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 Y88^T 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 Y88^T belongs to the genus *Novosphingobium*, it is distinct from all currently recognized *Novosphingobium* species. Therefore, strain Y88^T represents the first nitrogen-fixing species of the genus *Novosphingobium*, for which the name *Novosphingobium nitrogenifigens* sp. nov. is proposed. The type strain is Y88^T (=ICMP 16470^T=DSM 19370^T).

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; Tirola *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* (Tirola *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 Y88^T, 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 Y88^T was 30 °C; growth was observed at 25–35 °C but not at 37 °C.

Total genomic DNA was extracted as described by Tirola *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

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence and the *nifH* gene sequence of strain Y88^T are DQ448852 and DQ660368, respectively.

A transmission electron micrograph of Y88^T cells containing polyhydroxyalkanoate granules is available as a supplementary figure with the online version of this paper.

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, 1^{-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 Y88^T was obtained by demonstrating sustained growth in nitrogen-limited minimal medium (containing, 1^{-1} , 0.4 g KH_2PO_4 , 0.1 g K_2HPO_4 , 0.2 g MgSO_4 , 0.1 g NaCl, 10 mg FeCl_3 , 2 mg Na_2MoO_4 , 5 g glucose, 50 mg yeast extract and 0.1 g NH_4Cl ; pH 7.2 ± 0.1) concomitant with the detection of a positive acetylene-reduction assay (Sprent & Sprent, 1990), and by the detection of a *nifH* gene (encoding the iron protein of nitrogenase, a key enzyme in nitrogen fixation) in Y88^T 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, Y88^T accumulated

polyhydroxyalkanoate granules up to a level of $41 \pm 16\%$ 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 Y88^T was positive for catalase, nitrate reductase and urease activities and negative for arginine dehydrogenase activity. Y88^T 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 Y88^T from recognized members of the genus *Novosphingobium* are shown in Table 1.

The predominant fatty acids of Y88^T were 18:1 ω 7c (58.4%) and 16:1 ω 7c (17.1%), and, consistent with

Table 1. Differential characteristics of strain Y88^T from other *Novosphingobium* species

Strains: 1, Y88^T; 2, *N. aromaticivorans* SMCC F199^T; 3, *N. capsulatum* ATCC 14666^T; 4, *N. hassiacum* DSM 14552^T; 5, *N. rosa* IFO 15208^T; 6, *N. stygium* ATCC 700280^T; 7, *N. subarcticum* HAMBI 2110^T; 8, *N. subterraneum* DSM 12447^T; 9, *N. tardagens* JCM 11434^T; 10, *N. pentaromaticivorans* KCTC 10454^T; 11, *N. lentum* DSM 13663^T; 12, *N. taihuense* JCM 12465^T. 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 dehydrolase, oxidase, indole production, acid production from glucose, denitrification and for assimilation of adonitol, citrate, D-mannitol, D-sorbitol, L-alanine and L-phenylalanine.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12
Assimilation of:												
N-Acetyl-D-glucosamine	+	+	+	–	(+)	–	+	–	–	–	–	–
L-Arabinose	–	–	(+)	–	+	–	+	(+)	–	–	–	+
D-Cellobiose	+	+	+	+	+	–	–	+	–	–	–	+
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 17:1 ω 6c (3.0%) fatty acids were present. The fatty acid profile of Y88^T shares the same major 18:1 and 16:1 fatty acid classes as the recognized *Novosphingobium* species (Liu *et al.*, 2005; Tirola *et al.*, 2005). Y88^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^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^T showed that the highest level of sequence identity occurred with respect to *N. stygium* ATCC 700280^T (96% over 1190 bp); however, the complete sequence (1423 bp) of Y88^T showed a lower percentage identity with respect to other *Novosphingobium* species because of a 21 bp gap in the Y88^T 16S rRNA gene sequence commencing at base number 1192 (Y88^T numbering). The presence of this gap in the Y88^T 16S rRNA gene sequence was confirmed by repeat sequencing of the 16S rRNA PCR products from independent preparations of Y88^T DNA and by sequencing of DNA copies made from rRNA by reverse transcription from Y88^T RNA extracts. The 16S rRNA gene sequence from *N. capsulatum* ATCC 14666^T was used as a control sequence (i.e. one that does not contain this deletion). The gap present in the Y88^T 16S rRNA gene sequence represents a potential signature region by which Y88^T could be distinguished from recognized *Novosphingobium* species and sphingomonads. The complete 16S rRNA gene

sequence of Y88^T most closely matched those of *N. stygium* ATCC 700280^T (94.7%) and *N. taihuense* JCM 12465^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^T was *N. stygium* ATCC 700280^T.

Analysis of the partial sequence (319 bp minus the primer regions) of the *nifH* gene of Y88^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 *Sphingomonas azotifigens* as the first diazotrophic type strain belonging to the genus *Sphingomonas*. The *nifH* sequences of Y88^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 the storage 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^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^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^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^T was used as the outgroup. Bar, evolutionary distance (K_{nuc}) of 0.01.

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. Y88^T 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 Y88^T as a member of the *Novosphingobium* cluster. Takeuchi *et al.* (2001) described β -galactosidase activity (which is absent from Y88^T) as a phenotypic marker for the members of the *Novosphingobium* cluster; however, recently described *Novosphingobium* species (*N. hassiacum*, *N. tardaugens*, *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 Y88^T represents a novel species of the genus *Novosphingobium*: 16S rRNA gene sequence identities below 95 %, a 21 bp signature gap in the Y88^T 16S rRNA gene sequence and a positive result for urease activity. The name *Novosphingobium nitrogenifigens* sp. nov. is proposed for strain Y88^T.

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 18:1 ω 7c (58.4 %) and the major hydroxylated fatty acid is 2-OH 14:0 (15.1 %). The fatty acid profile also contains 16:1 ω 7c (17.1 %), 16:0 (4.3 %) and 17:1 ω 6c (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 (Y88^T numbering) when aligned with other *Novosphingobium* species.

The type strain, Y88^T (=ICMP 16470^T=DSM 19370^T), was isolated from pulp and paper wastewater in New Zealand.

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