Novosphingobium nitrogenifigens sp. nov., a polyhydroxyalkanoate-accumulating diazotroph isolated from a New Zealand pulp and paper wastewater

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A diazotroph capable of accumulating significant amounts of polyhydroxyalkanoate was isolated in New Zealand from a bioreactor treating nitrogen-deficient pulp and paper-mill effluent. Strain Y88T is Gram-negative, rod-shaped and positive for catalase, nitrate reductase and urease activities. The complete 16S rRNA gene sequence was most similar to those of other members of the genus Novosphingobium, the highest level of similarity (94.7 %) being found with respect to the type strain of Novosphingobium stygium. The combined phenotypic, chemotaxonomic and sequence data show that while strain Y88T belongs to the genus Novosphingobium, it is distinct from all currently recognized Novosphingobium species. Therefore, strain Y88T represents the first nitrogen-fixing species of the genus Novosphingobium, for which the name Novosphingobium nitrogenifigens sp. nov. is proposed. The type strain is Y88T (=ICMP 16470T = DSM 19370T).

The genus Sphingomonas was described by Yabuuchi et al. (1990) as comprising strictly aerobic, chemoheterotrophic, yellow-pigmented, Gram-negative, rod-shaped bacteria containing glycosphingolipids as cell-envelope components – a classification that does not take into account heterogeneity in polyamine patterns (Busse & Auling, 1988). Takeuchi et al. (1994) divided the group into four clusters on the basis of the 16S rRNA gene sequences, subsequently combining phylogenetic, chemotaxonomic and physiological analyses to divide the genus into the genera Sphingomonas, Sphingobium, Novosphingobium and Sphingopyxis (Takeuchi et al., 2001). Although Yabuuchi et al. (2002) suggested that the genus Sphingomonas should remain undivided, the genus Novosphingobium as proposed by Takeuchi et al. (2001) has been accepted by many 'sphingomonad' taxonomists (Kämpfer et al., 2002; Tiirola et al., 2005; Liu et al., 2005) because of the clear separation of Novosphingobium from the genus Sphingomonas sensu stricto demonstrated in phylogenetic and chemotaxonomic studies. The genus Novosphingobium includes a diverse group of bacteria displaying a number of unique traits that enable them to inhabit a variety of soil, sediment and aquatic environments. At the time of writing the genus Novosphingobium included 11 species: Novosphingobium aromaticivorans (Balkwill et al., 1997), Novosphingobium capsulatum (Leifson, 1962; Yabuuchi et al., 1990), Novosphingobium hassiacum (Kämpfer et al., 2002), Novosphingobium lentum (Tiirola et al., 2005), Novosphingobium pentaromativorans (Sohn et al., 2004), Novosphingobium rosa (Takeuchi et al., 1995), Novosphingobium stygium (Balkwill et al., 1997), Novosphingobium subarcticum (Nohynek et al., 1996), Novosphingobium subterraneum (Balkwill et al., 1997), Novosphingobium taihuense (Liu et al., 2005) and Novosphingobium tardaugens (Fujii et al., 2003).

We have isolated a bacterial strain from New Zealand pulp and paper-mill effluents (C/N ratio of 140:1) undergoing biological treatment in a bioreactor operated under nitrogen-limited conditions. The strain, designated Y88T, was isolated at 30 °C on nutrient agar (containing, 1−, 15 g purified agar, 3.0 g beef extract and 5.0 g peptone) with 5 mM NiCl2. The cells were Gram-negative, aerobic, non-spore-forming, non-motile rods that formed off-white/pale yellow colonies within 2–4 days on nutrient agar (lacking NiCl2). The colonies formed were circular, entire, convex and shiny in appearance. The optimum growth temperature for strain Y88T was 30 °C; growth was observed at 25–35 °C but not at 37 °C.

Total genomic DNA was extracted as described by Tiirola et al. (2002); total RNA was isolated using RNA-extraction kit according to the instruction of the manufacturer (Qiagen). The 16S rRNA gene was analysed as described by Lane (1991) and the sequence determined...
using an ABI 3100 sequencer (Applied Biosystems). 16S rRNA gene sequence alignments were performed using the CLUSTAL_X program (Thompson et al., 1997). A phylogenetic tree was constructed using the neighbour-joining method with bootstrap values based on 1000 replicates (Saitou & Nei, 1987). To determine the cellular fatty acid profile, cells were grown in nutrient broth (containing, l⁻¹, 1.0 g KH₂PO₄, 0.1 g MgSO₄, 0.1 g NaCl, 10 mg FeCl₃, 2 mg Na₂MoO₄, 5.0 g glucose, 50 mg yeast extract and 0.1 g NH₄Cl; pH 7.2 ± 0.1) concomitant with the detection of a nifH gene (encoding the iron protein of nitrogenase, a key enzyme in nitrogen fixation) in Y88ᵀ through the amplification of a 360 bp nifH fragment (with primers PolF and PolR) as described by Poly et al. (2001).

During growth on nitrogen-limited minimal medium with glucose as the sole carbon source, Y88ᵀ accumulated polyhydroxyalkanoate granules up to a level of 41 ± 16 % dry cell weight. The polyhydroxyalkanoate was composed of hydroxy-aliphatic esters of 3-hydroxybutyric acid and was extracted and analysed by gas chromatography using the method described by Riis & Mai (1988) to confirm the monomer composition. To visualize the granules, bacteria containing polyhydroxyalkanoate were centrifuged to a pellet, embedded in agar (2 %), fixed in glutaraldehyde (2 % in cacodylate buffer), dehydrated in an acetone series and embedded in Spurr’s resin. Ultrathin 120 nm sections were prepared with a diamond knife and mounted on copper support grids. Sections were stained with uranyl acetate/lead citrate and examined in a JEOL 6700 field emission scanning electron microscope using a transmission detector (see Supplementary Fig. S1 available in IJSEM Online).

The results of Microbact 24E and oxidase tests (Oxoid) showed that Y88ᵀ was positive for catalase, nitrate reductase and urease activities and negative for arginine dehydrogenase activity. Y88ᵀ was negative for indole production, acid production from glucose and assimilation of citrate, sorbitol, inositol, rhamnose, malonate, lactose, adonitol, raffinose and arabinose. Additional features that serve to differentiate strain Y88ᵀ from recognized members of the genus Novosphingobium are shown in Table 1.

The predominant fatty acids of Y88ᵀ were 18:1ω7c (58.4 %) and 16:1ω7c (17.1 %), and, consistent with

Table 1. Differential characteristics of strain Y88ᵀ from other Novosphingobium species

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Strains: 1, Y88ᵀ; 2, N. aromaticivorans SMCC F199ᵀ; 3, N. capsulatum ATCC 14666ᵀ; 4, N. hasiacum DSM 14552ᵀ; 5, N. rosa IFO 15208ᵀ; 6, N. stygium ATCC 700280ᵀ; 7, N. subarcticum HAMBI 2110ᵀ; 8, N. subterraneum DSM 12447ᵀ; 9, N. tardaugens JCM 11434ᵀ; 10, N. pentaromativorans KCTC 10454ᵀ; 11, N. lentum DSM 13663ᵀ; 12, N. taihuense JCM 12465ᵀ. Data for strains 4–12 were taken from Liu et al. (2005). +, Positive; −, negative; ( ), weakly positive; ND, not determined. All strains were positive for catalase and nitrate reduction. All strains were negative for arginine dehydrogenase, oxidase, indole production, acid production from glucose, denitrification and for assimilation of adonitol, citrate, D-mannitol, D-sorbitol, L-alanine and L-phenylalanine.
recognized *Novosphingobium* species, the only hydroxy fatty acid present was 2-OH 14:0 (15.1 %). Smaller quantities of 16:0 (4.3 %) and 17:1°v(3.0 %) fatty acids were present. The fatty acid profile of Y88T shares the same major 18:1 and 16:1 fatty acid classes as the recognized *Novosphingobium* species (Liu et al., 2005; Tiirilä et al., 2005). Y88T contained spermidine as the only polyamine compound, clearly differentiating this strain from *Sphingomonas sensu stricto* (Takeuchi et al., 2001).

Alignment of the 16S rRNA gene sequence of strain Y88T with those of members of the genus *Novosphingobium* confirmed the presence of the *Novosphingobium* signature nucleotides (52C, 134G, 359G, 593U, 987G, 990U, 1215A and 1218C; Takeuchi et al., 2001) in the isolate. Direct alignments of the 16S rRNA gene sequence of strain Y88T showed that the highest level of sequence identity occurred with respect to *N. stygium* ATCC 700280T (96 % over 1190 bp); however, the complete sequence (1423 bp) of Y88T showed a lower percentage identity with respect to other *Novosphingobium* species because of a 21 bp gap in the Y88T 16S rRNA gene sequence commencing at base number 1192 (Y88T numbering). The presence of this gap in the Y88T 16S rRNA gene sequence was confirmed by repeat sequencing of the 16S rRNA PCR products from independent preparations of Y88T DNA and by sequencing of DNA copies made from rRNA by reverse transcription from Y88T RNA extracts. The 16S rRNA gene sequence from *N. capsulatum* ATCC 14666T was used as a control sequence. The gap present in the Y88T 16S rRNA gene sequence represents a potential signature region by which Y88T could be distinguished from recognized *Novosphingobium* species and sphingomonads. The complete 16S rRNA gene sequence of Y88T most closely matched those of *N. stygium* ATCC 700280T (94.7 %) and *N. taihuense* JCM 12465T (94.5 %). The neighbour-joining tree constructed on the basis of the 16S rRNA gene sequences (Fig. 1) indicated that the closest relative of Y88T was *N. stygium* ATCC 700280T.

Analysis of the partial sequence (319 bp minus the primer regions) of the *nifH* gene of Y88T revealed that the sequence was 89 % identical to those obtained from uncultured bacteria (GenBank accession nos AF389709 and AF389707); lower levels of sequence identity were found for *nifH* sequences derived from species with validly published names. Recently, Xie & Yokota (2006) described *Sphingomonas azotifigens* as the first diazotrophic type strain belonging to the genus *Sphingomonas*. The *nifH* sequences of Y88T and *S. azotifigens* are 88.4 % identical (98 % for the corresponding amino acid sequence).

Sphingomonads have not been investigated extensively with respect to diazotrophy, or with respect to their ability to store polymer polyhydroxyalkanoate: therefore, it is not known how widespread these properties may be throughout this group. Examples of polyhydroxyalkanoate-accumulating sphingomonads include three strains of *Sphingopyxis* and *Sphingomonas* that have been shown to accumulate a polyhydroxyalkanoate content of up to 70 % (Godoy et al., 2003). Such properties are unlikely to be unique to strain Y88T, and, as sphingomonads are often isolated from low-nitrogen, high-carbon environments, diazotrophic sphingomonads are expected to be more common than is currently realized. To our knowledge, Y88T is the first type strain described as belonging to the genus *Novosphingobium* and capable of both diazotrophy and polyhydroxyalkanoate synthesis.
Three distinguishing features described by Takeuchi et al. (2001) can be used to differentiate Sphingomonas sensu stricto, Sphingobium, Novosphingobium and Sphingopyxis. These include hydroxy fatty acid profiles, polyamine patterns and nitrate reduction. Members of the genera Sphingobium and Novosphingobium contain 2-OH 14 : 0 as the only 2-hydroxy fatty acid (Takeuchi et al., 2001), although this is somewhat variable for different growth media (Yabuuchi et al., 2002). The predominant polyamine in Sphingomonas sensu stricto is sym-homospermidine, whereas members of the Novosphingobium, Sphingobium and Sphingopyxis clusters lack sym-homospermidine but contain spermidine as the main polyamine compound. Nitrate reduction was typical only for members of the Sphingobium and Novosphingobium clusters. Y88T contains 2-OH 14 : 0 as the major 2-hydroxy fatty acid component, has spermidine as the major polyamine and possesses nitrate reductase activity. These biochemical and chemotaxonomic data support the designation of Y88T as a member of the Novosphingobium cluster. Takeuchi et al. (2001) described β-galactosidase activity (which is absent from Y88T) as a phenotypic marker for the members of the Novosphingobium cluster; however, recently described Novosphingobium species (N. hassiaca, N. tardaegens, N. pentaromativorans and N. lentum) were also found to be negative for β-galactosidase activity.

From our polyphasic analysis of genotypic, phenotypic and chemotaxonomic traits we conclude that the following defining features indicate that strain Y88T represents a novel species of the genus Novosphingobium: 16S rRNA gene sequence identities below 95 %, a 21 bp signature gap in the Y88T 16S rRNA gene sequence and a positive result for urease activity. The name Novosphingobium nitrogenifigens sp. nov. is proposed for strain Y88T.

**Description of Novosphingobium nitrogenifigens sp. nov.**

Novosphingobium nitrogenifigens (ni.tro.gen.i’fi.gens. N.L. n. nitrogenum nitrogen; L. part. adj. figens fixing; N.L. part adj. nitrogenifigens referring to the ability of this organism to fix nitrogen).

Cells are Gram-negative, aerobic, non-spore-forming, non-motile rods. Colonies produced after 2–4 days cultivation on nutrient agar are off-white/pale yellow, circular, entire, convex and shiny. Growth is observed at 15–35 °C but not at 37 °C; the optimum growth temperature is 30 °C. Nitrogen-fixing occurs and polyhydroxyalkanoate granules are accumulated. Positive for catalase, nitrate reductase and urease, but negative for arginine dehydrogenase and β-galactosidase. Negative for indole production, acid production from glucose and assimilation of citrate, sorbitol, inositol, rhamnose, malonate, lactose, adonitol, raffinose and arabinose. The predominant fatty acid is 18:1ω7c (58.4 %) and the major hydroxyalkylated fatty acid is 2-ΟH 14 : 0 (15.1 %). The fatty acid profile also contains 16:1ω7c (17.1 %), 16:0 (4.3 %) and 17:1ω6c (3.0 %). Contains spermidine as the only polyamine. The 16S rRNA gene sequence of the strain matches the specific nucleotide signature bases for the genus Novosphingobium, as described by Takeuchi et al. (2001), and contains a 21 bp gap starting at base 1192 (Y88T numbering) when aligned with other Novosphingobium species.

The type strain, Y88T (=ICMP 16470T=DSM 19370T), was isolated from pulp and paper wastewater in New Zealand.

**Acknowledgements**

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**References**


