Hypnocyclicus thermotrophus gen. nov., sp. nov. isolated from a microbial mat in a hydrothermal vent field

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The bacterial strain, IR-2T, was isolated from a microbial mat sampled near a hydrothermal vent in the Greenland Sea. Phylogenetic analysis, based on the 16S rRNA gene, showed that the closest relatives of IR-2T were Ilyobacter tartaricus, Ilyobacter insuetus, Propionigenium modestum and Fusobacterium varium (91 % 16S rRNA gene sequence similarity). The cells of the novel strain were Gram-stain-negative and pleomorphic; changing from long motile rods to non-motile ring structures during the growth cycle. Growth occurred at 20–55 °C (optimally at 48 °C), with 1–6 % (w/v) NaCl (optimally with 2 %), and at pH 5.3–8.0 (optimally at pH 6.0–8.0). The strain had obligate fermentative growth on various sugars and yeast extract. The DNA G+C content of strain IR-2T was 25.7 mol%. The cell sugars comprised mainly ribose, mannose and glucose, while the main polar lipids were glycolipids, phospholipids, phosphatidylglycerol and diphosphatidylglycerol. The fatty acid content of strain IR-2 was dominated by saturated and unsaturated iso-branched or anteiso-branched forms. Strain IR-2 represents a novel genus and species, for which the name Hypnocyclicus thermotrophus gen. nov., sp. nov. is proposed. The type strain is IR-2T (=DSM 100055 = JCM 30901).

The phylum Fusobacteria includes pathogens isolated from both human and animal sources, in addition to non-pathogenic free-living species. The majority of the free-living members of Fusobacteriaceae, isolated from marine sediments and mud, seem to have a limited metabolic range where a few specific substrates are fermented such as succinate, hydroxybuturate, hydroaromatic compounds and tartrate (Brune et al., 2002; Janssen & Harfoot, 1990; Janssen & Liesack, 1995; Schink, 1984; Stieb & Schink, 1984). In this study we describe a novel bacterium, designated strain IR-2T, isolated from a microbial mat. The strain could only utilize sugars by fermentation, and we thereby suggest that the in situ metabolism of strain IR-2T is degradation of polysaccharides from exopolymeric substances (EPS) or of intracellular origin. Other free-living species within Fusobacteriaceae, such as Ilyobacter insuetus, Ilyobacter delafieldii and Propionigenium modestum, could not ferment sugars, probably as a result of a lifestyle in environments depleted of sugars (Both et al., 1991, Brune et al., 2002, Janssen & Harfoot, 1990). Strain IR-2T was isolated from a microbial mat situated in a hydrothermal vent field at the Northern Kolbeinsey Ridge, 166 km west of Jan Mayen in the Greenland Sea. The mat was collected at a depth of 130 m using a ROV Mariner XXL (Argus remote systems AS) equipped with a 1 l hydraulic sampling cylinder (biosyringe). The sample retrieved from the biosyringe, comprising mat material and ambient seawater, was a milky white homogeneous suspension. The sample was used directly as inoculum (5 %, v/v) in enrichment cultures supplied with 1.5 bar H2 : CO2 (80 : 20, v/v), 0.01 % (w/v) yeast extract and 5 mM nitrate, and was incubated at 50 °C for 7 days. The growth medium used for all culturing is described elsewhere (Myhr & Torsvik, 2000), and was based on an anaerobic mineral medium for nitrate reducers (NRB-medium) buffered with 0.3 M NaHCO3 (Merck) to a pH of 6.8–7.2 and supplied with trace element solution SL10 (Widdel et al., 1983), vitamin solution (Pfennig, 1978) and 2 mM Na2S (Sigma-Aldrich), unless specified otherwise. After three transfers, the enrichment

Abbreviation: EPS, exopolymeric substances.

The GenBank accession number for the 16S rRNA sequence of Hypnocyclicus thermotrophus is KP869170.
culture was adapted to a medium with 0.2 \% (w/v) yeast extract and 5 mM nitrate, and isolation of the strain was performed on plates. The plates, consisting of NRB medium with added 0.2 \% (w/v) yeast extract, 5 mM nitrate and 1.5 \% (w/v) agar, were made up and stored in an anaerobic atmosphere of H₂:CO₂:N₂ (10:10:80, by vol.) using a Whitley A35 anaerobic workstation (don Whitley). The inoculated plates were incubated anaerobically at 50 °C using the Anaerocult system (Merck). Colonies on the plates were white, glossy and circular with a diameter of approximately 1 mm. Inspection of morphology visually using a light microscope (Axioskop 40, Zeiss) and 16S rRNA gene sequencing confirmed a monoculture of the IR-2T strain. The 16S rRNA gene was amplified using the primers 8f (5'-AGAGTTTGATCCTGGCTCAG-3') (Edwards et al., 1989) and 1392r (5'-ACGAGTTGATCCTGGCAGGGC-3') (Lane et al., 1985) in addition to Hot Star Taq Plus Master Mix (Qiagen), followed by a BigDye (Applied Biosystems) Sanger sequencing. The closest relatives of IR-2T were *Ilyobacter tartaricus* strain GraTa2T (NR_028939), *Propionigenium modestum* strain GraSucc 2T (NR_029216) and *Ilyobacter insuetus* strain VenChi2T (NR_025454), which all had 91 \% identity to strain IR-2T (NR_113384), *Propionigenium modestum* (NR_028939), *Fusobacterium varium* (NR_025454), when a standalone BLASTN alignment was carried out using the Refseq_rna database. Phylogenetic analysis was based on 16S rRNA gene sequences of IR-2T (1213 bp) and sequences of selected species within the phylum *Fusobacteria* derived from the Silva database (release 119). A phylogenetic tree based on the neighbour joining method was reconstructed using ARB software v.5.0 (Ludwig et al., 2004). The IR-2T strain formed a distinct branch with a cluster comprising species of the genera *Ilyobacter* and *Propionigenium* as the closest relatives (Fig. 1). The 16S rRNA gene sequence of IR-2T has been submitted to the GenBank under accession number KP869170. In addition, the mol\% G+C content of the genomic DNA was calculated based on analyses of melting points of dsDNA, as described by Mandel et al. (1970). The DNA extraction protocol was based on the method of Marrmur (1961) and DNA dissolved in 0.1 x SSC solution with an OD₂₆₀ of 0.3 was used in the experiments. For comparison, genomic DNA from *Clostridium perfringens* (Sigma-Aldrich) was included in experiments. From parallel melting curves of DNA from the IR-2 strain, the genomic DNA G+C content was determined to be 25.7 mol\%.

During the early stages of growth, the cells were motile and rod-shaped: measuring 2–7 μm long and 0.5–0.7 μm wide (Fig. 2a). During the late stationary phase, the cells changed to a coccoid shape: measuring 2–7 μm in diameter. The 16S rRNA gene sequence of IR-2T has been submitted to the GenBank under accession number KP869170. In addition, the mol\% G+C content of the genomic DNA was calculated based on analyses of melting points of dsDNA, as described by Mandel et al. (1970). The DNA extraction protocol was based on the method of Marrmur (1961) and DNA dissolved in 0.1 x SSC solution with an OD₂₆₀ of 0.3 was used in the experiments. For comparison, genomic DNA from *Clostridium perfringens* (Sigma-Aldrich) was included in experiments. From parallel melting curves of DNA from the IR-2 strain, the genomic DNA G+C content was determined to be 25.7 mol\%.

![Fig. 1. A phylogenetic tree based on 16S rRNA gene sequence comparisons of strain IR-2 and its closest relatives within the phylum Fusobacteria. Sequences were obtained from the Silva database and a tree was reconstructed using the neighbour-joining method in ARB. Bar, 0.1 \% substitution per nucleotide position.](image-url)
morphology and became non-motile ring-structures, measuring 0.7–1.4 μm across, and seemed to be covered in an EPS (Fig. 2b). Gram staining was performed as described by Doetsch (1981) and the KOH string test was performed as described by Ryu (1938) showing that the strain was Gram-stain-negative. The temperature, NaCl concentration and pH ranges for growth were determined using parallel cultures in liquid NRB medium supplied with 0.1 % (w/v) glucose and 0.2 % (w/v) yeast extract. Positive results were based on evaluation of the general condition (cell density, motility and morphology) of the culture under each growth condition by light microscopy. In addition, maximal growth rate experiments for the determination of the optimal temperature, NaCl concentration and pH of the strain were performed in parallel: growth curves were generated by OD measurements every 10 min at 600 nm (Cary 3000 spectrophotometer, Varian) in 10 mm quartz glass cuvettes (Hellma). The temperature range was 20–55 °C, while the range of NaCl concentrations employed was 1–6 % (w/v). No growth was observed in cultures with <1 % (w/v) NaCl. Optimum growth conditions were achieved using 2 % (w/v) NaCl at 48 °C. The pH range tested in medium supplied with 30 mM bicarbonate (Sigma-Aldrich), 25 mM Tricine (Sigma-Aldrich) or 30 mM morpholino ethansulfonic acid (MES) (Sigma-Aldrich) showed that strain IR-2T grew over a wide pH range and also grew optimally over a wide pH range. Growth was observed at pH 5.3–8.0, but not at pH values above 8.2; and the optimum pH was between 6.0 and 8.0. An initial metabolic characterization was based on Anaerobe Identification Test Panel system AN Micro plate (Biolog), where inoculation was performed as suggested by the manufacturer. The plate was incubated under anaerobic conditions at 50 °C for 24 h, followed by a manual plate inspection where a colour change from clear to purple indicated a positive reaction. The results showed that IR-2T utilized mainly sugars, while amino acids and organic acids where not utilized. Furthermore, characterization of utilized substrates was also performed at 48 °C using NRB medium with 2 % (w/v) NaCl, pH of 7.0–7.3 and 0.1 % (w/v) or 20 mM of the different substrates. Cultures were incubated at 48 °C for 20–48 h. No growth in parallel cultures after 14 days of incubation was interpreted as a negative result. Strain IR-2T could ferment sugars, such as amylose (from potato), amylpectin (from potato starch), cellobiose, dextrin (from potato starch), D-fructose, D-galactose, D-glucose, maltose, D-mannitol, D-mannose, α-D-palatinose, starch (soluble) and sucrose, in addition to yeast extract and possibly tartrate (Micro plate) (Table 1). Substrates such as acetate, L-arabinose, α-D-cellulose, chitin (from shrimp shells), citrate, formate, 3-hydroxybutyrate (Micro plate), α-lactose, L-rhamnose, peptone, propionate, pyruvate, D-ribose, succinate (Micro plate), tryptone, xylan (from beechwood) and D-xylose were not utilized (Table 1). Microaerophilic growth conditions with 3 % (v/v) and 5 % (v/v) oxygen were tolerated, but did not enhance growth rates. Nitrate was not used as a terminal electron acceptor. Enzyme activity of oxidase and urease were tested with Diatabs (Roche), while catalase activity was tested by applying 30 % (w/w) H2O2 (Sigma). Strain IR-2T could easily be distinguished from species within the closest related genera, Propionigenium and Ilyobacter, based on metabolic characterization (Table 1).

H2 production from fermentation of 0.1 % (w/v) glucose, maltose, dextrin or yeast extract after 24 h was quantified using headspace gas from parallel batch cultures that were injected into a SRI 8610C gas chromatograph (SRI Instruments). The gas chromatograph was equipped with a 30 m long MXT-Plot column (Restek) to separate the gases (0.53 mmID) during a 4 min long run. H2 concentrations were then determined with a highly sensitive helium-pulsed discharge detector. H2 standard gases of 100 % (v/v) H2 and 5 % (v/v) oxygen were tolerated, but did not enhance growth rates. Nitrate was not used as a terminal electron acceptor. Enzyme activity of oxidase and urease were tested with Diatabs (Roche), while catalase activity was tested by applying 30 % (w/w) H2O2 (Sigma). Strain IR-2T could easily be distinguished from species within the closest related genera, Propionigenium and Ilyobacter, based on metabolic characterization (Table 1).
Table 1. Differential characteristics that distinguish strain IR-2 from selected members of the phylum Fusobacteria

<table>
<thead>
<tr>
<th>Source</th>
<th>Morphology</th>
<th>Optimum temperature (°C)</th>
<th>G+C (mol%)</th>
<th>Motility</th>
<th>Utilization of:</th>
<th>Enzyme activity</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>mat</td>
<td>sediment</td>
<td>sediment</td>
<td>mud</td>
<td>sediment</td>
<td>sediment</td>
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<tr>
<td></td>
<td>long rods</td>
<td>short rods</td>
<td>oval rods</td>
<td>short rods</td>
<td>coccoid</td>
<td>long rods</td>
</tr>
<tr>
<td>1</td>
<td>IR-2 (this study)</td>
<td>48</td>
<td>25.7</td>
<td>+</td>
<td>Acetate</td>
<td>Catalase</td>
</tr>
<tr>
<td>2</td>
<td>Ilyobacter tartaricus (Schink, 1984)</td>
<td>28</td>
<td>33.1</td>
<td>+</td>
<td>Citrate</td>
<td>Oxidase</td>
</tr>
<tr>
<td>3</td>
<td>Propionigenium maris (Janssen &amp; Liesack, 1995)</td>
<td>34–37</td>
<td>40.0</td>
<td>+</td>
<td>Fructose</td>
<td>Oxidase</td>
</tr>
<tr>
<td>4</td>
<td>Propionigenium modestum (Schink &amp; Pfennig, 1982)</td>
<td>33</td>
<td>33.9</td>
<td>+</td>
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<td>Urease</td>
</tr>
<tr>
<td>5</td>
<td>Ilyobacter insuetus (Brune et al., 2002)</td>
<td>30</td>
<td>35.7</td>
<td>+</td>
<td>Succinate</td>
<td>Urease</td>
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<tr>
<td>6</td>
<td>Ilyobacter delafeldii (Janssen &amp; Harfoot, 1990)</td>
<td>40</td>
<td>29.0</td>
<td>+</td>
<td>Tartrate</td>
<td>Urease</td>
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<tr>
<td>7</td>
<td>Ilyobacter polytropus (Stieb &amp; Schink, 1984)</td>
<td>30</td>
<td>32.2</td>
<td>+</td>
<td>Pyruvate</td>
<td>Oxidase</td>
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<tr>
<td>8</td>
<td>Fusobacterium varium (Eggerth &amp; Gagnon, 1933; Moore et al., 1969)</td>
<td>37</td>
<td>29.2</td>
<td>+</td>
<td>Yeast extract</td>
<td>Oxidase</td>
</tr>
</tbody>
</table>

*Data from GenBank: ACIE00000000.2.
†Results from AN Micro plates (Biolog).

Cells are Gram-stain-negative, non-spore-forming and pleomorphic, occurring as rods or ring structures. During active growth the cells are motile rods, while cells form non-motile ring structures covered in EPS during the resting state. Thermophilic, neutrophilic and moderately halophilic. They are obligately anaerobic and ferment sugars and complex organic rich substrates. Members of the genus are catalase-, urease- and oxidase-negative. The predominant fatty acids are saturated and unsaturated iso-branched or anteiso-branched. The cell membrane contains glycolipids, phospholipids and glycerols. 16S rRNA gene sequence analysis places the genus Hypnocyclicus as a separate branch within the family Fusobacteriaceae. The type species is Hypnocyclicus thermotrophus.

Description of Hypnocyclicus thermotrophus sp. nov.

Hypnocyclicus thermotrophus (ther.mo.tro'phus. Gr. adj. thermos warm; Gr. masc. n. trophos feeder; N.L. masc. adj. thermotrophus feeding under warm conditions).

Displays the following properties in addition to those given in the genus description. Cells are motile rods, measuring 2–7 μm × 0.5–0.7 μm. Growth occurs at 20–55 °C (optimally at 48 °C), with 1–6% (w/v) NaCl (optimally with 2%), and at pH 5.3–8.0 (optimally at pH 6.0–8.0).
Amylose, amylpectin, cellulose, dextrin, fructose, galactose, glucose, maltose, mannitol, mannose, palatinitose, starch, sucrose tartate and yeast extract are fermented. Acetate, arabinoce, cellulose, chitin, citrate, formate, 3-hydroxybutyrate, lactose, rhamnose, peptone, propionate, pyruvate, ribose, succinate, tryptone, xylan and xylose are not utilized. Oxygen and nitrate are not used as electron acceptors. The major fatty acids are C_{16:0}, C_{14:0}, C_{16:1\alpha7c} or C_{15:0 iso 2OH} and C_{12:0 3OH}. The whole cell sugars are ribose, mannose and glucose, in addition to trace amounts of galactose.

The type strain is IR-2^{T} (=DSM 100055=JCM 30901), isolated from a bacterial mat situated in a hydrothermal venting field in the Greenland Sea. The genomic DNA G+C content of the type strain is 25.7 mol%.

Acknowledgements

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References


