Sphingosinicella microcystinivorans gen. nov.,sp.nov,a microcystin-degrading bacterium

Proposal of *Sphingosinicella microcystinivorans* gen. nov., sp. nov., a cyanotoxin microcystin-degrading bacterium

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Running title: *Sphingosinicella microcystinivorans* gen. nov., sp. nov.

The GenBank/EMBL/DDBJ/ accession number for the 16S rRNA gene sequence of strain $Y2^T$, MDB2 and MDB3 are AB084247, AB219940 and AB219941.

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Abstract

Three strains of cyanobacterial hepatotoxin microcystin-degrading bacteria, $Y2^T$, MDB2 and MDB3, were isolated from a eutrophic lake, Lake Suwa, and Tenryu River, Japan, and characterized. These strains were aerobic chemoorganotrophic and had gram-negative, non-spore-forming, motile by means of single polar flagella, rod-shaped cells. Yellow-pigmented colonies were formed on nutrient agar media. These strains assimilated only citrate among the organic compounds tested as carbon sources. The G+C content of genomic DNA ranged from 63.6 to 63.7 mol%. A phylogenetic analysis based on 16S rRNA gene sequences indicated that the new isolates formed a tight cluster within the family Sphingomonadaceae but were clearly separate from any established genera of this family, i.e., *Sphingomonas, Sphingobium, Novosphingobium* and *Sphingopyxis*. Sequence similarities between the new isolates and type strains of established genera ranged from 90.9 to 94.9%. Chemotaxonomic and phenotypic data supported that these

strains were members of the family Sphingomonadaceae. The major components of cellular fatty acids were 18:1ω7c (36-41%) and 16:1ω7c (33-36%). Hydroxy fatty acid was mainly 2-OH 14:0 (11-13%) and 3-OH fatty acids were absent. Glycosphingolipids were detected. Ubiquinone-10 and homospermidine were present as the major quinine and polyamine, respectively. Thus, we propose to classify the three strains as a new genus and species of the family Sphingomonadaceae with the name *Sphingosinicella microcystinivorans*. The type strain is Y2^T (=KTCT 12019, =JCM13185)

Hepatotoxin microcystins, produced by several members of cyanobacteria belonging to the genera *Microcystis, Anabaena, Nostoc* and *Oscillatoria* (=*Planktothrix*), may causes serious disease in humans and animals (Jochimsen *et al.*, 1998; Kuiper-Goldman *et al.*, 1999). A microcystin-degrading bacterium designated strain Y2^T was isolated using diluted Nutrient Agar (Nissui Pharmaceutical, Japan) from a eutrophic lake, Lake Suwa, Japan, during the blooming period of toxic *Microcystis* (Park *et al.*, 2001). This strain was able to degrade microcystin-RR, -YR, –LR, and its isomer [6(Z)-Adda microcystin-LR] and to grow in inorganic media containing microcystin as the sole carbon sources as well as in diluted nutrient broth (Park *et al.*, 2001). A phylogenetic analysis of strain Y2^T based on 16S rRNA gene sequences revealed that it represents a deeply branching lineage within the cluster of the sphingomonads, including the genera *Blastomonas*, *Novosphingobium*, *Sphingobium*, *Sphingopyxis* and *Sphingomonas* (Park *et al.*, 2001). Later, we isolated two other strains (MDB2 and MDB3) of microcystin-degrading bacteria from Tenryu River in Japan. These strains were phylogenetically similar to strain Y2^T. In the present study, we describe the taxonomic

properties of these three strains of microcystin degraders and propose to classify them into a novel genus and species with the name *Sphingosinicella microcystinivorans*.

General cell morphology, gram reaction, spore forming and motility by means of flagella were studied under an Olympus light microscope (U-LH 1000) by NCIMB Japan Co. (Shizuoka, Japan). Colony shape was observed after the cells were incubated at 30°C for 48 hr on Nutrient Agar (Oxoid, England, UK). Biochemical tests were performed by NCIMB Japan Co. (Shizuoka, Japan) using an API 20NE kit according to the manufacturer's instructions (API bioMerieux), as well as by conventional tests, which were for activity of catalase and oxidase, gas/acid production from glucose and oxidation/fermentation from glucose, as described previously (Barrow and Feltham, 1993). The analysis of cellular fatty acids was performed by NCIMB Japan Co. (Shizuoka, Japan) using the Sherlock Microbial Identification system (version 5.0, MIDI, DE, USA) according to the manufacturer's instructions. Cellular fatty acids were extracted from cells grown on Tripticase Soy (SCD) Agar (Becton Dickinson, NJ, USA) at 30 °C for 24 hours, and analyzed at methyl esters. Glycosphingolipids were analyzed by TLC as described previously (Takeuchi *et al.*, 2001). Respiratory quinone profiles were studied as described previously (Hiraishi *et al.*, 1996; Iwasaki & Hiraishi 1998). Polyamines were analyzed as previously reported (Hamana & Takeuchi, 1998; Hamana *et al.*, 2003). Genomic DNA was extracted and purified by the phenol extraction method as described previously (Saitou & Miura, 1963), and DNA base composition was determined by the HPLC method of Katayama-Fujiwara *et al.* (1984). After genomic DNA was prepared by the PrepMan method (Applied Biosystems, CA, USA), 16S rRNA genes were amplified by PCR and sequenced with a MicroSeq® Full 16S rDNA Bacterial Sequencing kit (Applied

Biosystems) by NCIMB Japan Co. (Shizuoka, Japan). Sequence similarities were studied using the BLAST program (Altschul *et al.*, 1997). Relative sequences including type strains of established genera of the family Sphingomonadaceae were obtained from the GenBank/EMBL/DDBJ. Multiple alignments of sequence data, calculation of evolutionary distances and construction of a neighbor-joining phylogenetic tree (Saitou & Nei, 1987) were performed with the Clustal W program (Thompson *et al.*, 1994) using bootstrap values based on 1000 replications.

Strains Y2^T, MDB2 and MDB3 were gram-negative, non-spore-forming rods measuring 0·6–0·7 μm in width and 0·8–1·0 μm in length. Cells were motile by means of single polar flagella. All these strains formed yellow colonies on Nutrient Agar (Oxoid, England, UK) after 48 hr incubation at 30 °C. The temperature range for growth was 10-37 °C and optimum temperature was 30 °C. No growth occurred at 45 °C. The pH range for growth was 7–9. Strains Y2^T, MDB2 and MDB3 were strictly aerobic and chemoorganotrophic. They exhibited positive reactions for oxidase and catalase but negative reactions in the oxidation/fermentation test and gas/acid production test with glucose. Other physiological and biochemical characteristics of strains Y2^T, MDB2 and MDB3 were compared with those of the type strains of the phylogenetically related genera, *Sphingomonas, Sphingobium, Novosphingobium* and *Sphingopyxis* (Table 1.). As the result of assimilation test using twelve carbon sources, strains Y2^T, MDB2 and MDB3 were shown to only assimilate citrate. These strains did not assimilate glucose, L-arabinose, D-mannose, *N*-acetyl-D-glucosamine, maltose, gluconate, *n*-caproate, adipate, DL-malic acid, and phenylacetate. Strains Y2^T, MDB2 and MDB3 exhibited all negative reactions for other phenotypic tests: nitrate reduction, β-galactosidase, aesculin hydrolysis,

urease, gelatin hydrolysis, indole production, glucose fermentation, arginine dihydrolase. Negative reaction of nitrate reduction, which was proposed one of the phenotypic makers to distinguish four genera of the family Sphingomonadaceae (Takeuchi *et al.*, 2001), was the character of *Sphingobium, Novosphingobium* and some of *Sphingomonas*.

As shown in Table 2, the major fatty acids of the three strains were 18:1ω7c (36–41%) and 16:1ω7c (33–36%). The minor compositions of fatty acids were 16:0 (7–8%), 16:1 ω5c (3%) and 14:0 (1–2%). The main component of hydroxy fatty acids was 2-OH 14:0 (11-13%), and 3-OH fatty acids were absent. The analysis of the lipid extracts by thin-layer chromatography revealed the presence of glycosphingolipids in all three strains. The major respiratory quinone was Q-10. The polyamine detected was homospermidine (1.5 μmol (g wet cell)$^{-1}$) as same as genus *Sphingomonas*. The G+C content of the three strains ranged from 63·6 to 63·7 mol%.

The 16S rRNA gene sequences of strains Y2^{T}, MDB2 and MDB3 determined by using MicroSeq® Full 16S rDNA Bacterial Sequencing kit were a continuous stretch of 1449, 1482 and 1482 bp, respectively. The three strains had a sequence similarity of 99·9% to each other, suggesting that they form a genetically coherent group at the species level. Homology search of the sequences using the BLAST program indicated that the closest relatives of our strains were unidentified strains 7CY (99·5%, AB076083, Ishii *et al.*, 2004), B9 (99·3%, AB159609, Harada *et al.*, 2004) and IC075 (99·3%, AB196249, Inoue *et al.*, 2005). Strains 7CY and B9 were also microcystin-degrading bacteria isolated independently from Lake Suwa. The microcystins-degrading process of strains 7CY and B9 were quite similar to that of strain Y2^{T}, as several common degradation products

were detected (Park *et al.*, 2001; Harada *et al.*, 2004; Ishii *et al.*, 2004). Saito *et al.* (2003) reported that strain Y2^T possessed one of microcystin hydrolytic enzyme gene, *mlrA*, to open cyclic peptide of microcystins (Bourne *et al.*, 2001) . These findings suggest that strains Y2^T, 7CY and B9 were identical. Strain IC075 was able to degrade carbazole, which was one of aromatic compounds and similar structure to dioxins. Although there has been no report on the capability of strain IC075 to degrade microcystin, a high sequence similarity between strains Y2^T and IC075 implied the potential ability of the latter strain to degrade microcystin. A phylogenetic tree based on 16S rRNA gene sequences revealed that strains Y2, MDB2 and MDB3 formed a distinct clade together with strains 7CY, B9 and IC075 within the family Sphingomonadaceae (Kosako *et al.* 2000). However, this clade was separate from any of the established genera of this family, e.g., *Sphingomonas, Sphingobium*, *Novosphingobium* and *Sphingopyxis* (Fig. 1). For example, strains Y2^T, MDB2 and MDB3 showed a sequence similarity of 90·9–94·4% to the type strains of the respective type species.

To find nucleotide signatures specific to 16S rRNA of the four genera of the family Sphingomonadaceae (Takeuchi *et al.*, 2001), we aligned the sequences of strains Y2^T, MDB2, MDB3, 7CY, B9 and IC075. The nucleotide signatures specific to the 16S rRNAs of strains Y2^T, MDB2 and MDB3 were the same as those of the genus *Sphingomonas* sensu stricto reported by Takeuchi *et al.* (2001), i.e., C:G at position 52:359, G at position 134, G at position 593, G:C at position 987:1218, U:G at position 990:1215 (in *Escherichia coli* numbering (Brosius *et al.* 1978)). The same nucleotide signatures were found in the other microcystin degraders, strains 7CY (Ishii *et al.*, 2004) and B9 (Harada *et al.*, 2004), and a carbazole-utilizing bacterium, strain IC075 (Inoue *et al.*, 2005).

As described above, the phylogenetic data clearly have demonstrated that strains Y2^T, MDB2 and MDB3 are members of the family Sphingomonadaceae. However, since strains Y2^T, MDB2 and MDB3 form a distinct phylogenetic cluster within this family, it is difficult to allocate them to any of the previously described genera, *Sphingomonas, Sphingobium, Novosphingobium* and *Sphingopyxis* (Fig.1). Similarities of 16S rRNA gene sequence between strains Y2^T, MDB2, MDB3 and type strains of established genera were low, ranging from 90.9 to 94.9%. Takeuchi *et al.* (2001) reported that the four genera of the family Sphingomonadaceae were separated approximately <95% sequence similarity. Chemotaxonomic and phenotypic data support that these strains are members of the family Sphingomonadaceae (Table 1, 2). Glycosphingolipids and ubiquinone-10 were present. Strains Y2^T, MDB2, MDB3 contained 18:1ω7c and 16:1ω7c as the dominant fatty acids and 2-OH 14:0 as the major hydroxyl fatty acid (Takeuchi *et al.*, 1993; Kämpfer *et al.*, 1997; Takeuchi *et al.*, 2001; Tiirola *et al.*, 2005). And 3-OH fatty acids were absent (Takeuchi *et al.*, 1993) (Table 2). The polyamine of the microcystin-degrading strains was homospermidine, as was the case in the genus *Sphingomonas sensu stricto*, whereas all other genera noted above contained spermidine (Takeuchi *et al.*, 2001; Hamana *et al.*, 2003). The ability to reduce nitrate was absent in our strains as well as in *Sphingobium* and *Sphingopyxis* strains.

By a combination of a number of chemotaxonomic and phenotypic characteristics listed above (see Table 1 and 2), together with the phylogenetic information of distinct clade within the family Sphingomonadaceae and low sequence similarity (<95%) to related genera, it is most appropriate to conclude that novel microcystin-degrading strains studied should be classified in a novel genus and novel species of the family Sphingomonadaceae.

Thus, the name *Sphingosinicella microcystinivorans* gen. nov., sp. nov. is proposed for the three strains.

Description of *Sphingosinicella* gen. nov.

Sphingosinicella (Sphin.go.si.ni.cel'la. Gr. gen. n. sphingos, of sphinx (from the mysteries sphingosine presented to early observers); N.L. n. sphingosinum, sphingosine; L. fem. n. cella, a store-room and in biology a cell; N.L. fem. n. *Sphingosinicella*, sphingosine-containing cell)

Cells are gram-negative, non-spore-forming, and motile by means of polar flagella. rod-shaped cells. Colonies are yellow. Strictly aerobic and chemoorganotrophic. Catalase and oxidase positive. Nitrate is not reduced. The major fatty acids are 18:1 ω7c and 16:1 ω7c. 2-hydroxy fatty acid is present with 2-OH 14:0 predominating. 3-hydroxy fatty acid is absent. Glycosphingolipids are produced. Respiratory quinone is predominantly Q-10. Homospermidine is the major polyamine component as same as the genus *Sphingomonas*. The DNA G+C content is 63·6-63·7 mol%. The phylogenetic position is in the family of Sphingomonadaceae. The characteristic 16S rRNA signatures are the same as the genus *Sphingomonas*, 52:359(C:G), 134(G), 593(G), 987:1218(G:C) and 990:1215(U:G). The type species is *Sphingosinicella microcystinivorans*.

Description of *Sphingosinicella microcystinivorans* sp. nov.

Sphingosinicella microcystinivorans (mi.cro.cys.ti.ni.vo'rans. *microcystinivorans* microcystin-degrading)

Cell size is 0·3-0·7x0·6-1·0μm. Citrate is only assimilated. Nitrate is not reduced to nitrite.

Negative reactions are: hydrolysis of aesculin, gelatin and urease, activity of β-galactosidase, indole production, glucose fermentation, arginine dihydrolase, and assimilation of glucose, L-arabinose, D-mannose, *N*-acetyl-D-glucosamine, maltose, gluconate, *n*-caproate, adipate, DL-malic acid and phenylacetate. Glycosphingolipids are produced. Respiratory quinone is predominantly Q-10. Major fatty acids are 18:1 ω7c (33-36%) and 16:1 ω7c (36-41%). As minor components, 16:0 (7-8%), 16:1 ω5c (3%) and 14:0 (1-2%) are produced. Major 2-hydroxy fatty acid is 2-OH 14:0 (11-13%). As minor component, 2-OH 16:0 (1%) is produced. Polyamine is homospermidine [1.5 μmol (g wet cell)$^{-1}$]. The DNA G+C content is 63·6-63·7 mol%. Isolated from toxic *Microcystis* blooming lake, Lake Suwa, Japan.

The type strain is strain Y2^{T} (=KTCT 12019, =JCM13185).

The accession number for the 16S rRNA gene sequence of the type strain is AB084247.

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Table 1. Biochemical characteristics of strain Y2^T, MDB2, MDB3 and several type strains of *Sphingomonas, Sphingobium, Nobosphingobium* and *Sphingopyxis.*

Data for strains 4, 7-9 are from Takeuchi *et al*.(2001), and data for strain 5, 6 are from Ushiba *et al.* (2003).
All strains were positive for assimilation of citrate. All strains were negative for assimilation of phenylacetate, urease activity, gelatin hydrolysis, indole production, glucose fermentation, and arginine dihydrolase.

Characteristic	1 strain Y2^T	2 strain MDB2	3 strain MDB3	4 *Sphingomonas adhaesiva* IFO15099^T	5 *Sphingomonas paucimobilis* IFO13935^T	6 *Sphingobium yanoikuyae* IFO15102^T	7 *Novosphingobium capsulatum* IFO12533^T	8 *Sphingopyxis terrae* IFO15098^T	9 *Sphingopyxis macrogoltabida* IFO15033^T
Assimilation of									
Glucose	-	-	-	-	+	+	-	-	-
L-Arabinose	-	-	-	+	+	+	+	+	+
D-Mannose	-	-	-	+	+	-	+	+	+
D-Mannitol	-	-	-	+	-	-	+	+	+
N-Acetyl-D-glucosamine	-	-	-	+	+	+	+	+	+
Maltose	-	-	-	+	+	+	+	+	+
Gluconate	-	-	-	+	-	+	+	+	+
n-caproate	-	-	-	+	-	-	+	+	+
Adipate	-	-	-	+	-	-	+	+	+
DL-malic acid	-	-	-	+	+	+	+	+	+
Citrate	+	+	+	+	+	+	+	+	+
Phenylacetate	-	-	-	-	-	-	-	-	-
Activity of									
Nitrate reduction	-	-	-	-	+	-	+	-	-
β-Galactosidase	-	-	-	+	+	+	+	-	+
Aesculin hydrolysis	-	-	-	+	+	+	+	-	+
Urease	-	-	-	-	-	-	-	-	-
Gelatin hydrolysis	-	-	-	-	-	-	-	-	-
indole production	-	-	-	-	-	-	-	-	-
Glucose fermentation	-	-	-	-	-	-	-	-	-
Arginine dihydrolase	-	-	-	-	-	-	-	-	-

Table 2. Major fatty acid composition of strain Y2^{T}, MDB2 and MDB3.

											2-OH					ISO 3-OH
	12:0	14:0	16:0	16:1 w5c	16:1 w7c	17:1 w6c	18:0	18:1 w5c	18:1 w7c	11methyl 18:1w7c	12:0	14:0	15:0	16:0	16:1	16:0
Strain Y2^{T}		1	8	3	34	t	t	1	38	t	t	12	t	1	t	t
Strain MDB2		2	7	3	33	t	t	1	41			11		1		t
Strain MDB3	t	2	7	3	36	1	t	1	36	t	t	13	t	1	t	t

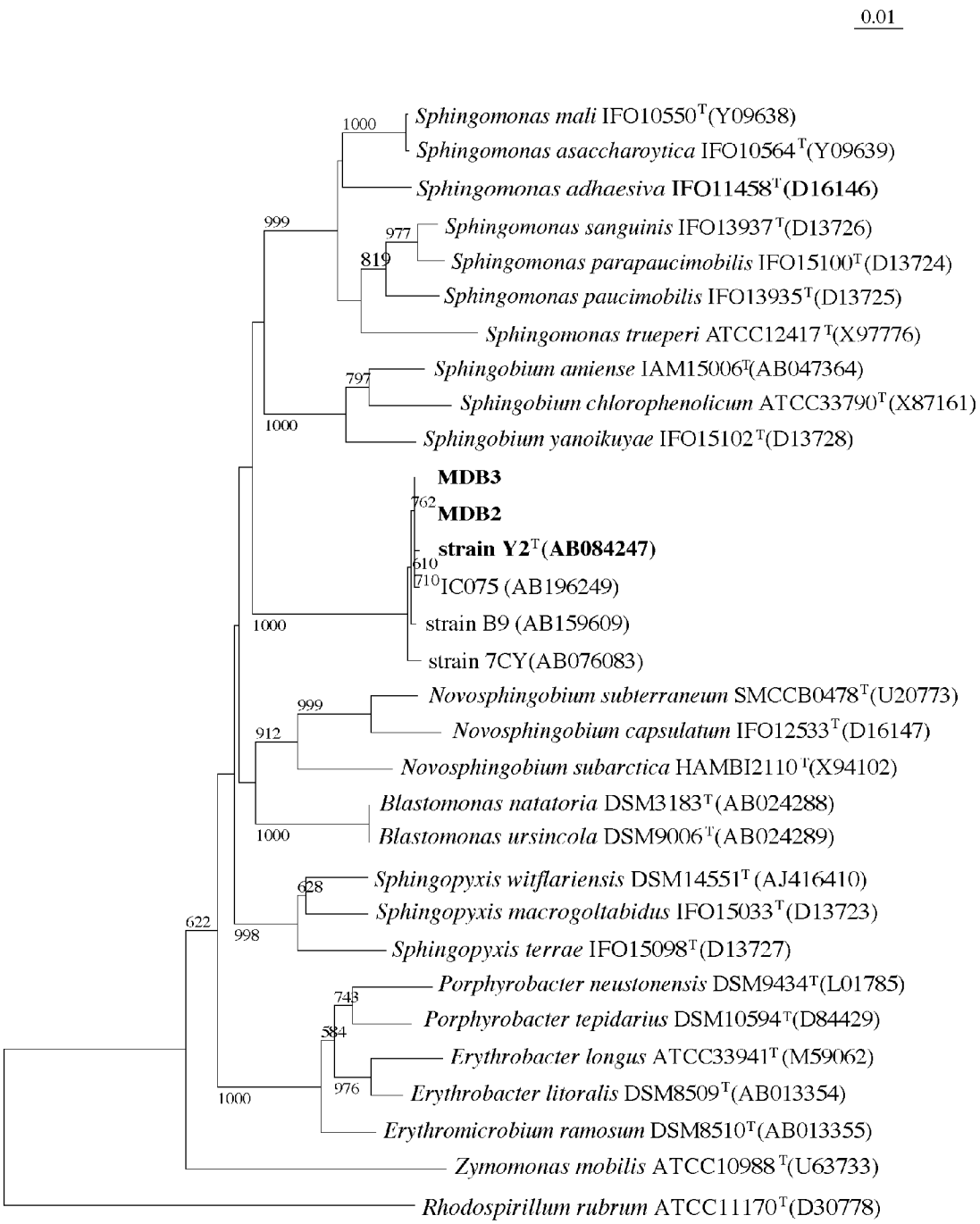
Values are percentages of total fatty acid content.

t: trace (<1%)

Legend

Figure 1

Distance matrix tree showing phylogenetic relationships between strains Y2^T, MDB2 and MDB3 (bold) and the type species of representative genera of the family Sphingomonadaceae. The sequence of *Rhodospirillum rubrum* was used as an outgroup to root the tree. The phylogenetic tree was constructed by the neighbor-joining method (Saitou & Nei 1987). Bootstrap values with 1000 trials are shown at branching points of interest. Scale bar=1% nucleotide substitution.


0.01
Sphingomonas mali IFO10550T(Y09638)
Sphingomonas asaccharoytica IFO10564T(Y09639)
Sphingomonas adhaesiva IFO11458T(D16146)
Sphingomonas sanguinis IFO13937T(D13726)
Sphingomonas parapaucimobilis IFO15100T(D13724)
Sphingomonas paucimobilis IFO13935T(D13725)
Sphingomonas trueperi ATCC12417T(X97776)
Sphingobium amiense IAM15006T(AB047364)
Sphingobium chlorophenolicum ATCC33790T(X87161)
Sphingobium yanoikuyae IFO15102T(D13728)
MDB3
MDB2
strain Y2T(AB084247)
IC075 (AB196249)
strain B9 (AB159609)
strain 7CY(AB076083)
Novosphingobium subterraneum SMCCB0478T(U20773)
Novosphingobium capsulatum IFO12533T(D16147)
Novosphingobium subarctica HAMBI2110T(X94102)
Blastomonas natatoria DSM3183T(AB024288)
Blastomonas ursincola DSM9006T(AB024289)
Sphingopyxis witflariensis DSM14551T(AJ416410)
Sphingopyxis macrogoltabidus IFO15033T(D13723)
Sphingopyxis terrae IFO15098T(D13727)
Porphyrobacter neustonensis DSM9434T(L01785)
Porphyrobacter tepidarius DSM10594T(D84429)
Erythrobacter longus ATCC33941T(M59062)
Erythrobacter litoralis DSM8509T(AB013354)
Erythromicrobium ramosum DSM8510T(AB013355)
Zymomonas mobilis ATCC10988T(U63733)
Rhodospirillum rubrum ATCC11170T(D30778)
1000
999
977
819
797
1000
762
610
710
1000
999
912
1000
628
622
998
743
584
976
1000