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 SIT, 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 \( \Delta H \). 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 SIT contained MK-7 and MK-7(H4) as the major quinones and contained iso-C15:0 as the major fatty acid. Based on 16S rDNA sequence analysis, strain SIT 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 SIT\(^{T}\) (= 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

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

The GenBank/EMBL/DDBJ accession number for the 16S rDNA sequence of strain SIT\(^{T}\) is AB035723.
Smithella [Syntrophobacter wolinii (Boone & Bryant, 1980), Syntrophobacter pﬁnnigii (Wallrabenstein et al., 1995), Syntrophobacter fumaroxidans (Harmsen et al., 1998) and Smithella propionica (Liu et al., 1999)] and two thermophilic members of the genus Desulfothermus (Desulfothermus thermocisternum (Nilsen et al., 1996) and Desulfothermus 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\textsuperscript{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 Methano-thermobacter-like cells (Imachi et al., 2000). In our previous study, strain SI\textsuperscript{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 Desulfothermus, 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\textsuperscript{T} and propose a new genus and species for this strain.

Strain SI\textsuperscript{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\textsuperscript{T} were prepared as described previously (Imachi et al., 2000). Cells of strain SI\textsuperscript{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\textsuperscript{T} was cultivated on propionate medium in tri-culture with Methano-thermobacter thermautotrophicus strain ΔH\textsuperscript{T} and Methanothrix ('Methanoseta') thermophila strain P\textsuperscript{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\textsuperscript{T} as inoculum. All measurements were performed in triplicate and growth was quantitated by measuring the OD\textsubscript{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\textsubscript{2} gas. Under these conditions, the pH range for growth of strain SI\textsuperscript{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 1-65 ± 0-17 day\textsuperscript{-1}. The strain could grow at NaCl concentrations ranging from 0 to 4 g l\textsuperscript{-1}. Growth was inhibited completely by 5 g NaCl l\textsuperscript{-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\textsuperscript{T} grew on pyruvate (20 mM) and fumarate (20 mM) in pure culture. For determination of the fermentation products of strain SI\textsuperscript{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;...
Pelotomaculum thermopropionicum gen. nov., sp. nov.

Interspecies formate transfer, as well as hydrogen transfer, has been considered important for certain syntrophic cultures (Boone et al., 1989; Thieie & 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-utilizing methanogen. To investigate whether formate transfer could be involved in the syntrophic growth of strain ST on propionate, we replaced the hydrogenotrophic partner with the hydrogen-/formate-utilizing methanogen Methanothermobacter thermautotrophicus strain type-

(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 (20 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 K₃ (50 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 ST could not reduce sulphate in the presence of these compounds.

Strain ST was able to grow on the following substrates in co-culture with Methanothermobacter thermautotrophicus strain ΔH⁷⁻: 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), 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-butadienol (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 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; 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%), H₂/CO₂ (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...
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\textsuperscript{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; Pluge et al., 2002). The closest relatives of strain SI\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{T}, Desulfotomaculum thermosapovorans DSM 6562\textsuperscript{T} and Desulfotomaculum thermocisternum DSM 10259\textsuperscript{T}. The FAME analysis (Hanada et al., 2002) showed that strain SI\textsuperscript{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\textsuperscript{T} contained MK-7 and MK-7(H\textsubscript{4}) as the major quinones.

Table 1. Fatty acid compositions of strain SI\textsuperscript{T} and thermophilic Desulfotomaculum species

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
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<tr>
<td>C11:0</td>
<td>-</td>
<td>-</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
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<tr>
<td>iso-C14:0</td>
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<td>0.8</td>
<td>1.4</td>
<td>-</td>
<td>-</td>
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<tr>
<td>C14:0</td>
<td>3.2</td>
<td>6.1</td>
<td>2.1</td>
<td>6.2</td>
<td>2.0</td>
</tr>
<tr>
<td>iso-C15:1</td>
<td>-</td>
<td>1.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>iso-C15:0</td>
<td>76.4</td>
<td>21.1</td>
<td>23.1</td>
<td>63.3</td>
<td>79.4</td>
</tr>
<tr>
<td>anteiso-C15:0</td>
<td>-</td>
<td>1.1</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C15:0</td>
<td>-</td>
<td>1.6</td>
<td>1.4</td>
<td>3.9</td>
<td>-</td>
</tr>
<tr>
<td>C16:0 aldehyde</td>
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<td>-</td>
<td>0.6</td>
<td>-</td>
<td>-</td>
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<tr>
<td>iso-C16:0</td>
<td>-</td>
<td>3.4</td>
<td>2.6</td>
<td>2.4</td>
<td>-</td>
</tr>
<tr>
<td>C16:1</td>
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<td>5.4</td>
<td>-</td>
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<tr>
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<td>5.8</td>
<td>15.7</td>
<td>10.4</td>
<td>7.4</td>
</tr>
<tr>
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<td>-</td>
<td>2.0</td>
<td>5.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>iso-C17:0</td>
<td>2.9</td>
<td>41.8</td>
<td>8.9</td>
<td>11.3</td>
<td>-</td>
</tr>
<tr>
<td>branched C17:0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2.4</td>
<td>-</td>
</tr>
<tr>
<td>anteiso-C17:0</td>
<td>-</td>
<td>1.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C17:0</td>
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<td>0.5</td>
<td>8.9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>iso-C18:0</td>
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<td>-</td>
<td>-</td>
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<tr>
<td>C18:1o9</td>
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<td>0.7</td>
<td>-</td>
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</tr>
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<td>0.9</td>
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<td>6.7</td>
<td>1.5</td>
<td>3.2</td>
<td>2.4</td>
<td>-</td>
</tr>
</tbody>
</table>

Fig. 3. Phylogenetic tree of strain SI\textsuperscript{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.
whereas almost all its thermophilic relatives possess only MK-7 as the major component (Table 2).

Strain SIᵀ 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 SIᵀ 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 contain MK-7 as the major quinone, while the strain possesses MK-7(H₂) in addition to MK-7; and (ii) strain SIᵀ could not; (ii) almost all members of the genus Desulfotomaculum contain MK-7 as the major quinone, while the strain possesses MK-7(H₂) in addition to MK-7; and (iii) strain SIᵀ could reduce fumarate with the concomitant oxidation of organic substances instead of sulphate reduction (Table 2). The most striking feature of strain SIᵀ is the lack of ability to reduce sulphate (Fig. 3; Table 2).
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<sup>T</sup>. 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; Flugge 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<sup>T</sup>. Among these, strain SI<sup>T</sup> 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<sup>T</sup>. *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<sup>T</sup> as a novel species of a new genus. Hence, we propose the name *Pelotomaculum thermopropionicum* gen. nov., sp. nov., for strain SI<sup>T</sup>.

**Description of Pelotomaculum gen. nov.**

*Pelotomaculum* (Pel.o.to.ma'cu.lum. Gr. adj. pelos dark-coloured, hence anaerobic mud; L. neut. n. toma'culum sausage; N.L. neut. n. Pelotoma'culum 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<sub>2</sub>). Forms a lineage in the Clostridium/Bacillus subclass of the Gram-positive bacteria. The type species is *Pelotomaculum thermopropionicum*.

**Description of Pelotomaculum thermopropionicum**

*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<sup>T</sup> (DSM 13744<sup>T</sup> = JCM 10971<sup>T</sup>).

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**References**


Pelotomaculum thermopropionicum gen. nov., sp. nov.


