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 delafiedii 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), vitamine solution (Pfenning, 1978) and 2 mM Na2S (Sigma-Aldrich), unless specified otherwise. After three transfers, the enrichment
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 monocolony of the IR-2T strain. The IR-2T strain formed a distinct branch with a high similarity content of the genomic DNA was calculated to be 25.7 mol%.

The DNA extraction protocol was based on the method of Marmur (1961) and DNA dissolved in 0.1 M SSC solution with an OD260 content of 0.1 × 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 filamentous form with a maximum diameter of approximately 1 mm. Examination of the cells under a light microscope (Axioskop 40, Zeiss) and 16S rRNA gene sequencing confirmed a monocolony of the IR-2T strain. The IR-2T strain formed a distinct branch with a high similarity content of the genomic DNA was calculated to be 25.7 mol%.

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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), cellulose, 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 100 p.p.m. H2 were mixed with He to achieve a standard

![Fig. 2.](http://ijs.microbiologyresearch.org) Electron micrographs of strain IR-2. The morphology of the cells change during growth: from rods during active growth (a) to ring structures covered in EPS during the stationary phase (b). Bar, 1 μm.
The fatty acid content of strain IR-2 was dominated by C16:0 (38.2%), C16:1 and C17:1 iso or ante iso. The cell membrane comprises two glycolipids and three phospholipids, in addition to phosphatidylglycerol and diphosphatidylglycerol.

In total, seven unidentified polar lipids were detected, containing 0.1% (w/v) substrate decreased considerably for maltose, 1.19 mM for dextrin, 0.52 mM for glucose and 0.48 mM for yeast extract. In addition, the pH in medium within 24 h. Fermentation of yeast extract, dextrin, cellobiose, glucose 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.

Analyses of whole cell sugars, polar lipids and fatty acid composition were performed by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). The cell sugars comprised mainly ribose, in addition to some mannose and glucose and trace amounts of galactose. In total, seven unidentified polar lipids were detected, comprising two glycolipids and three phospholipids, in addition to phosphatidylglycerol and diphosphatidylglycerol. The fatty acid content of strain IR-2 was dominated by C16:0 (38.2%), C16:1ω7c or C15:0 iso 2OH (24.5%), C14:0 (14.4%), C12:0 3OH (10.6%), C17:1iso or ante iso (4.0%) C12:0 (3.0%) and C18:0 (1.1%).

**Table 1. Differential characteristics that distinguish strain IR-2 from selected members of the phylum Fusobacteria**

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<td>40</td>
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<td>37</td>
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*Data from GenBank: ACIE00000000.2.
†Results from AN Micro plates (Biolog).

The mean H₂ production after 24 h incubation was 2.47 mM for yeast extract. In addition, the pH in medium 0.48 mM for yeast extract. In addition, the pH in medium within 24 h. Fermentation of yeast extract, dextrin, cellobiose, glucose 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, amyllopectin, cellulose, dextrin, fructose, galactose, glucose, maltose, mannitol, mannose, palatinose, starch, sucrose tartarate and yeast extract are fermented. Acetate, arabinose, cellulose, chitin, citrate, formate, 3-hydroxybutyrate, lactose, rhamnose, peptone, propionate, pyruvate, ribose, succinate, tryptone, xylose and xylose are not utilized. Oxygen and nitrate are not used as electron acceptors. The major fatty acids are C₁₆:₀, C₁₄:₀, C₁₆:₁₀₇c or C₁₅:₀ iso 2OH and C₁₂:₀ 3OH. The whole cell sugars are ribose, mannose and glucose, in addition to trace amounts of galactose.

The type strain is IR-2ᵀ (= 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


