Hyphomicrobium nitrativorans sp. nov., isolated from the biofilm of a methanol-fed denitrification system treating seawater at the Montreal Biodome

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A budding prosthecate bacterial strain, designated NL23T, was isolated from a methanol-fed denitrification system treating seawater at the Montreal Biodome, Canada. Phylogenetic analysis based on 16S rRNA (rRNA) gene sequences showed that the strain was affiliated with the genus Hyphomicrobium of the Alphaproteobacteria and was most closely related to Hyphomicrobium zavarzii with 99.4% sequence similarity. Despite this high level of 16S rRNA gene sequence similarity, DNA–DNA hybridization assays showed that strain NL23T was only distantly related to H. zavarzii ZV-622T (12%). Strain NL23T grew aerobically, but also had the capacity to grow under denitrifying conditions in the presence of nitrate without nitrite accumulation. Growth occurred at pH 7.0–9.5, with 0–1% NaCl and at temperatures of 15–35 °C. Major fatty acids were C18:1ω7c or ω6c (84.6%) and C19:0 (8.5%), and major quinones were Q8 (5%) and Q9 (95%). The complete genome of the strain was sequenced and showed a DNA G+C content of 63.8 mol%. Genome analysis predicted open reading frames (ORF) encoding the key enzymes of the serine pathway as well as enzymes involved in methylotherotrophy. Also, ORF encoding a periplasmic nitrate reductase (Nap), a nitrite reductase (Nir), a nitric oxide reductase (Nor) and a nitrous oxide reductase (Nos) were identified. Our results support that strain NL23T represents a novel species within the genus Hyphomicrobium, for which the name Hyphomicrobium nitrativorans sp. nov. is proposed. The type strain is NL23T (=ATCC BAA-2476T=LMG 27277T).

The genus Hyphomicrobium Stutzer and Hartleb 1898 is, at the time of writing, composed of nine species with validly published names (Gliesche et al., 2005): H. vulgare (Hirsch, 1989), H. aestuarii (Hirsch, 1989), H. chloromethanica (McDonald et al., 2001), H. coagulans (Hirsch, 1989) H. denitrificans (Urakami et al., 1995), H. facile (Hirsch, 1989), H. hollandica (Hirsch, 1989), H. methylovorum (Izumi et al., 1982) and H. zavarzii (Hirsch, 1989). Members of this genus are restricted facultative methylo- trophs, reproduce by budding at the tip of a polar prosthecae and are ubiquitous in water and soils, but can also be found in sewage treatment plants (Gliesche et al., 2005). Some strains are characterized by their denitrification capacities (Fesefeldt et al., 1998; Kloos et al., 1995; Timmermans & Van Haute, 1983; Urakami et al., 1995). In previous work, we isolated strain NL23T from the denitrifying biofilm of a methanol-fed denitrification system treating the seawater of the St. Lawrence mesocosm at the Montreal Biodome, Canada (Labbé et al., 2003). Analysis of the 16S rRNA gene sequence indicated that strain NL23T was affiliated with the genus Hyphomicrobium. Here, we report the taxonomic characterization of this strain.

Strain NL23T was isolated on R2A agar medium but growth on this culture medium was slow. Strain NL23T was preserved in 25% glycerol at −80 °C and routinely cultured in a modified version of Hyphomicrobium medium 337a (Atlas, 2010) (composition per litre: 1.3 g KH2PO4, 1.13 g Na2HPO4, 0.5 g (NH4)2SO4, 0.2 g MgSO4·7H2O, 3.09 mg CaCl2, 2H2O, 2.0 mg FeSO4·7H2O, 1.0 mg Na2MoO4·2H2O, 0.88 mg MnSO4·4H2O, 3 ml methanol, 0.1 mg vitamin B12, pH 7.5–8.0) at an incubation temperature of 30 °C. Unless otherwise specified, these culture conditions were used for all assays.

A summary of the main characteristics of strain NL23T compared to the other species of the genus Hyphomicrobium is presented in Table 1. When grown on modified Hyphomicrobium medium 337a agar plates [1.5% (w/v)
Table 1. Selected physiological and molecular properties of species of the genus Hyphomicrobiun

<table>
<thead>
<tr>
<th>Characteristic</th>
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<th>3</th>
<th>4</th>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>ND</td>
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<td>Catalase*</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>ND</td>
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<td>Optimal growth pH</td>
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<td>&gt;7.5</td>
<td>6.0</td>
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<td>ND</td>
<td>3</td>
<td>ND</td>
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<td>−</td>
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*Data for taxa 3–10 are from Table 5 in Urakami et al. (1995).
†Nitrate reduction was reported as negative by Gliesche et al. (2005).
‡Optimal growth temperature is indicated in parentheses when the temperature growth range was not available.
strains NL23T was positive for esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, trypsin, and naphthol-AS-BI-phosphohydrolase activities. Methanol (as a control), methylvamine hydrochloride, acetate, formate, succinate, ethanol, glycerol, peptones, aspartate, and asparagine were further tested as sole carbon sources in liquid medium. Growth was observed with methanol, methylvamine hydrochloride and formate.

The draft genome of strain NL23T was determined and a single copy of the 16S rRNA gene was found in the genome of strain NL23T (GenBank accession number JX131369) and its sequence was used to determine the phylogenetic position of strain NL23T compared to the other species of the genus Hyphomicrobium. Trees were constructed in MEGA version 5 (Tamura et al., 2011) using the neighbour-joining (Saitou & Nei, 1987) (Fig. 2), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony algorithms (Fitch, 1971) with 1000 bootstrap iterations. In all trees, strain NL23T was more closely related to H. zavarzini (99.4 % similarity) and grouped with species from Cluster I (Rainey et al., 1998), which also includes the type species of the genus, H. vulgare (98.1 % similarity), and the species H. hollandicum (98.7 %) and H. aestuarii (97.7 %) (Fig. 2). All species from Cluster I share high 16S rRNA gene sequence similarities (>97 %), although they were identified as different species based on other criteria (Hirsch, 1989; Urakami et al., 1995). DNA–DNA hybridization analysis was performed by the Identification Service of the DSMZ to evaluate the degree of relatedness between strain NL23T and its most closely related species H. zavarzini (strain ZV-622T = ATCC 27496T). The results showed a DNA–DNA relatedness value of 12 % between these two strains, which is much lower than the threshold value of 70 % defining members from the same species (Wayne et al., 1987). Previous studies have also reported low levels of DNA–DNA relatedness between species of the genus Hyphomicrobium sharing high 16S rRNA gene sequence similarities (Gliesche et al., 2005; Urakami et al., 1995), revealing the disparity between 16S rRNA gene sequence similarity and genome similarity in this bacterial genus.

In order to confirm that strain NL23T was phenotypically distinct from H. zavarzini ZV-622T (= ATCC 27496T), analysis confirmed the presence in strain NL23T of ORF encoding reductases associated with each of the four steps necessary to a complete denitrification of nitrate to gaseous nitrogen (Nap, NirK, cNor, and Nos), as previously reported by our group (Auclair et al., 2012). Interestingly, strain NL23T is the first member of the genus Hyphomicrobium in which the periplasmic nitrate reductase (Nap) gene was detected, while membrane-bound nitrate reductase (Nar) genes were found in the genomes of H. denitrificans (GenBank accession number YP_003755061) and Hyphomicrobium sp. strain MC1 (YP_004677027).

Denitrification capacities of strain NL23T were tested by cultivation of the strain under anoxic conditions in liquid medium supplemented with 14.1 mM NaNO3 (200 mg NO3−·N L−1) for 188 h (Fig. S1, available in IJSEM Online). The nitrate and nitrite concentrations in the culture medium were determined by ion chromatography using the 850 Professional IC (Metrohm). Strain NL23T had the capacity to grow under these incubation conditions and nitrate was completely eliminated without nitrite accumulation.

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In order to confirm that strain NL23T was phenotypically distinct from H. zavarzini ZV-622T (= ATCC 27496T),
relevant phenotypic tests (catalase, carbon sources, nitrate respiration, optimal growth pH and NaCl concentration) were performed in parallel experiments for both strains as previously described for strain NL23T. These assays revealed several phenotypic differences between the two strains. While strain NL23T grew exclusively with methanol, methylamine hydrochloride and formate as carbon sources, *H. zavarzinii* ZV-622T also had the capacity to utilize acetate, ethanol, glycerol, peptones and aspartate, as previously reported in the literature (Gliesche et al., 2005). Unlike strain NL23T, *H. zavarzinii* ZV-622T was catalase-negative and grew between pH 6 and 8.5 (optimum, pH 7.0). Surprisingly, *H. zavarzinii* ZV-622T did not grow at NaCl concentrations higher than 1%, although growth at NaCl concentrations up to 3% has previously been reported in the literature (Gliesche et al., 2005). This discrepancy might be related to differences in experimental conditions, although it is impossible to confirm this hypothesis because of the lack of methodological details in the cited reference. Characteristics distinguishing strain NL23T from strain ZV-622T and from the other species of the genus *Hyphomicrobium* are outlined in Table 1. These specificities indicate that strain NL23T represents a novel species of the genus *Hyphomicrobium*, for which the name *Hyphomicrobium nitrativorans* sp. nov. is proposed.

**Description of *Hyphomicrobium nitrativorans* sp. nov.**


Cells are Gram-stain-negative rods with pointed ends (oval), 0.5–0.8 × 1.5–2.0 μm, with bud forming at the tip of a prosthecæ. Colonies are small (<1 mm), white to light beige and circular after incubation at 30 °C for 10 days on modified *Hyphomicrobium* medium 337a agar (1.5 % w/v agar). Catalase- and oxidase-positive. Reduces nitrate to nitrite. Grows in the absence of oxygen with nitrate without nitrite accumulation. Grows at temperatures between 15 and 35 °C with optimal growth at 30–35 °C. Grows between pH 7.0 and 9.5, with optimum growth at pH 7.5–8.5. The optimum NaCl concentration is 0–0.5%; no growth is observed at concentrations >1.0 %. NaCl. Vitamin B12 stimulates growth but is not required. Methanol, methylamine hydrochloride and formate are used as carbon sources. The major fatty acids are C18:1ω7c or ω6c and C18:ω3c and major quinones are Q8 and Q9.

The type strain, NL23T (=ATCC BAA-2476T=LMG 27277T) was isolated from a methanol-fed denitrification system treating seawater at the Montreal Biodome, Canada.
The G+C content of the DNA of the type strain is 63.8 mol%.

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References


