53·7
Optimum temperature (°C)*	55 (45–65)	55 (30–70)	50 (35–60)	62 (41–75)	62 (40–70)	55 (45–62)
Optimum pH*	7·0 (6·5–8·0)	(6·0–8·0)	7·2–7·5	6·7 (6·2–8·9)	7·2 (6·0–8·0)	7·0
Motility	−	+	+	+	+	+
Major quinone	MK-7, MK-7(H_4)	MK-7	MK-7†	MK-7†	MK-7†	ND
Sulphate reduction	−	+	+	+	+	+
Fumarate reduction	+	ND	ND	ND	ND	−
Substrate utilization in pure culture:						
Lactate	−	−	+	ND	+	+
Fumarate	+	−	ND	ND	−	+
3-Hydroxybenzoate	−	ND	−	ND	−	−
Substrate utilization in pure culture with sulphate reduction:						
Propionate	−	−	−	+	+	+
Lactate	−	+	+	+	+	+
Ethanol	−	+	+	+	+	−
Butyrate	−	−	+	+	+	−
Substrate utilization in co-culture with hydrogenotrophic methanogens:						
Propionate	+	ND	ND	+/−‡	−	+
Lactate	+	+	ND	ND	ND	+
Ethanol	+	+	ND	ND	ND	−
Butyrate	−	ND	ND	ND	ND	−

* Mean with range in parentheses.

† Data from this study.

‡ Data from Nilsen *et al*. (1996), Imachi *et al*. (2000) and Plugge *et al*. (2002).

whereas almost all its thermophilic relatives possess only MK-7 as the major component (Table 2).

Strain SI^T tolerated ampicillin (50 μg ml^{-1}), chloramphenicol (50 μg ml^{-1}), rifampicin (50 μg ml^{-1}) and vancomycin (50 μg ml^{-1}) under optimum cultivation conditions (pH 7·0, 55 °C on 20 mM pyruvate plus 0·01% yeast extract medium). Kanamycin (50 μg ml^{-1}) and neomycin (50 μg ml^{-1}) completely inhibited growth.

Based on its morphological, physiological, chemotaxonomic and molecular phylogenetic traits, strain SI^T was found to be related to members of the genus *Desulfotomaculum*. However, extended physiological and chemotaxonomic studies showed that there are significant differences between the strain and other known species of the genus *Desulfotomaculum*. Major differences that distinguish the strain from members of the genus *Desulfotomaculum* are: (i) all *Desulfotomaculum* species can conduct dissimilatory sulphate reduction, while strain SI^T could not; (ii) almost all members of the genus *Desulfotomaculum* contain MK-7 as the major quinone, while the strain possesses MK-7(H_4) in addition to MK-7; and (iii) strain SI^T could reduce fumarate with the concomitant oxidation of organic substances instead of sulphate reduction (Table 2). The most striking feature of strain SI^T is the lack of ability to reduce sulphate (Fig. 3;

Table 2). Similar findings were reported for the 3-hydroxybenzoate-degrading anaerobe *Sporotomaculum hydroxybenzoicum*, which is closely affiliated with the genus *Desulfotomaculum* (Brauman *et al*., 1998). However, *Sporotomaculum hydroxybenzoicum* is mesophilic and clearly different from this isolate in phylogenetic and physiological respects. Syntrophic growth is also a unique and important trait that characterizes strain SI^T. Only three thermophilic members of the genus *Desulfotomaculum* have been reported to perform syntrophic substrate oxidation, of which two are known to grow with syntrophic propionate oxidation (Table 2). The syntrophic growth of *Desulfotomaculum thermocisternum* on propionate is doubtful, however, since other researchers have reported that no syntrophic growth of this species was observed (Imachi *et al*., 2000; Plugge *et al*., 2002).

With respect to mesophilic, syntrophic propionate-oxidizing species, all the microbes known to date have been classified phylogenetically in the *δ-Proteobacteria*, i.e. the genera *Syntrophobacter* and *Smithella*. Hence, they are phylogenetically distant from strain SI^T. Among these, strain SI^T resembles *Syntrophobacter fumaroxidans* in its fumarate utilization; both syntrophs can grow on fumarate and pyruvate in pure culture (Harmsen *et al*., 1998). In addition, both organisms can oxidize propionate with reduction of fumarate in pure culture. However, all members of the genus *Syntrophobacter* can grow with sulphate reduction, unlike strain SI^T. *Smithella propionica* is a mesophilic, propionate-oxidizing syntroph unable to grow by sulphate reduction (Liu *et al*., 1999), but the species can grow only on crotonate in pure culture.

This phenotypic and phylogenetic distinctiveness justifies the description of strain SI^T as a novel species of a new genus. Hence, we propose the name *Pelotomaculum thermopropionicum* gen. nov., sp. nov., for strain SI^T.

Description of *Pelotomaculum* gen. nov.

Pelotomaculum (Pe.lo.to.ma′cu.lum. Gr. adj. *pelos* dark-coloured, hence anaerobic mud; L. neut. n. *tomaculum* sausage; N.L. neut. n. *Pelotomaculum* sausage-shaped bacteria living in anaerobic environments).

A strictly anaerobic and thermophilic organism. Non-motile and sausage-shaped cells. Gram reaction is negative, but shows Gram-positive cell-wall structure. Spherical endospores are formed. The organism can oxidize propionate, lactate or several alcohols in syntrophic association with hydrogenotrophic methanogens. Sulphate, sulphite, thiosulphate, elemental sulphur, nitrate and ferric ion cannot be reduced. The major cellular fatty acid is iso-C15:0. Major quinones are MK-7 and MK-7(H_4). Forms a lineage in the *Clostridium*/*Bacillus* subclass of the Gram-positive bacteria. The type species is *Pelotomaculum thermopropionicum*.

Description of *Pelotomaculum thermopropionicum* sp. nov.

Pelotomaculum thermopropionicum (ther.mo.pro.pi.o′ni.cum. Gr. adj. *thaemos* hot; N.L. n. *propionatum* propionate; L. suff. *-icus* pertaining to; N.L. adj. *thermopropionicum* thermophilic and pertaining to propionate).

Cells are 1·7–2·8 μm long and 0·7–0·8 μm wide. Spores are spherical and central. In syntrophic association with hydrogenotrophic methanogens, can utilize propionate, ethanol, lactate, ethylene glycol, 1-butanol, 1-propanol, 1-pentanol and 1,3-propanediol. In pure culture, can ferment pyruvate and fumarate. Fumarate can also be used as an electron acceptor in the presence of propionate, ethanol or lactate as an electron donor. Growth occurs in the presence of 0–0·4% NaCl but does not occur in the presence of more than 0·5% NaCl. The temperature range for growth is 45–65 °C (optimum 55 °C). The pH range for growth is 6·7–7·5 (optimum 7·0). The G+C content of the DNA is 52·8 mol%. The type strain is SI^T (= DSM 13744^T = JCM 10971^T).

Acknowledgements

We thank the following researchers in the National Institute of Advanced Industrial Science and Technology: Xian-Ying Meng for transmission electron microscopy, Yoko Ueda for measurement of the DNA base composition, Aiko Sukegawa for the determinations of quinones and Satoshi Hattori for his useful advice on the cultivation of recalcitrant syntrophs. This study was supported financially by a research grant no. 11794002 of the Grant-in-aid for University and Society Collaboration subsidized by the Ministry of Education, Culture, Sports, Science and Technology, Japan, and Research Fellowships of the Japan Society for the Promotion of Science (JSPS) for Young Scientists (no. 00004157).

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