Description of *Sphingobium fuliginis* sp. nov., a phenanthrene-degrading bacterium from a fly ash dumping site, and reclassification of *Sphingomonas cloacae* as *Sphingobium cloacae* comb. nov.

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A phenanthrene-degrading bacterium, strain TKP^T, was isolated from a fly ash dumping site of the thermal power plant in Panki, Kanpur, India, by an enrichment culture method using phenanthrene as the sole source of carbon and energy. Phylogenetic analysis based on 16S rRNA gene sequences indicated that the strain belonged to the genus *Sphingobium*, as it showed highest sequence similarity to *Sphingobium herbicidovorans* DSM 11019^T (97.3%) and *Sphingomonas cloacae* JCM 10874^T (96.5%), compared with only 91–93% similarity to members of other genera such as *Sphingomonas sensu stricto*, *Novosphingobium*, *Sphingopyxis* and *Sphingosinicella*. In DNA–DNA hybridization experiments with strains that were closely related phylogenetically and in terms of 16S rRNA gene sequences, i.e. *Sphingobium herbicidovorans* DSM 11019^T and *Sphingomonas cloacae* JCM 10874^T, strain TKP^T showed less than 70% relatedness. Strain TKP^T contained sphingoglycolipids SGL-1 and SGL-2 and 18:1 ω 7c as the predominant fatty acid, with 16:0 as a minor component and 14:0 2-OH as the major 2-hydroxy fatty acid. Thus, phylogenetic analysis, DNA–DNA hybridization, fatty acid and polar lipid profiles and differences in physiological and morphological features from the most closely related members of the *Sphingobium* group showed that strain TKP^T represents a distinct species of *Sphingobium*. The name *Sphingobium fuliginis* sp. nov. is proposed, with the type strain TKP^T (=MTCC 7295^T=CCM 7327^T). *Sphingomonas cloacae* JCM 10874^T formed a coherent cluster with members of *Sphingobium*, did not reduce nitrate to nitrite and had a fatty acid profile similar to those of *Sphingobium* species; hence *Sphingomonas cloacae* should be transferred to the genus *Sphingobium* as *Sphingobium cloacae* comb. nov., with the type strain JCM 10874^T (=DSM 14926^T).

Phenanthrene is a member of the polycyclic aromatic hydrocarbon (PAH) group, a class of hydrophobic organic compounds. It is a constituent of petroleum hydrocarbons and coal, originating from incomplete combustion of organic materials such as coal and oil, and of tobacco smoke. Phenanthrene is distributed abundantly in higher concentrations around coal gasification sites, fly ash dumping sites of coal-fired thermal power plants and petroleum-contaminated sites of oil refineries. It is persistent in nature, it is a human skin photosensitizer and a mild allergen and is toxic to aquatic organisms (Pipe & Moore, 1986). Contamination of

the environment by phenanthrene has created several environmental problems.

In the present study, a phenanthrene-degrading, yellow-pigmented bacterium, strain TKP^T, was isolated from a fly ash dumping site of the thermal power plant in Panki, Kanpur, India, by an enrichment culture approach using phenanthrene as the sole source of carbon and energy. Phylogenetic and taxonomic characterization of the strain using a polyphasic approach revealed that the strain represents a novel species of *Sphingobium*. We also reclassify *Sphingomonas cloacae* as *Sphingobium cloacae* comb. nov. based on its greater similarity to members of *Sphingobium* than to members of other related genera.

Strain TKP^T was screened for phenanthrene degradation as described by Kiyohara *et al.* (1982). Colonies producing a clear zone by degradation of phenanthrene were picked and

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain TKP^T is DQ092757.

Details of the polar lipid and fatty acid profiles of strain TKP^T and related strains and a two-dimensional TLC of polar lipids of strain TKP^T are available as supplementary material in IJSEM Online.

purified by restreaking several times on nutrient agar (NA) plates. Strain TKP^T produced a clear zone of phenanthrene degradation after 48 h of incubation and utilized more than 200 mg phenanthrene l⁻¹ within 24 h in liquid culture (data not shown).

16S rRNA gene sequencing and analysis

Genomic DNA from strain TKP^T was extracted using the method of Kaur *et al.* (2001). The 16S rRNA gene sequence of strain TKP^T was obtained from the Sherlock Microbial Identification System (Microbial ID Inc.). Similarity searches were done using the sequence match program of the Ribosomal Database Project (<http://rdp.cme.msu.edu/html/>) and the BLAST program of the National Center for Biotechnological Information (<http://www.ncbi.nlm.nih.gov>).

16S rRNA gene sequences (>1200 bp) of 38 established species of the genera *Sphingomonas sensu stricto*, *Sphingobium*, *Novosphingobium*, *Sphingopyxis* and *Sphingosinicella* were retrieved and their similarity to the 16S rRNA gene sequence of strain TKP^T was analysed. For construction of the tree, 16S rRNA gene sequences of strain TKP^T, *Blastomonas ursincola* DSM 9006^T, *Blastomonas natatoria* DSM 3183^T and all members of *Sphingobium* with validly published names along with type strains of the genera *Sphingomonas sensu stricto*, *Novosphingobium*, *Sphingopyxis* and *Sphingosinicella* (Maruyama *et al.*, 2006) were selected. The 16S rRNA gene sequence of *Zymomonas mobilis* ATCC 10988^T was used as an outgroup. Selected sequences were aligned using the CLUSTAL X program (Thompson *et al.*, 1997), gaps common to all the selected sequences were removed and the alignment was checked manually for quality. Terminal nucleotides not common to all the sequences were removed. Phylogenetic analysis was carried out using the PHYLIP package version 3.5c (Felsenstein, 1993). An evolutionary distance matrix was calculated using the distance model of Jukes & Cantor (1969). The evolutionary tree (Fig. 1) was constructed using the neighbour-joining method (Saitou & Nei, 1987) and the resultant tree topology was evaluated by bootstrap analysis based on 100 resamplings, using the SEQBOOT and CONSENSE programs in the PHYLIP package. Parsimony analysis was also performed for the aligned sequence data using DNAPARS including bootstrap analysis with 100 resamplings.

Evaluation of the tree topology (Fig. 1) revealed that strain TKP^T clustered with species represented by the genus *Sphingobium* and formed a monophyletic clade with *Sphingobium herbicidovorans* DSM 11019^T. Similar tree topology and clustering were also obtained by the maximum-parsimony method of the PHYLIP package (data not shown). Strain TKP^T showed 95.9–97.3 % 16S rRNA gene sequence similarity to members of *Sphingobium*, in contrast with only 91–93 % sequence similarity to members of *Sphingomonas sensu stricto*, *Novosphingobium*, *Sphingopyxis* and *Sphingosinicella*. This indicated that strain TKP^T is a member of *Sphingobium*. The highest 16S

Fig. 1. Phylogenetic tree based on nearly complete 16S rRNA gene sequences showing the relationship of strain TKP^T to *Sphingobium herbicidovorans* DSM 11019^T and *Sphingomonas cloacae* JCM 10874^T and related species. The tree was constructed by the neighbour-joining method and was rooted by using *Zymomonas mobilis* ATCC 10988^T as the outgroup. Numbers at nodes represent bootstrap values (based on 100 resamplings). Bar, 0.1 nucleotide substitution per nucleotide position.

rRNA gene sequence similarity of strain TKP^T was found to the sequences of *Sphingobium herbicidovorans* DSM 11019^T (97.3 %), the nearest relative of strain TKP^T in the phylogenetic tree, followed by *Sphingomonas cloacae* JCM 10874^T (96.5 %) (Fig. 1).

DNA–DNA hybridization

DNA–DNA hybridization was carried out in order to check the delineation of strain TKP^T from its closest phylogenetic relative, *Sphingobium herbicidovorans* DSM 11019^T, as well as *Sphingomonas cloacae* JCM 10874^T, which is closely related phylogenetically and in terms of 16S rRNA gene sequence similarity. DNA extraction, purification and hybridization were done as described by Pal *et al.* (2005). The amount of bound probe DNA was estimated by using a scintillation counter (Beckman Instruments) and levels of hybridization were expressed as the percentage of probe bound relative to the homologous reaction.

In DNA–DNA hybridization experiments, strain TKP^T showed only 11 % relatedness with *Sphingobium herbicidovorans* DSM 11019^T and 14 % relatedness with *Sphingomonas cloacae* JCM 10874^T. Similar results were obtained when labelled DNA of *Sphingobium herbicidovorans* DSM 11019^T or *Sphingomonas cloacae* JCM 10874^T was used as the probe. These levels of DNA–DNA hybridization are much less than the threshold value (70 %) suggested for bacterial species delineation by Wayne *et al.* (1987). Thus, DNA–DNA hybridization clearly delineated strain TKP^T from the most

closely related strains, *Sphingobium herbicidovorans* DSM 11019^T and *Sphingomonas cloacae* JCM 10874^T.

Polar lipid and fatty acid methyl ester analysis

Fatty acid profiles of strain TKP^T and *Sphingomonas cloacae* JCM 10874^T were obtained from the Sherlock Microbial Identification System (Microbial ID Inc.). For this purpose, the bacterium was grown on trypticase soya broth agar (TSBA) at 28 °C and fatty acids were saponified, methylated and extracted as described by Miller (1982) and Kuykendall *et al.* (1988). Polar lipid analysis was carried out by the identification service of the DSMZ (Braunschweig, Germany) as described by Tindall (1990a, b).

Fatty acids of strain TKP^T along with phylogenetically close members of *Sphingobium* are detailed in Supplementary Table S1 (available in IJSEM Online). The predominance of 18:1 ω 7c and high levels of 16:0 in strain TKP^T indicated that the strain is a member of the *Alphaproteobacteria*. The presence of 2-hydroxy fatty acids and the absence of 3-hydroxy fatty acids (features common to sphingomonads) further indicated that strain TKP^T is a member of the family *Sphingomonadaceae* (Busse *et al.*, 1999). Like other members of *Sphingobium*, *Novosphingobium*, *Sphingopyxis* and *Sphingosinicella*, it also contains 14:0 2-OH as the major 2-hydroxy fatty acid. However, the presence of 16:0 2-OH differentiated strain TKP^T from members of the genera *Sphingomonas* and *Novosphingobium*, since 16:0 2-OH is not found in members of these genera (Takeuchi *et al.*, 2001), and indicated that the strain could be a member of *Sphingobium* or *Sphingopyxis*. Further, the presence of only a minor amount of 16:0 2-OH (a major component in *Sphingopyxis*) and the lower level of 16S rRNA gene sequence similarity of strain TKP^T with members of *Sphingopyxis* (91–93 %) compared with *Sphingobium* (95–97 %) justified the clustering of strain TKP^T in a clade represented by the genus *Sphingobium*.

The polar lipids phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylcholine and sphingoglycolipids, commonly found in other sphingomonads, were also detected in strain TKP^T (see Supplementary Fig. S1 and Supplementary Table S2 available in IJSEM Online). From comparison of lipid profiles, it appears that SGL-1 of strain TKP^T probably corresponds to SGL of *Sphingobium yanoikuyae* IFO 15102^T and either GL-4 or SGL of *Sphingomonas macrogoltabidus* IFO 15033^T, while SGL-2 represents GL-1 of *Sphingobium yanoikuyae* IFO 15102^T and SGL or GL-1 of *Sphingomonas macrogoltabidus* IFO 15033^T (Busse *et al.*, 1999). It was also noted that the aminophospholipid (PN) of strain TKP^T probably corresponds to phosphatidylmonomethylethanolamine (PME) and the unidentified phospholipid (PL) to phosphatidyl dimethylethanolamine (PDE) of Busse *et al.* (1999).

The presence of sphingoglycolipids confirms only that strain TKP^T is a member of the family *Sphingomonadaceae*

(Yabuuchi *et al.*, 1990; Busse *et al.*, 1999; Takeuchi *et al.*, 2001), but comparison of the polar lipids and fatty acids of strain TKP^T with phylogenetically close members of *Sphingobium* showed the presence of similar profiles and confirmed that strain TKP^T is a member of genus *Sphingobium*.

Phenotypic characterization

Morphological features of the colonies (shape, size, colour, contour and pigment production) were studied on NA and Luria–Bertani (LB) agar plates after 72 h of incubation at 30 °C. Strain TKP^T formed yellow-coloured, circular, smooth colonies, 1.5 and 2.0 mm in diameter, respectively, on NA and LB agar plates. Gram staining and spore staining were done using a Himedia kit. The cell size was measured by micrometry. Motility of the organism was studied by the hanging drop method as well as on motility agar (Table 1). Antibiotic sensitivity tests were performed on Mueller–Hinton II medium using Readymade Sensi-Discs (Himedia). Growth at different temperatures was examined and the catalase test was carried out as described by McCarthy & Cross (1984). Biochemical tests were performed as described by Pal *et al.* (2005). Hydrolysis of Tween 20 and 80 and the ability of the strain to grow in the presence of NaCl were tested as described by Arden-Jones *et al.* (1979). Urease activity was detected as described by Christensen (1946). Acid production from carbohydrates and degradation of xanthine and hypoxanthine were tested as described by Gordon *et al.* (1974). The other physiological tests and methods were described by Collins *et al.* (1989). Phenanthrene-degrading activity of the strain was tested by gas chromatography (Samanta *et al.*, 1999).

Pigments were extracted in chloroform/methanol (2:1) (Goel *et al.*, 2001) and in acetone (Jenkins *et al.*, 1979). Absorption maxima (λ_{max}) of the pigment in chloroform/methanol and in acetone extracts were 254 and 211 nm, respectively. Strain TKP^T also produced a water-soluble yellow pigment (λ_{max} 230 nm) distinct from the water-soluble brown pigment of *Sphingobium herbicidovorans* DSM 11019^T. Nitrate reduction is common to species of *Sphingomonas* and *Novosphingobium* but has not been reported so far for *Sphingobium*. However, unlike other members of *Sphingobium*, strain TKP^T showed a weakly positive test for nitrate reduction. In conclusion, 16S rRNA gene sequence analysis, comparative study of fatty acid and lipid profiles, pigment analysis, morphological features, biochemical tests (Table 1) and DNA–DNA hybridization with the most closely related members of *Sphingobium* differentiated strain TKP^T from these species and indicated that strain TKP^T represents a novel species of *Sphingobium*, for which the name *Sphingobium fuliginis* sp. nov. is proposed.

During the classification of strain TKP^T, it was found that *Sphingomonas cloacae* JCM 10874^T clustered with members of *Sphingobium* and not with *Sphingomonas*. It also showed the highest 16S rRNA gene sequence similarity (95–97 %) to

Table 1. Differential phenotypic characteristics of strain TKP^T and phylogenetically close members of the genus *Sphingobium*

Strains: 1, strain TKP^T (data from this study); 2, *Sphingomonas cloacae* JCM 10817^T; 3, *Sphingobium herbicidovorans* DSM 11019^T; 4, *Sphingobium yanoikuyae* IFO 15102^T (unless indicated otherwise, data in columns 2–4 were taken from Yabuuchi *et al.*, 2002); 5, *Sphingobium amiense* IAM 15006^T (data from Ushiba *et al.*, 2003). All strains showed positive results for oxidase, catalase and assimilation of maltose and negative results for urease and assimilation of lactose, inositol and sorbitol. +, Positive; –, negative; w, weakly positive; ND, no data available.

Character	1	2	3	4	5
Colour	Yellow	Creamy white	Yellow	Light yellow	Creamy yellow
Motility	–	–	+	+	+
Cell size (µm)	0.5 × 1.5	1.1 × 3.1	0.4 × 2.1	0.7 × 3.5	1.1 × 1.7
Water-soluble pigment	Yellow	ND	Brown	ND	ND
Optimum growth temperature (°C)	37	25	28	28	27
Degradation of nonylphenol	ND	+	ND	ND	+
Hydrolysis of:					
Gelatin	–	–	+	–	–
Aesculin	–	+	+	+	–
Nitrate reductase	w	–*	–*	–	–
Acid production from:					
Adonitol	w	–	+	–	ND
Dulcitol	–	–	+	–	ND
Glucose	+	–	+	+	–
Mannitol	–	–	+	–	ND
Ribose	w	–	–	+	ND
Xylose	+	–	+	+	ND
Arabinose	+	–	–	–	–

*Data from this study.

members of *Sphingobium*, in contrast to only 91–94% similarity to members of other genera such as *Sphingomonas sensu stricto*, *Novosphingobium*, *Sphingopyxis*, *Sphingosinicella* and *Blastomonas*. Phylogenetic trees published previously (Fujii *et al.*, 2001; Yabuuchi *et al.*, 2002; Pal *et al.*, 2005) revealed a similar position for *Sphingomonas cloacae* JCM 10874^T. In addition, examination of the fatty acid profile of *Sphingomonas cloacae* JCM 10874^T (from this study) showed that, in common with most members of *Sphingobium*, it also contains 18:1ω7c as the dominant fatty acid with 16:0 as a minor component and 14:0 2-OH as the major 2-hydroxy fatty acid (Supplementary Table S1). It did not reduce nitrate to nitrite, a characteristic feature of all members of *Sphingobium* (Takeuchi *et al.*, 2001), supporting its position with members of *Sphingobium*. The paper by Takeuchi *et al.* (2001) on the division of *Sphingomonas* into *Sphingomonas sensu stricto* and three new genera, *Sphingobium*, *Novosphingobium* and *Sphingopyxis*, and the description of *Sphingomonas cloacae* by Fujii *et al.* (2001) were published in the same volume of the *International Journal of Systematic and Evolutionary Microbiology*. Thus, data for *Sphingomonas cloacae* JCM 10874^T were not available to Takeuchi *et al.* (2001) for analysis and reclassification. Therefore, we also propose the transfer of *Sphingomonas cloacae* to the genus *Sphingobium* as *Sphingobium cloacae* comb. nov.

Description of *Sphingobium fuliginis* sp. nov.

Sphingobium fuliginis (fu.li'gi.nis. L. gen. n. *fuliginis* of soot, referring to the coal fly ash from which the type strain was isolated).

Gram-negative, strictly aerobic, non-spore-forming, non-motile, small rod (0.7–1.0 µm). Colonies are yellow-pigmented, small (diameter 1.5 mm on NA and 2.0 mm on LB agar after 72 h of incubation at 30 °C), entire, smooth and circular. Positive in tests for oxidase, catalase and nitrate reductase but gives negative results in tests for gelatinase, urease and amylase. Acids are produced from glucose, maltose, D-ribose, xylose and adonitol (after a long incubation) but not from inositol, sucrose, dulcitol, mannitol or sorbitol. Grows at 20–37 °C but not at 10 or 40 °C. The optimum temperature for growth is 37 °C. Sensitive to 5% NaCl and does not grow at pH 10. Sensitive to discs containing nalidixic acid (30 µg), tetracycline (30 µg), gentamicin (10 µg), chlortetracycline (30 µg), rifamycin (5 µg), oxytetracycline (30 µg), neomycin (30 µg), kanamycin (30 µg) and novobiocin (30 µg) and resistant to vancomycin (30 µg), penicillin G (10 µg), ampicillin (10 µg), streptomycin (10 µg), amoxicillin (10 µg) and erythromycin (15 µg). Together with glycosphingolipids (SGL-1 and SGL-2), the polar lipid profile also

contains phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylcholine, an unidentified glycolipid, unidentified phospholipids and an amino-phospholipid. The fatty acid profile of the type strain contains 14:0 (0.45 %), 15:0 (0.24 %), 16:0 (8.33 %), 18:0 (0.30 %), 20:0 (0.24 %), 14:0 2-OH (10.51 %), 16:0 2-OH (0.52 %), 16:1 ω 5c (1.29 %), 15:0 2-OH (0.44 %), 17:1 ω 8c (0.28 %), 17:1 ω 6c (1.70 %), 18:1 ω 7c (65.80 %), 18:1 ω 5c (0.97 %) and 11-methyl 18:1 ω 7c (1.16 %).

The type strain, strain TKP^T (=MTCC 7295^T=CCM 7327^T), was isolated from a fly ash dumping site of the thermal power plant at Panki, Kanpur, India, and degrades phenanthrene efficiently on solid medium (plates sprayed with phenanthrene) as well as in liquid culture.

Description of *Sphingobium cloacae* (Fujii *et al.* 2001) comb. nov.

Sphingobium cloacae (clo.a'cae. L. gen. n. *cloacae* of a sewer, the source of the type strain).

Basonym: *Sphingomonas cloacae* Fujii *et al.* 2001.

The description is identical to that of *Sphingomonas cloacae* as given by Fujii *et al.* (2001). The type strain is JCM 10874^T (=DSM 14926^T=CIP 107076^T=IAM 14885^T).

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