Sphingomonas rubra sp. nov., isolated from bioreactor wastewater

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A Gram-reaction-negative, rod-shaped, motile, neutrophilic bacterium, designated strain BH3^T, was isolated from wastewater of a sequential batch reactor treating wastewater taken from a leather plant. The isolate grew in 0–8 % (w/v) NaCl, at pH 6–9 and at 4–45 °C. Chemotaxonomic analysis showed that strain BH3^T had characteristics typical of members of the genus *Sphingomonas*, such as the presence of sphingolipids, Q-10 and 2-hydroxymyristic acid and the absence of 3-hydroxy fatty acids. The presence of $C_{18:1}\omega7c$ (39.2 %) and $C_{16:0}$ (11.2 %) as major fatty acids, $C_{14:0}$ 2-OH (20.6 %) as the major 2-hydroxy fatty acid and homospermidine as the major polyamine indicated that strain BH3^T belonged to the genus *Sphingomonas sensu stricto*. The genomic DNA G+C content of strain BH3^T was 65.6 mol%. 16S rRNA gene sequence similarities between the isolate and closely related members of the genus *Sphingomonas sensu stricto* ranged from 92.6 to 97.3 %, the highest sequence similarities being to *Sphingomonas melonis* DSM 14444^T (97.3 %) and *Sphingomonas aquatilis* DSM 15581^T (97.3 %). Based on its phenotypic characteristics and the results of DNA–DNA hybridization studies and 16S rRNA gene sequence comparisons, strain BH3^T represents a novel species of the genus *Sphingomonas sensu stricto*, for which the name *Sphingomonas rubra* sp. nov. is proposed. The type strain is BH3^T (=CGMCC 1.9113^T =JCM 16230^T).

The genus *Sphingomonas* was first proposed by Yabuuchi *et al.* (1990) and the genus description was later emended by Takeuchi *et al.* (1993, 2001), Yabuuchi *et al.* (1999, 2002) and Busse *et al.* (2003). Based on their phylogenetic, chemotaxonomic and phenotypic differences, Takeuchi *et al.* (2001) divided members of the genus *Sphingomonas* into four genera: *Sphingomonas sensu stricto*, *Sphingobium*, *Novosphingobium* and *Sphingopyxis*. Cells of members of the genus *Sphingomonas* are aerobic, Gram-reaction-negative, chemoheterotrophic, non-motile or motile rods, which form yellow-pigmented colonies, have single polar flagella and are characterized by the presence of sphingolipids, Q-10, 2-hydroxymyristic acid and the absence of 3-hydroxy fatty acids. Members of the genus *Sphingomonas sensu stricto* contain $C_{18:1}$, saturated $C_{16:0}$ and/or $C_{17:1}$ as major fatty acids, $C_{14:0}$ 2-OH or $C_{15:0}$ 2-OH as major 2-hydroxy fatty acids and homospermidine as the major polyamine, by which they can be differentiated from members of the genera *Sphingobium*, *Novosphingobium* and *Sphingopyxis*. At the time of writing, the genus *Sphingomonas* comprised over 60 recognized species. Here we present a polyphasic study describing a novel strain, designated BH3^T, of the genus *Sphingomonas sensu stricto* which was isolated from wastewater in a bioreactor and formed red-pigmented colonies.

Strain BH3^T was isolated from wastewater of a sequential batch reactor treating wastewater from a leather plant. A sample of the wastewater was diluted using a tenfold dilution series method and spread on DSMZ 756 plates. The DSMZ 756 medium contained (l^{-1} distilled water): 1.5 g yeast extract, 1.5 g peptone, 0.55 g sodium pyruvate, 1 ml trace element solution, 0.007 g $CaCO_3$, 0.5 g NaCl,

Abbreviation: FAMEs, fatty acid methyl esters.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain BH3^T is FJ834325.

Two supplementary figures and two supplementary tables are available with the online version of this paper.

0.05 g $MgSO_4 . 7H_2O$ and 0.15 g KH_2PO_4 and was adjusted to pH 7.5 by using NaOH. The trace element solution contained (l^{-1} distilled water): 33.8 mg $MnSO_4 . H_2O$, 49.4 mg H_3BO_3, 43.1 mg $ZnSO_4 . 7H_2O$, 37.1 mg $(NH_4)_6 Mo_7O_{24}$, 97.3 mg $FeSO_4 . 7H_2O$ and 25.0 mg $CuSO_4 . 5H_2O$. After 3 days of incubation at 30 °C, a red-coloured colony was picked and a pure culture was obtained by repeated restreaking. The isolate was routinely cultured on DSMZ 756 medium and maintained in a 30 % (v/v) glycerol suspension at −80 °C.

Growth in 0, 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12 and 15 % (w/v) NaCl was determined in DSMZ 756 medium. Growth at pH 5.0–10.5 (intervals of 0.5) was determined in DSMZ 756 medium using the following buffers (BBI) at a concentration of 40 mM: 2-(*N*-morpholino)ethanesulfonic acid (MES; pH 5.0–6.0), piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.5–7.0), *N*-tris(hydroxymethyl)methylglycine (Tricine; pH 7.5–8.5), 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid (CAPSO; pH 9.0–10.0) and 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS; pH 10.5). Growth at 4, 10, 15, 20, 25, 30, 35, 37, 42, 45, 48 and 50 °C was determined. Cell morphology and motility were examined by optical microscopy (BX40; Olympus) and transmission electron microscopy (JEM-1230; JEOL).

Single carbon source assimilation tests were performed in DSMZ 756 medium without yeast extract, peptone and pyruvate and with a corresponding filter-sterilized sugar (0.2 %), alcohol (0.2 %), organic acid (0.1 %) or amino acid (0.1 %) added into the liquid medium. Acid production was tested by using oxidation–fermentation (OF) medium supplemented with 1.0 % sugar or alcohol (Hugh & Leifson, 1953). The OF medium contained (l^{-1} distilled water): 2.0 g casein, 5.0 g NaCl, 0.3 g KH_2PO_4 and 0.08 g bromothymol blue and was adjusted to pH 7.1 by using NaOH. Physiological and biochemical characteristics of strain BH3^T were determined on DSMZ 756 agar using API ZYM, API 20 NE and API 20 E (bioMérieux) tests. API ZYM strips were read after 12 h and API 20 E and API 20 NE strips after 24 h. Susceptibility to antibiotics was determined on agar plates using discs containing the following antibiotics (μg per disc unless stated otherwise): amoxicillin (10), ampicillin (10), bacitracin (0.04 IU), carbenicillin (100), cefotaxime (30), cefoxitin (30), chloramphenicol (30), erythromycin (15), gentamicin (10), kanamycin (30), neomycin (30), novobiocin (30), nystatin (100), penicillin (10), rifampicin (5), streptomycin (10), tetracycline (30) and tobramycin (10).

For pigment analysis, pigment was extracted from freeze-dried cells of strain BH3^T using methanol, according to the methods of Hildebrand *et al.* (1994), and was scanned by using a Beckman Coulter DU800 spectrophotometer. Fatty acid methyl esters (FAMEs) were obtained from freeze-dried cells as described by Kuykendall *et al.* (1988). Identification and quantification of the FAMEs were performed automatically by using the Sherlock Microbial Identification System with the standard MIS Library Generation Software (MIDI). Isoprenoid quinones were analysed as described previously (Komagata & Suzuki, 1987) using reversed-phase HPLC. Polar lipids were separated on silica gel plates (10 × 10 cm) by TLC according to the methods of Xu *et al.* (2007a) and observed after staining with 10 % ethanolic molybdophosphoric acid. Polyamines were extracted as described by Scherer & Kneifel (1983) and analysed according to Ducros *et al.* (2009). Genomic DNA was obtained using the method described by Marmur & Doty (1962) and the DNA G+C content was determined by reversed-phase HPLC and calculated from the ratio of deoxyguanosine (dG) to deoxythymidine (dT) (Mesbah & Whitman, 1989).

The 16S rRNA gene of strain BH3^T was amplified and analysed as described previously (Xu *et al.*, 2007b). PCR products were cloned into vector pMD 19-T (TaKaRa) and then sequenced to determine the almost-complete sequence of the 16S rRNA gene, which was compared with sequences of closely related reference organisms obtained from the EzTaxon database (Chun *et al.*, 2007). Sequence data were aligned using CLUSTAL W version 1.8 (Thompson *et al.*, 1994) and phylogenetic trees were constructed by using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods in the MEGA 4 program package (Tamura *et al.*, 2007) and the maximum-likelihood method (Felsenstein, 1981) using the RAXML web server (Stamatakis *et al.*, 2008). Distances were calculated for the neighbour-joining method according to the algorithm of the Kimura two-parameter model (Kimura, 1980).

Cells of strain BH3^T were Gram-reaction-negative, rod-shaped and ~0.6–1.1 × 1.1–3.6 μm in size. Cells were motile by means of a polar flagellum (Supplementary Fig. S1, available in IJSEM Online). The detailed phenotypic characteristics of strain BH3^T are given in the species description. Differential phenotypic properties of strains BH3^T, *Sphingomonas melonis* DSM 14444^T and *Sphingomonas aquatilis* DSM 15581^T are shown in Table 1. Differential characteristics of other closely related species are provided in Supplementary Table S1.

A comparison between the almost-complete 16S rRNA gene sequence (1448 nt) of strain BH3^T obtained in this study and those of closely related bacteria indicated that strain BH3^T belonged to the family *Sphingomonadaceae*. 16S rRNA gene sequence similarities between the novel isolate and members of the genus *Sphingomonas sensu stricto* ranged from 92.6 to 97.3 %, the highest sequence similarities being to *S. melonis* DSM 14444^T (97.3 %) and *S. aquatilis* DSM 15581^T (97.3 %). Sequence similarities between strain BH3^T and other members of the genus *Sphingomonas sensu stricto* were <97.0 % and were <94.6 % when compared with other species of the genus *Sphingomonas*. Phylogenetic analyses based on 16S rRNA gene sequences showed that strain BH3^T formed a cluster with *Sphingomonas aurantiaca* and *Sphingomonas faeni* within the genus *Sphingomonas sensu stricto* (Fig. 1).

The pigment extracted with methanol from strain BH3^T gave a major peak at 451 nm and two shoulder peaks at

Table 1. Differential characteristics of strain BH3^T and closely related strains

Strains: 1, BH3^T; 2. *S. melonis* DSM 14444^T; 3. *S. aquatilis* DSM 15581^T. Data were obtained from this study under identical growth conditions. +, Positive; −, negative; W, weakly positive; R, red; Y, yellow.

Characteristic	1	2	3
Colour of colony	R	Y	Y
ONPG	−	+	+
Utilization of:			
Cellobiose	−	+	+
Fumarate	−	+	−
L-Glutamate	−	+	+
Lactate	−	+	+
Rhamnose	−	+	+
Sucrose	−	+	+
D-Xylose	−	+	+
Utilization of (API 20 NE):			
N-Acetylglucosamine	−	+	+
Capric acid	−	−	+
Phenylacetic acid	−	+	−
Acid production from:			
Glucose	−	+	+
Rhamnose	−	+	+
Sucrose	−	+	+
Maltose	−	+	+
D-Xylose	−	+	+
Sensitivity to:			
Amoxicillin	+	−	−
Cefotaxime	+	−	−
Cefoxitin	+	−	−
Enzyme activities (API ZYM)			
α-Chymotrypsin	+	−	−
β-Galactosidase	−	+	+
β-Glucuronidase	−	+	−
N-Acetyl-β-glucosaminidase	−	+	+
Fermentation/oxidation of (API 20 E):			
Amygdalin	−	+	+
L-Arabinose	−	+	+
D-Glucose	−	W	W
L-Rhamnose	−	W	W
Sucrose	−	+	+

423 and 475 nm, typical of carotenoids, whereas the major peaks of the most closely related species were at 448 nm for *S. melonis* DSM 14444^T and 447 nm for *S. aquatilis* DSM 15581^T. To further verify that strain BH3^T was a novel species of the genus *Sphingomonas*, DNA–DNA hybridizations were performed using the thermal denaturation and renaturation method of De Ley *et al.* (1970) with modifications (Huß *et al.*, 1983), using a Beckman DU 800 spectrophotometer. The hybridization temperature used was 78 °C and the experiments were carried out in triplicate. DNA–DNA relatedness values of 42 % and 33 % between strain BH3^T and reference strains *S. melonis* DSM 14444^T and *S. aquatilis* DSM 15581^T, respectively, were significantly lower than 70 %, the recommended threshold value for the delineation of bacterial species (Wayne *et al.*, 1987). Strain BH3^T had chemotaxonomic characteristics typical of members of the genus *Sphingomonas*, such as the presence of sphingoglycolipids (see Supplementary Fig. S2), Q-10, 2-hydroxymyristic acids and the absence of 3-hydroxy fatty acids (Busse *et al.*, 1999). The presence of $C_{18:1}\omega7c$ (39.2 %) and $C_{16:0}$ (11.2 %) as major fatty acids, $C_{14:0}$ 2-OH (20.6 %) as the major 2-hydroxy fatty acid and homospermidine as the major polyamine also indicated that strain BH3^T belonged to the genus *Sphingomonas sensu stricto* (Takeuchi *et al.*, 2001). However, the proportion of $C_{18:1}\omega7c$ of strain BH3^T was lower than that of *S. melonis* DSM 14444^T (61.2 %), *S. aquatilis* DSM 15581^T (56.1 %), *S. pruni* (73.1 %) and *S. mali* (70.5 %) and the proportion of iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$ of strain BH3^T (18.9 %) was higher than that of *S. melonis* DSM 14444^T (5.0 %) and *S. aquatilis* DSM 15581^T (4.9 %). Compared with *S. melonis* DSM 14444^T and *S. aquatilis* DSM 15581^T, strain BH3^T also contained a small amount of 10-methyl $C_{19:0}$ and $C_{15:1}\omega6c$ but no $C_{18:1}\omega5c$ or $C_{18:1}$ 2-OH was detected (Supplementary Table S2).

The comparison of phenotypic properties between strain BH3^T and *S. melonis* DSM 14444^T and *S. aquatilis* DSM 15581^T also indicated differences in colony colour, the utilization of carbon sources, acid production from sugars and several enzyme activities (Table 1).

On the basis of the phylogenetic, genotypic, chemotaxonomic and phenotypic data, strain BH3^T represents a novel species of the genus *Sphingomonas sensu stricto*, for which the name *Sphingomonas rubra* sp. nov. is proposed.

Description of *Sphingomonas rubra* sp. nov.

Sphingomonas rubra (ru′bra. L. fem. adj. *rubra* red).

Cells are Gram-reaction-negative, rod-shaped, 0.6–1.1 × 1.1–3.6 μm and motile. Colonies are 1–2 mm in diameter, circular, elevated and red when grown on DSMZ 756 medium for 2 days at 28 °C. Growth occurs in 0–8 % (w/v) NaCl (optimum 0 %), at pH 6–9 (optimum pH 7.5) and at 4–37 °C (optimum 28 °C). Oxidase- and catalase-positive. Aesculin is hydrolysed. Casein, DNA, gelatin, starch, Tweens 20 and 80 and tyrosine are not hydrolysed. Negative for indole production and arginine dihydrolase, lysine carboxylase, ornithine carboxylase, tryptophan deaminase, urease and *o*-nitrophenyl-β-D-galactopyranosidase activities. Hydrogen sulfide is not produced. L-Cysteine, D-galactose and L-valine are utilized and glucose and maltose are weakly utilized. The following compounds are not utilized as sole carbon sources: acetate, L-arginine, L-aspartate, cellobiose, citrate, ethanol, formate, fumarate, gluconate, L-glutamate, glycine, glycerol, L-histidine, *myo*-inositol, isoleucine, lactate, malonate, mannitol, L-methionine, L-ornithine, propionate, pyruvate, raffinose, D-ribose, rhamnose, L-serine, D-sorbitol, L-sorbose, sucrose, starch and D-xylose. Acid is produced from L-arabinose but not from adonitol, ethanol, D-fructose, glucose, glycerol, *myo*-inositol,

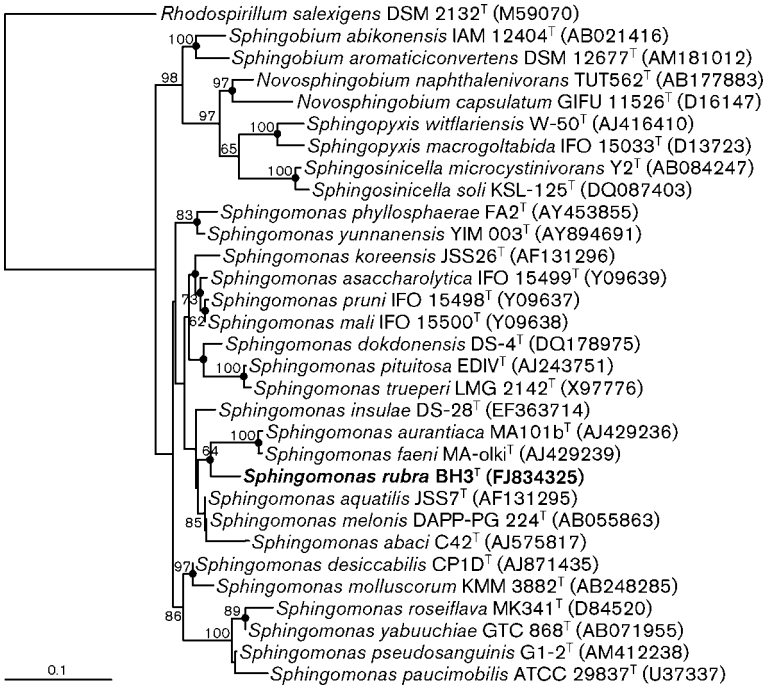

Fig. 1. Maximum-likelihood tree based on 16S rRNA gene sequences showing the phylogenetic relationships between strain BH3^T and related taxa. Bootstrap values >50 % (based on 100 replicates) are given at branch points. *Rhodospirillum salexigens* DSM 2132^T was used as an outgroup. Bar, 0.1 substitutions per nucleotide position. Filled circles indicate nodes also recovered in the neighbour-joining and maximum-parsimony trees.

maltose, mannitol, raffinose, rhamnose, D-salicin, D-sorbitol, L-sorbose, sucrose, trehalose or D-xylose. In the API ZYM system, acid phosphatase, alkaline phosphatase, α-chymotrypsin, esterase (C4), esterase lipase (C8), α-glucosidase, leucine arylamidase, naphthol-AS-BI-phosphohydrolase and valine arylamidase activities are present, whereas *N*-acetyl-β-glucosaminidase, β-fucosidase, α- and β-galactosidase, β-glucuronidase, lipase (C14), α-mannosidase and trypsin activities are absent. Susceptible to (μg unless otherwise stated) amoxicillin (10), cefataxime (30), cefoxitin (30), chloramphenicol (30), erythromycin (15), gentamicin (10), kanamycin (30), neomycin (30), novobiocin (30), rifampicin (5), tetracycline (30) and tobramycin (10) but not to ampicillin (10), bacitracin (0.04 IU), carbenicillin (100), nystatin (100), penicillin (10) or streptomycin (10). The predominant quinone is Q-10. The major polyamine is homospermidine. The major polar lipids of strain BH3^T are phosphatidylethanolamine, phosphatidylglycerol, phosphatidyldimethylethanolamine and diphosphatidylglycerol as well as two sphingoglycolipids and an unidentified lipid. Small amounts of two unidentified lipids are also present. The major fatty acids (>5 %) include $C_{18:1}\omega7c$, $C_{14:0}$ 2-OH, iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$ and $C_{16:0}$.

The type strain, BH3^T (=CGMCC 1.9113^T =JCM 16230^T), was isolated from wastewater of a sequential batch reactor treating wastewater taken from a leather plant. The DNA G+C content of the type strain is 65.6 mol%.

Acknowledgements

This work was supported by grants from the Ministry of Science and Technology of China (863 Program, 2007AA021305), the National Natural Science Foundation of China (30970002; 40806066), the Zhejiang Provincial Natural Science Foundation of China (Y5080060) and the Key Project of Zhejiang Science and Technology (2006C13053).

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