Sphingobium amiense sp. nov., a novel nonylphenol-degrading bacterium isolated from a river sediment

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A nonylphenol-degrading bacterial strain (YT^T) was isolated previously from a river sediment sample obtained in Ami-machi, Ibaraki, Japan, and identified as a *Sphingomonas* species. In this study, the taxonomic relationship between strain YT^T, a recently described nonylphenol-degrading strain, *Sphingomonas cloacae*, and *Sphingobium yanoikuyae*, which is phylogenetically related, was examined. Their phenotypic characteristics were compared and levels of DNA–DNA relatedness between these strains were determined. Based on the results of physiological and biochemical tests and DNA–DNA hybridization, it is proposed that strain YT^T (=IAM 15006^T = JCM 11777^T = CIP 107839^T) represents a novel species of the genus *Sphingobium*, *Sphingobium amiense* sp. nov.

Nonylphenol (NP) and the shorter homologues of non-ionic surfactants, nonylphenol polyethoxylates (NPEO_x), are known to exert oestrogenic effects in aquatic organisms (Soto *et al.*, 1991; White *et al.*, 1994; Brown *et al.*, 1999; Kloas *et al.*, 1999; Kinnberg *et al.*, 2000). In the aquatic environment, microbial breakdown of NPEO_x yields shorter homologues under aerobic conditions and further NP under anaerobic conditions (Rudling & Solyom, 1974; Giger *et al.*, 1984; Kveštek & Ahel, 1995). Although NP is rather recalcitrant in the environment, especially in anoxic sediments (Ahel *et al.*, 1996; Heinis *et al.*, 1999), both Tanghe *et al.* (1999) and Fujii *et al.* (2000) have recently isolated *Sphingomonas* strains that were able to grow on a commercial mixture of branched NP isomers as a sole carbon and energy source in pure culture. Strain S-3^T, described by Fujii *et al.* (2000), degraded 1000 p.p.m. NP, i.e. a concentration 10 000–1 000 000-fold higher than that found in urban environments, almost completely within 10 days. In their further taxonomic study, this strain was placed in a novel species of the genus *Sphingomonas*, *Sphingomonas cloacae* (Fujii *et al.*, 2001).

Recently, a conventional enrichment culture was carried out on 10 p.p.m. NP, with diluted nutrient broth (NB) for additional organic nutrients, to isolate bacterial strains able to degrade NP at low levels. From this enrichment culture, an NP-degrading strain (YT^T) was isolated and characterized chemotaxonomically and phylogenetically (de Vries *et al.*, 2001).

Strain YT^T is a Gram-negative, yellow-pigmented rod that contains ubiquinone with 10 isoprene units (Q-10) and alkali-stable sphingoglycolipid. In addition, 16S rDNA sequence analysis revealed that strain YT^T belonged to *Sphingobium* (*Sphingomonas* cluster II), recently proposed by Takeuchi *et al.* (2001). The closest known relatives of strain YT^T were *Sphingobium yanoikuyae* and the recently described NP-degrading *Sphingomonas cloacae*, with 16S rDNA similarity values of 98.0 and 97.8%, respectively (de Vries *et al.*, 2001). Based on these results, strain YT^T was classified as a member of the genus *Sphingomonas* (de Vries *et al.*, 2001). In the present study, other phenotypic and chemotaxonomic characteristics of strain YT^T and the DNA–DNA relatedness between YT^T and closely related strains of *Sphingobium yanoikuyae* and *Sphingomonas cloacae* were examined.

Sphingobium chlorophenolicum JCM 10275^T, *Sphingomonas cloacae* JCM 10874^T, *Sphingomonas paucimobilis* JCM 7516^T and *Sphingobium yanoikuyae* JCM 7371^T were obtained from the Japan Collection of Microorganisms. *Sphingobium chlorophenolicum* DSM 8671, obtained from the DSMZ, was also used in some experiments. NB [1% (w/v) Ehrlich meat extract (Kyokuto Seiyaku), 1% (w/v) Trypticase peptone (Becton Dickinson) and 0.5% (w/v) NaCl] or its 10-fold dilution (NB/10) was used for cultivation. The pH was adjusted to 7.0 with 1 M NaOH. Unless otherwise indicated, strains were grown aerobically at 27 °C on NB/10 or NB agar medium.

For electron microscopy, cells of strain YT^T were grown on NB/10 for 3 days, suspended in 50 µl 20 mM ammonium acetate and dried on a carbon-coated mesh. Cells were

Abbreviations: NP, nonylphenol; NPEO_x, nonylphenol polyethoxylates.

The DDBJ accession number for the 16S rDNA sequence of strain YT^T is AB047364.

stained with 0.1% (w/v) phosphotungstic acid and observed under a Hitachi H-800 transmission electron microscope at 100 kV. Growth of strain YT^T at 4, 30 and 42 °C on NB was monitored for 7 days. Nitrate reduction was tested by growing strain YT^T in NB and NB/10 supplemented with 0.1% (w/v) KNO₃ and then adding Griess–Ilosvay reagents (Skerman, 1967) to the culture. Assimilation of organic compounds, including carbohydrates and organic acids, activities of arginine dihydrolase, urease and β -galactosidase, hydrolysis of aesculin and gelatin and production of indole were tested using API 20NE (bioMérieux). The tests were run according to the manual and incubated at 27 °C for 4 days. Oxidase and catalase tests were carried out as described previously (Ohta & Hattori, 1983). Cellular fatty acids were analysed as described previously (Ohta & Hattori, 1983). In brief, cellular fatty acid methyl esters were prepared by heating dried cells in anhydrous methanolic HCl at 100 °C for 3 h (Ikemoto *et al.*, 1978) and then analysed by GLC. A column (4.1 m $\times$ 2.6 mm) with 10% dimethyleneglycol succinate on Chromosorb W (AW-DMCS, 80–100 mesh) was used. For the detection of hydroxy fatty acids, TLC (Kieselgel F-254; Merck) was used with a hexane/diethyl ether (4:1, v/v) solvent system. To compare cellular protein profiles, whole-cell lysates with SDS were prepared essentially as described by Ohta *et al.* (1993) and one-dimensional analytical SDS-PAGE was performed by the method of Laemmli (1970) with a 12.5% separating gel and a 4.5% stacking gel. Proteins were visualized by silver staining with a commercial kit (Daiichi Pure Chemicals).

In a previous work, the 16S rDNA sequence of strain YT^T was determined, indicating that the strain belongs to the genus *Sphingomonas* (de Vries *et al.*, 2001). In the present study, sequences that exhibited close relatedness in a FASTA (Pearson & Lipman, 1988) search and those of representative species in *Sphingomonas sensu lato* were used for phylogenetic analysis. Multiple alignment, calculation of nucleotide substitution rates (K_{nuc} values) as described by Kimura (1980) and construction of a phylogenetic tree by the neighbour-joining method (Saitou & Nei, 1987) were performed using CLUSTAL W (Thompson *et al.*, 1994). Sequence similarity calculations indicated that the closest relatives of strain YT^T were *Sphingobium yanoikuyae* (98.0%), *Sphingomonas cloacae* (97.8%), *Sphingobium chlorophenolicum* (97.1%), '*Sphingomonas agrestis*' (96.8%) (Pinyakong *et al.*, 2003), *Sphingobium herbicidovorans* (96.8%) and *Sphingomonas chungbukensis* (96.4%). For determination of DNA base composition, DNA was extracted, purified by phenol treatment (Saitou & Miura, 1963) and enzymically degraded into nucleosides. The nucleoside mixture was separated by reverse-phase HPLC as described by Tamaoka & Komagata (1984). DNA–DNA hybridization experiments were carried out with photobiotin-labelled probes in microplate wells as described by Ezaki *et al.* (1989) using a Wallac 1420 ARVOsx multilabel counter for determination of chemiluminescence. For enzymic development,

Fig. 1. Electron micrograph of a negatively stained cell of strain YT^T. Bar, 1 μ m.

alkaline phosphatase–streptavidin conjugate (Vector) was used with CDP-Star (Tropix) as substrate.

Cells of strain YT^T were straight rods, about 1.1–1.7 μ m long and 0.5 μ m wide, and motile by means of a polar flagellum (Fig. 1). Colonies on NB and NB/10 agar were circular, entire, convex, opaque and creamy yellow. Strain YT^T grew at 27 °C, but not at 4 or 42 °C. The organism was catalase- and oxidase-positive, but negative for nitrate reduction. Strain YT^T contained C_{18:1} as the primary fatty acid component (62% total fatty acid content) and C_{16:1} as the secondary fatty acid component (13%). Other fatty acids detected were C_{16:0} (8%), 2-OH C_{14:0} (7%), C_{17:1} (2%) and C_{14:0} (1%); the summed amount of unknown acids was 7%. No visible 3-hydroxy fatty acid spot was detected in TLC of the fatty acid methyl esters. The G + C

Fig. 2. Unrooted tree showing the phylogenetic relationships of strain YT^T and representative species of *Sphingomonas sensu stricto*, *Sphingobium*, *Novosphingobium* and *Sphingopyxis*. *Escherichia coli* was used as the outgroup. The tree, constructed using the neighbour-joining method, was based on a comparison of a region corresponding to *E. coli* 16S rDNA positions 37–1417. Bootstrap values, expressed as percentages of 1000 replications, are given at branching points. Bar, 0.05 nucleotide substitution rate (K_{nuc}) units.

content of strain YT^T was 66.40 ± 0.47 mol% (mean ± SD of five independent determinations).

The phylogenetic tree, based on 16S rDNA sequences, shows that strain YT^T belongs to the genus *Sphingobium* (Fig. 2). In addition, the nucleotide signatures specific to the 16S rRNA of *Sphingobium*, i.e. a U:A pair at position 52:359, the presence of U at position 593 and a U:G pair at position 987:1218 (Takeuchi *et al.*, 2001), were all found in the sequence of strain YT^T. Takeuchi *et al.* (2001) have shown that polyamine patterns and nitrate reduction are good diagnostic markers for differentiation of *Sphingobium* and *Sphingomonas sensu stricto*. Although polyamines were not analysed in this study, nitrate reduction data suggest that strain YT^T should be classified in the genus *Sphingobium*.

A comparison of the biochemical characteristics of strain YT^T with those of phylogenetically related species of *Sphingobium* and *Sphingomonas* and two other *Sphingobium* species is shown in Table 1. Of the 11 substrates and five enzyme activities tested, strain YT^T was positive for only two tests (assimilation of glucose and maltose) and, by this unique catabolic profile, the strain can be clearly differentiated from the other species. Moreover, the whole-cell protein profile of strain YT^T was compared with those of phylogenetically related *Sphingomonas* and *Sphingobium*

strains. As shown in Fig. 3, strain YT^T was distinct from the other strains with respect to the overall protein-banding pattern. To provide further clear evidence for the low similarity between strain YT^T, *Sphingomonas cloacae* JCM 10874^T and *Sphingobium yanoikuyae* JCM 7371^T, DNA–DNA hybridization experiments were performed. The relatedness values between strain YT^T and all other type strains tested were below 21%. Therefore, it is proposed that strain YT^T represents a novel species, *Sphingobium amiense* sp. nov.

Description of *Sphingobium amiense* sp. nov.

Sphingobium amiense (a.mi.en'se. N.L. neut. adj. *amiense* of Ami, Ibaraki, Japan, where the type strain of this organism was isolated).

Gram-negative, aerobic rod. Cells are about 1.1–1.7 × 0.5 µm. Colonies on NB agar are circular, entire, convex, opaque and creamy yellow. Able to grow in NB at 27 °C, but not at 4 or 42 °C. Oxidase- and catalase-positive. Indole, urease and arginine dihydrolase are not produced. Aesculin

Table 1. Biochemical characteristics of strain YT^T, two *Sphingomonas* species, three *Sphingobium* species and the type strain of *Sphingomonas paucimobilis*

Strains: 1, strain YT^T; 2, *Sphingomonas cloacae* JCM 10874^T; 3, *Sphingobium yanoikuyae* JCM 7371^T; 4, *Sphingobium chlorophenolicum* DSM 8671; 5, *Sphingomonas paucimobilis* JCM 7516^T; 6, *Sphingobium herbicidovorans* DSM 11019^T; 7, *Sphingomonas chungbukensis* KCTC 2955^T. Data for strains 1–5 are from this study and data for strains 6 and 7 are from Kim *et al.* (2000). None of the strains assimilated D-mannitol, caprate or adipate. Arginine dihydrolase and gelatin hydrolysis activities were not observed in the strains tested. Acid production from glucose was not detected in any strain.

Characteristic	1	2	3	4	5	6	7
Assimilation of:							
D-Glucose	+	–	+	+	+	+	+
L-Arabinose	–	–	+	–	+	+	+
D-Mannose	–	–	–	–	+	–	+
N-Acetyl-D-glucosamine	–	–	+	–	+	–	–
Maltose	+	–	+	–	+	+	+
Gluconate	–	–	+	–	–	+	+
Malate	–	–	+	–	+	+	–
Citrate	–	–	+	–	–	–	–
Activity of:							
Urease	–	–	–	+	–	–	–
β-Galactosidase	–	–	+	–	+	–	–
Aesculin hydrolysis	–	–	+	+	+	+	–
Nitrate reduction	–	–	–	–	+	–	–

Fig. 3. SDS-PAGE protein profiles of strain YT^T and phylogenetically related *Sphingomonas* and *Sphingobium* species. Lanes: 1, molecular mass markers; 2, strain YT^T; 3, *Sphingomonas cloacae* JCM 10874^T; 4, *Sphingobium yanoikuyae* JCM 7371^T; 5, *Sphingobium chlorophenolicum* DSM 8671; 6, *Sphingomonas paucimobilis* JCM 7516^T. The molecular mass markers are (from top to bottom): macroglobulin, 170 000; β-galactosidase, 116 400; fructose-6-phosphate kinase, 85 200; glutamate dehydrogenase, 55 600; aldolase, 39 200; triosephosphate isomerase, 26 600; trypsin inhibitor, 20 100; lysozyme, 14 300.

and gelatin are not hydrolysed. Nitrate is not reduced to nitrate. Glucose and maltose are assimilated, but L-arabinose, D-mannose, D-mannitol and N-acetyl-D-glucosamine are not. Nonylphenol, an endocrine disrupter chemical, is degraded in the presence of organic nutrients such as yeast extract, but is not used as a growth substrate. Major non-polar fatty acids are C_{18:1} and C_{16:1}; the major hydroxy fatty acid is 2-OH C_{14:0}. Sphingoglycolipid is present. The major isoprenoid quinone is ubiquinone Q-10.

The type strain, YT^T (=IAM 15006^T=JCM 11777^T=CIP 107839^T), was isolated from a river sediment sample from Ami-machi, Ibaraki, Japan. The DNA G + C content of this strain is 66.4 mol%.

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