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 tardauges (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, 1−1, 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 an 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 phylo-
genetic 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,
3.0 g beef extract and 5.0 g peptone) and harvested in the
late exponential phase; whole-cell fatty acid methyl esters
were prepared and analysed commercially (MIDI Inc.)
and polyamines were extracted and analysed as described by
Busse & Auling (1988) and Busse et al. (1997).
Indirect evidence for diazotrophy in Y88T was obtained by
demonstrating sustained growth in nitrogen-limited mini-
Table 1. Differential characteristics of strain Y88T from other Novosphingobium species

| 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. tardaengus JCM 11434T; 10, N. pentaromativorans KTCC 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 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
| Characteristic | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| Assimilation of: | | | | | | | | | | | | |
| N-Acetyl-d-glucosamine | + | + | + | + | + | + | + | + | + | + | + | + |
| l-Arabinose | – | – | ( + ) | – | – | – | – | – | – | – | – | – |
| d-Cellulbiose | + | + | + | + | + | – | – | – | – | – | – | – |
| d-Galactose | + | – | – | + | – | – | – | – | – | – | – | – |
| d-Glucose | + | + | + | + | – | – | – | – | – | – | – | – |
| d-Fructose | – | – | – | + | + | – | – | – | – | – | – | – |
| d-Mannose | + | + | + | + | – | – | – | – | – | – | – | – |
| Maltose | + | + | + | + | + | + | + | + | + | + | + | + |
| d-Melibiose | – | – | – | – | – | – | – | – | – | – | – | – |
| l-Rhamnose | – | – | – | – | – | – | – | – | – | – | – | – |
| Sucrose | + | + | + | + | – | – | – | – | – | – | – | – |
| Trehalose | + | + | + | + | – | – | – | – | – | – | – | – |
| d-Xylose | – | + | + | + | + | – | – | – | – | – | – | – |
| l-Proline | – | + | + | + | + | – | – | – | – | – | – | – |
| Enzyme activity: | | | | | | | | | | | | |
| Urease | + | – | – | – | – | – | – | – | – | – | – | – |
| β-Galactosidase | – | + | + | – | + | + | + | + | – | – | – | ND |
| Gelatin hydrolysis | – | – | – | – | – | – | – | – | – | – | – | – |
recognized *Novosphingobium* species, the only hydroxy fatty acid present was 2-OH 14:0 (15.1%). Smaller quantities of 16:0 (4.3%) and 17:1ω6c (3.0%) fatty acids were present. The fatty acid profile of Y88\(^\mathrm{T}\) shares the same major 18:1 and 16:1 fatty acid classes as the recognized *Novosphingobium* species (Liu et al., 2005; Tiirola et al., 2005). Y88\(^\mathrm{T}\) 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 Y88\(^\mathrm{T}\) 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 Y88\(^\mathrm{T}\) showed that the highest level of sequence identity occurred with respect to *N. stygium* ATCC 700280\(^\mathrm{T}\) (96% over 1190 bp); however, the complete sequence (1423 bp) of Y88\(^\mathrm{T}\) showed a lower percentage identity with respect to other *Novosphingobium* species because of a 21 bp gap in the Y88\(^\mathrm{T}\) 16S rRNA gene sequence commencing at base number 1192 (Y88\(^\mathrm{T}\) numbering). The presence of this gap in the Y88\(^\mathrm{T}\) 16S rRNA gene sequence was confirmed by repeat sequencing of the 16S rRNA PCR products from independent preparations of Y88\(^\mathrm{T}\) DNA and by sequencing of DNA copies made from rRNA by reverse transcription from Y88\(^\mathrm{T}\) RNA extracts. The 16S rRNA gene sequence from *N. capsulatum* ATCC 14666\(^\mathrm{T}\) was used as a control sequence. The 16S rRNA gene sequence of Y88\(^\mathrm{T}\) was most closely matched those of *N. stygium* ATCC 700280\(^\mathrm{T}\) (94.7%) and *N. taihuense* JCM 12465\(^\mathrm{T}\) (94.5%). The neighbour-joining tree constructed on the basis of the 16S rRNA gene sequences (Fig. 1) indicated that the closest relative of Y88\(^\mathrm{T}\) was *N. stygium* ATCC 700280\(^\mathrm{T}\).

Analysis of the partial sequence (319 bp minus the primer regions) of the *nifH* gene of Y88\(^\mathrm{T}\) 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 *Sphingomomas azotifigens* as the first diazotrophic type strain belonging to the genus *Sphingomomas*. The *nifH* sequences of Y88\(^\mathrm{T}\) 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 Y88\(^\mathrm{T}\), 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, Y88\(^\mathrm{T}\) is the first type strain described as belonging to the genus *Novosphingobium* and capable of both diazotrophy and polyhydroxyalkanoate synthesis.

![Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, for strain Y88\(^\mathrm{T}\) and related taxa. The final analysis included 1377 bp; gaps and alignment uncertainties were omitted from the analysis. Numbers at nodes indicate percentages of bootstrap support (based on 1000 replicates); only values greater than 50% are shown. *Sphingomonas suberifaciens* IFO 15211\(^\mathrm{T}\) was used as the outgroup. Bar, evolutionary distance (*K*\textsubscript{nuc}) of 0.01.](http://ijs.sgmjournals.org)
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. hassiacum, N. tardoagens, N. pentoromativorans 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. 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 polyhydroxylanoate 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 hydroxylated fatty acid is 2-OH 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.

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### References


