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

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Three strains of bacteria that degrade the cyanobacterial hepatotoxin microcystin, Y2^T, MDB2 and MDB3, were isolated from a eutrophic lake, Lake Suwa, and the Tenryu River, Japan, and characterized. These strains were aerobic and chemo-organotrophic and their cells were Gram-negative, non-spore-forming rods, motile by means of single polar flagella. Yellow-pigmented colonies were formed on nutrient agar media. The 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%. 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 established genera of this family, e.g. *Sphingomonas*, *Sphingobium*, *Novosphingobium* and *Sphingopyxis*; sequence similarities between the new isolates and type strains from established genera ranged from 90·9 to 94·9 %. Chemotaxonomic and phenotypic data supported the conclusion that these strains were members of the family *Sphingomonadaceae*. The major components of the cellular fatty acids were 18 : 1ω7*c* (36–41 %) and 16 : 1ω7*c* (33–36 %). Hydroxy fatty acids were 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, it is proposed that the three strains represent a new genus and species of the family *Sphingomonadaceae* with the name *Sphingosinicella microcystinivorans* gen. nov., sp. nov. The type strain is Y2^T (=KCTC 12019^T=JCM 13185^T).

The hepatotoxic microcystins, produced by several members of the cyanobacterial 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) 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 and -LR and its isomer 6(*Z*)-Adda microcystin-LR and to grow in inorganic media containing microcystin as the sole carbon source 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

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The GenBank/EMBL/DDBJ/accession numbers for the 16S rRNA gene sequences of strains Y2^T, MDB2 and MDB3 are AB084247, AB219940 and AB219941.

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 the 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 in a novel genus and species.

General cell morphology, Gram reaction, spore formation and motility by means of flagella were studied under an Olympus light microscope (U-LH 1000) by NCIMB Japan (Shizuoka, Japan). Colony shape was observed after the cells were incubated at 30 °C for 48 h on nutrient agar (Oxoid). Biochemical tests were performed by NCIMB Japan using an API 20NE kit according to the manufacturer's instructions (API bioMérieux) and by conventional tests for activity of catalase and oxidase, gas/acid production from glucose and oxidation/fermentation from glucose, as described previously (Barrow & Feltham, 1993). Analysis of cellular fatty acids was performed by NCIMB Japan using the Sherlock Microbial Identification system (version 5.0; MIDI Inc.) according to the manufacturer's instructions. Cellular fatty acids were extracted from cells grown on trypticase soy (SCD) agar (Becton Dickinson) at 30 °C for 24 h and analysed as methyl esters. Glycosphingolipids were analysed 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 analysed as previously reported (Hamana & Takeuchi, 1998; Hamana *et al.*, 2003). Genomic DNA was extracted and purified by the phenol extraction method as described previously (Saito & Miura, 1963) and DNA base composition was determined by the HPLC method of Katayama-Fujimura *et al.* (1984). After genomic DNA was prepared by the PrepMan method (Applied Biosystems), 16S rRNA genes were amplified by PCR and sequenced with a MicroSeq Full 16S rDNA Bacterial Sequencing kit (Applied Biosystems) by NCIMB Japan. Sequence similarities were studied using the BLAST program (Altschul *et al.*, 1997). Related sequences including type strains of established genera of the family *Sphingomonadaceae* were obtained from GenBank/EMBL/DDBJ. Multiple alignments of sequence data, calculation of evolutionary distances and construction of a neighbour-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 three strains formed yellow colonies on nutrient agar (Oxoid) after 48 h incubation at 30 °C. The temperature range for growth was 10–37 °C and the optimum temperature was 30 °C. No growth occurred at 45 °C. The pH range for growth was 7–9. The strains were strictly aerobic and chemo-organotrophic. 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 type strains of the phylogenetically related genera *Sphingomonas*, *Sphingobium*, *Novosphingobium* and *Sphingopyxis* (Table 1). In assimilation tests using 12 carbon sources, strains Y2^T, MDB2 and MDB3 were shown to assimilate citrate only. The strains did not assimilate glucose, L-arabinose, D-mannose, *N*-acetyl-D-glucosamine, maltose, gluconate, n-caproate, adipate, DL-malic acid or phenylacetate. Strains Y2^T, MDB2 and MDB3 exhibited negative reactions for all other phenotypic tests: nitrate reduction, β-galactosidase, aesculin hydrolysis, urease, gelatin hydrolysis, indole production, glucose fermentation and arginine dihydrolase. A negative reaction for nitrate reduction, which was proposed as a phenotypic marker to distinguish the four genera of the family *Sphingomonadaceae* (Takeuchi *et al.*, 2001), was characteristic of *Sphingobium*, *Novosphingobium* and some species of *Sphingomonas*.

As shown in Table 2, the major fatty acids of the three strains were 18 : 1ω7*c* (36–41 %) and 16 : 1ω7*c* (33–36 %). Minor fatty acids were 16 : 0 (7–8 %), 16 : 1ω5*c* (3 %) and 14 : 0 (1–2 %). The main component of the hydroxy fatty acids

Table 1. Biochemical characteristics of strains Y2^T, MDB2, MDB3 and related type strains

Strains: 1, strain Y2^T (strains MDB2 and MDB3 showed identical results); 2, *Sphingomonas adhaesiva* IFO 15099^T; 3, *Sphingomonas paucimobilis* IFO 13935^T; 4, *Sphingobium yanoikuyae* IFO 15102^T; 5, *Novosphingobium capsulatum* IFO 12533^T; 6, *Sphingopyxis terrae* IFO 15098^T; 7, *Sphingopyxis macrogoltabida* IFO 15033^T. Data in columns 2 and 5–7 are from Takeuchi *et al.* (2001) and data in columns 3 and 4 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	2	3	4	5	6	7
Assimilation of:							
Glucose	−	−	+	+	−	−	−
L-Arabinose	−	+	+	+	+	+	+
D-Mannose	−	+	+	−	+	+	+
D-Mannitol	−	+	−	−	+	+	+
N-Acetyl-D-glucosamine	−	+	+	+	+	+	+
Maltose	−	+	+	+	+	+	+
Gluconate	−	+	−	+	+	+	+
n-Caproate	−	+	−	−	+	+	+
Adipate	−	+	−	−	+	+	+
DL-Malic acid	−	+	+	+	+	+	+
Nitrate reduction	−	−	+	−	+	−	−
β-Galactosidase	−	+	+	+	+	−	+
Aesculin hydrolysis	−	+	+	+	+	−	+

Table 2. Major fatty acids of strains Y2^T, MDB2 and MDB3

Values are percentages of total fatty acid content. tr, Trace (<1 %); −, not detected.

Fatty acid	Y2^T	MDB2	MDB3
12:0	−	−	tr
14:0	1	2	2
16:0	8	7	7
16:1ω5*c*	3	3	3
16:1ω7*c*	34	33	36
17:1ω6*c*	tr	tr	1
18:0	tr	tr	tr
18:1ω5*c*	1	1	1
18:1ω7*c*	38	41	36
11-Methyl 18:1ω7*c*	tr	−	tr
2-OH 12:0	tr	−	tr
2-OH 14:0	12	11	13
2-OH 15:0	tr	−	tr
2-OH 16:0	1	1	1
2-OH 16:1	tr	−	tr
iso 3-OH 16:0	tr	tr	tr

was 2-OH 14:0 (11–13 %), and 3-OH fatty acids were absent. Analysis of lipid extracts by TLC 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 cells)$^{-1}$], as reported for the genus *Sphingomonas*. The DNA 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 were continuous stretches of 1449, 1482 and 1482 bp, respectively. The three strains showed 99·9 % sequence similarity to each other, suggesting that they form a genetically coherent group at the species level. Similarity searches with the sequences using the BLAST program indicated that the closest relatives of our strains were unidentified strains 7CY (99·5 %; GenBank accession no. 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 microcystin-degrading processes 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)

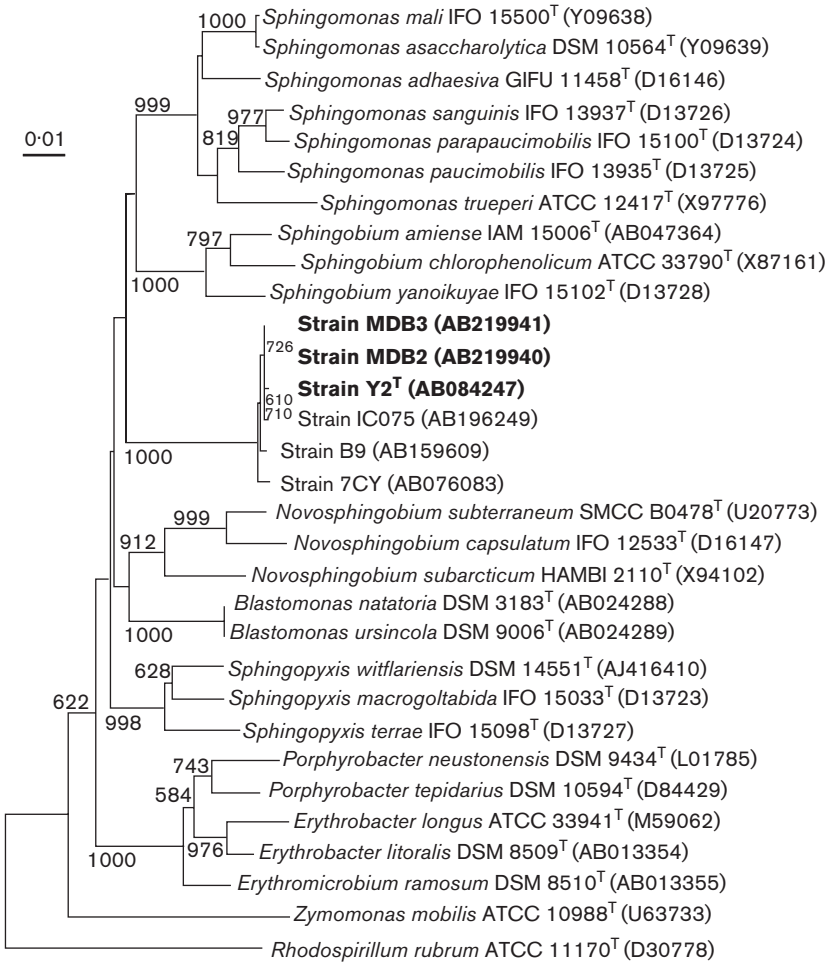

Fig. 1. Distance-matrix tree based on 16S rRNA gene sequences showing phylogenetic relationships between strains Y2^T, MDB2 and MDB3 and the type species of representative genera of the family *Sphingomonadaceae*. The sequence of *Rhodospirillum rubrum* ATCC 11170^T was used as an outgroup to root the tree. The phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987). Bootstrap values from 1000 trials are shown at branch points of interest. Bar, 1 % nucleotide substitution.

reported that strain Y2^T possessed a gene, *mlrA*, that encodes a hydrolytic enzyme to open the cyclic peptide of microcystins (Bourne *et al.*, 2001). These findings suggest that strains Y2^T, 7CY and B9 are highly similar. Strain IC075 was able to degrade carbazole, which is an aromatic compound similar in structure to dioxins. Although there has been no report of the ability of strain IC075 to degrade microcystin, the high 16S rRNA gene sequence similarity between strains Y2^T and IC075 implies that the latter strain may be able to degrade microcystin. A phylogenetic tree based on 16S rRNA gene sequences revealed that strains Y2^T, 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, in particular *Sphingomonas*, *Sphingobium*, *Novosphingobium* and *Sphingopyxis* (Fig. 1); strains Y2^T, MDB2 and MDB3 showed 16S rRNA gene sequence similarity of 90·9–94·4 % to the type strains of the respective type species.

To find nucleotide signatures specific to the 16S rRNA of the genera of the family *Sphingomonadaceae* (Takeuchi *et al.*, 2001), we aligned the sequences of strains Y2^T, MDB2, MDB3, 7CY, B9 and IC075. 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 and U : G at position 990 : 1215 (*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 the carbazole-utilizing strain IC075 (Inoue *et al.*, 2005).

As described above, the phylogenetic data demonstrate clearly 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 (Fig. 1). 16S rRNA gene sequence similarities between strains Y2^T, MDB2, MDB3 and the type strains of species of established genera were low, ranging from 90·9 to 94·9 %. Takeuchi *et al.* (2001) reported that the genera of the family *Sphingomonadaceae* were separated at approximately $<$95 % 16S rRNA gene sequence similarity. Chemotaxonomic and phenotypic data support the conclusion that these strains are members of the family *Sphingomonadaceae* (Tables 1 and 2). Glycosphingolipids and ubiquinone-10 were present. Strains Y2^T, MDB2 and MDB3 contained 18 : 1ω7*c* and 16 : 1ω7*c* as the dominant fatty acids and 2-OH 14 : 0 as the major hydroxy fatty acid (Takeuchi *et al.*, 1993, 2001; Kämpfer *et al.*, 1997; 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 for 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 from our strains as well as from *Sphingobium* and *Sphingopyxis* strains.

By a combination of a number of chemotaxonomic and phenotypic characteristics listed above (see Tables 1 and 2), together with phylogenetic information of the formation of a distinct clade within the family *Sphingomonadaceae* and low 16S rRNA gene sequence similarity ($<$95 %) to related genera, it is most appropriate to conclude that these novel microcystin-degrading strains should be classified in a novel genus and species of the family *Sphingomonadaceae*. 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. 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 rods, motile by means of polar flagella. Colonies are yellow. Strictly aerobic and chemo-organotrophic. Catalase- and oxidase-positive. Nitrate is not reduced to nitrite. The major fatty acids are 18 : 1ω7*c* and 16 : 1ω7*c*. 2-Hydroxy fatty acids are present, with 2-OH 14 : 0 predominating. 3-Hydroxy fatty acids are absent. Glycosphingolipids are produced. Respiratory quinone is predominantly Q-10. Homospermidine is the major polyamine component, as for the genus *Sphingomonas*. Placed phylogenetically in the family *Sphingomonadaceae*. The characteristic 16S rRNA signatures are the same as for 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. N.L. n. *microcystinum* microcystin; L. part. adj. *vorans* devouring; N.L. part. adj. *microcystinivorans* microcystin-degrading).

Shows the following properties in addition to those given in the genus description. Cells are 0·3–0·7 × 0·6–1·0 μm. Citrate only is assimilated. Negative reactions are observed for 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. Major fatty acids are 18 : 1ω7*c* (33–36 %) and 16 : 1ω7*c* (36–41 %); 16 : 0 (7–8 %), 16 : 1ω5*c* (3 %) and 14 : 0 (1–2 %) are produced as minor components. Major 2-hydroxy fatty acid is 2-OH 14 : 0 (11–13 %); 2-OH 16 : 0 (1 %) is produced as a minor component. Polyamine is homospermidine [1·5 μmol (g wet cells)$^{-1}$]. The DNA G+C content is 63·6–63·7 mol%.

The type strain, strain Y2^T (=KCTC 12019^T=JCM 13185^T), was isolated from a toxic *Microcystis* blooming lake, Lake Suwa, Japan. Strains MDB2 and MDB3, isolated from the Tenryu River, Japan, are reference strains.

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