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

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A nonylphenol-degrading bacterial strain (YT) 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, 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 (=IAM 15006T = JCM 11777T = CIP 107839T) represents a novel species of the genus Sphingobium, Sphingobium amiense sp. nov.

Nonylphenol (NP) and the shorter homologues of non-ionic surfactants, nonylphenol polyethoxylates (NPEOs), 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 NPEOs yields shorter homologues under aerobic conditions and further NP under anaerobic conditions (Rudling & Solyom, 1974; Giger et al., 1984; Kvešták & 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-3T, 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) was isolated and characterized chemotaxonomically and phylogenetically (de Vries et al., 2001).

Abbreviations: NP, nonylphenol; NPEOs, nonylphenol polyethoxylates.

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

Strain YT 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 belonged to Sphingobium (Sphingomonas cluster II), recently proposed by Takeuchi et al. (2001). The closest known relatives of strain YT 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 was classified as a member of the genus Sphingobium (de Vries et al., 2001). In the present study, other phenotypic and chemotaxonomic characteristics of strain YT and the DNA–DNA relatedness between YT and closely related strains of Sphingobium yanoikuyae and Sphingomonas cloacae were examined.

Sphingobium chlorophenicolicum JCM 10275T, Sphingomonas cloacae JCM 10874T, Sphingomonas paucimobilis JCM 7516T and Sphingobium yanoikuyae JCM 7371T were obtained from the Japan Collection of Microorganisms. Sphingobium chlorophenicolicum 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 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
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 \textsuperscript{T} at 4, 30 and 42 °C on NB was monitored for 7 days. Nitrate reduction was tested by growing strain YT \textsuperscript{T} in NB and NB/10 supplemented with 0·1 % (w/v) KNO\textsubscript{3} 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 \(\beta\)-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 \textit{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 \textit{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 \textsuperscript{T} was determined, indicating that the strain belongs to the genus \textit{Sphingomonas} (de Vries \textit{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 \textit{Sphingomonas sensu lato} were used for phylogenetic analysis. Multiple alignment, calculation of nucleotide substitution rates (\(K_{\text{nuc}}\) 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 \textit{et al}., 1994). Sequence similarity calculations indicated that the closest relatives of strain YT \textsuperscript{T} were \textit{Sphingobium yanoikuyae} (98·0 %), \textit{Sphingomonas cloacae} (97·8 %), \textit{Sphingobium chlorophenolicum} (97·1 %), \textit{Sphingomonas agrestis} (96·8 %) (Pinyakong \textit{et al}., 2003), \textit{Sphingobium herbicidovorans} (96·8 %) and \textit{Sphingomonas chungbukensis} (96·4 %). For determination of DNA base composition, DNA was extracted, purified by phenol treatment (Saitou & Miura, 1963) and enzymically degraded into nucleotides. The nucleotide sequence 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 \textit{et al}. (1989) using a Wallac 1420 ARVOx multilabel counter for determination of chemiluminescence. For enzymic development, alkaline phosphatase–streptavidin conjugate (Vector) was used with CDP-Star (Tropix) as substrate.

Cells of strain YT \textsuperscript{T} were straight rods, about 1·1–1·7 \(\mu\)m long and 0·5 \(\mu\)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 \textsuperscript{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 \textsuperscript{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 %), \(2\text{-OH} \: C_{14:0}\) (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

\[\text{Fig. 1.} \text{ Electron micrograph of a negatively stained cell of strain YT}^{\text{T}}. \text{ Bar, 1 \mu m.} \]
content of strain YT<sup>T</sup> was 66.40 ± 0.47 mol% (mean ± SD of five independent determinations).

The phylogenetic tree, based on 16S rRNA sequences, shows that strain YTT 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 YTT. 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 YTT should be classified in the genus Sphingobium.

A comparison of the biochemical characteristics of strain YTT with those of phylogenetically related species of Sphingobium and Sphingomonas and another Sphingobium species is shown in Table 1. Of the 11 substrates and five enzyme activities tested, strain YTT 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 YTT was compared with those of phylogenetically related Sphingomonas and Sphingobium strains. As shown in Fig. 3, strain YTT 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 YTT, Sphingomonas cloacae JCM 10874<sup>T</sup> and Sphingobium yanoikuyae JCM 7371<sup>T</sup>, DNA–DNA hybridization experiments were performed. The relatedness values between strain YTT and all other type strains tested were below 21%. Therefore, it is proposed that strain YTT 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

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

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**Fig. 3. SDS-PAGE protein profiles of strain YTT and phylogenetically related Sphingomonas and Sphingobium species.**

Lanes: 1, molecular mass markers; 2, strain YTT; 3, Sphingomonas cloacae JCM 10874<sup>T</sup>; 4, Sphingobium yanoikuyae JCM 7371<sup>T</sup>; 5, Sphingobium chlorophenolicum DSM 8671<sup>T</sup>; 6, Sphingomonas paucimobilis JCM 7516<sup>T</sup>. 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, 14 900.
and gelatin are not hydrolysed. Nitrate is not reduced to nitrite. 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 C₁₈:₁ and C₁₆:₁; the major hydroxy fatty acid is 2-OH C₁₄:₀. Sphingoglycolipid is present. The major isoprenoid quinone is ubiquinone Q-10.

The type strain, YT<sup>T</sup> (= IAM 15006<sup>T</sup> = JCM 11777<sup>T</sup> = CIP 107839<sup>T</sup>), 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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References


