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<sup>T</sup>, 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<sup>T</sup> (97.3%) and *Sphingomonas cloacae* JCM 10874<sup>T</sup> (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<sup>T</sup> and *Sphingomonas cloacae* JCM 10874<sup>T</sup>, strain TKP<sup>T</sup> showed less than 70% relatedness. Strain TKP<sup>T</sup> contained sphingoglycolipids SGL-1 and SGL-2 and 18:1<sup>v7c</sup> 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<sup>T</sup> represents a distinct species of *Sphingobium*. The name *Sphingobium fuliginis* sp. nov. is proposed, with the type strain TKP<sup>T</sup> (=MTCC 7295<sup>T</sup> = CCM 7327<sup>T</sup>). *Sphingomonas cloacae* JCM 10874<sup>T</sup> 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<sup>T</sup> (=DSM 14926<sup>T</sup>).

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<sup>T</sup>, 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 TKP<sup>T</sup> 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<sup>T</sup> was screened for phenanthrene degradation as described by Kiyohara *et al.* (1982). Colonies producing a clear zone by degradation of phenanthrene were picked and
purified by restreaking several times on nutrient agar (NA) plates. Strain TKPT 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 TKPT was extracted using the method of Kaur et al. (2001). The 16S rRNA gene sequence of strain TKPT 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 TKPT was analysed. For construction of the tree, 16S rRNA gene sequences of strain TKPT, *Blastomonas ursincola* DSM 9006², *Blastomonas natatoria* DSM 3183² 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 10988³ 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 and was rooted by using *Zymomonas mobilis* ATCC 10988³ as the outgroup. Numbers at nodes represent bootstrap values (based on 100 resamplings). Bar, 0-1 nucleotide substitution per nucleotide position.

Fig. 1. Phylogenetic tree based on nearly complete 16S rRNA gene sequences showing the relationship of strain TKPT to *Sphingobium herbicidovorans* DSM 11019⁴ and *Sphingomonas cloacae* JCM 10874⁵ and related species. The tree was constructed by the neighbour-joining method and was rooted by using *Zymomonas mobilis* ATCC 10988³ as the outgroup. Numbers at nodes represent bootstrap values (based on 100 resamplings). Bar, 0-1 nucleotide substitution per nucleotide position.

DNA–DNA hybridization

DNA–DNA hybridization was carried out in order to check the delineation of strain TKPT from its closest phylogenetic relative, *Sphingobium herbicidovorans* DSM 11019⁴, as well as *Sphingomonas cloacae* JCM 10874⁵, 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 TKPT showed only 11 % relatedness with *Sphingobium herbicidovorans* DSM 11019⁴ and 14 % relatedness with *Sphingomonas cloacae* JCM 10874⁵. Similar results were obtained when labelled DNA of *Sphingobium herbicidovorans* DSM 11019⁴ or *Sphingomonas cloacae* JCM 10874⁵ 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 TKPT from the most
closely related strains, *Sphingobium herbicidovorans* DSM 11019<sup>T</sup> and *Sphingomonas cloacae* JCM 10874<sup>T</sup>.

**Polar lipid and fatty acid methyl ester analysis**

Fatty acid profiles of strain TKP<sup>T</sup> and *Sphingomonas cloacae* JCM 10874<sup>T</sup> 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<sup>T</sup> along with phylogenetically close members of *Sphingobium* are detailed in Supplementary Table S1 (available in IJSEM Online). The predominance of 18:1<sub>ω7c</sub> and high levels of 16:0 in strain TKP<sup>T</sup> 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 sphingomonoinds) further indicated that strain TKP<sup>T</sup> is a member of the family *Sphingomonadaceae* (Busse et al., 1999). Like other members of *Sphingobium*, *Novosphingobium*, *Sphingopyxis* and *Sphingobacterium*, 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<sup>T</sup> 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<sup>T</sup> with members of *Sphingobium* (91–93%) compared with *Sphingomonas* (95–97%) justified the clustering of strain TKP<sup>T</sup> in a clade represented by the genus *Sphingobium*.

The polar lipids phosphatidylethanolamine, phosphatidyglycerol, diphasphatidylglycerol, phosphatidylcholine and sphingoglycolipids, commonly found in other sphingomonoids, were also detected in strain TKP<sup>T</sup> (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<sup>T</sup> probably corresponds to SGL of *Sphingobium yanoikuyae* IFO 15102<sup>T</sup> and either GL-4 or SGL of *Sphingomonas macrogoltabidus* IFO 15033<sup>T</sup>, while SGL-2 represents GL-1 of *Sphingobium yanoikuyae* IFO 15102<sup>T</sup> and SGL or GL-1 of *Sphingomonas macrogoltabidus* IFO 15033<sup>T</sup> (Busse et al., 1999). It was also noted that the aminophospholipid (PN) of strain TKP<sup>T</sup> probably corresponds to phosphatidylmonomethylethanolamine (PME) and the unidentified phospholipid (PL) to phosphatidyldimethylethanolamine (PDE) of Busse et al. (1999).

The presence of sphingoglycolipids confirms only that strain TKP<sup>T</sup> 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<sup>T</sup> with phylogenetically close members of *Sphingobium* showed the presence of similar profiles and confirmed that strain TKP<sup>T</sup> 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<sup>T</sup> 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 Hemedia 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<sub>max</sub>) of the pigment in chloroform/methanol and in acetone extracts were 254 and 211 nm, respectively. Strain TKP<sup>T</sup> also produced a water-soluble yellow pigment (λ<sub>max</sub> 230 nm) distinct from the watersoluble brown pigment of *Sphingobium herbicidovorans* DSM 11019<sup>T</sup>. 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<sup>T</sup> 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<sup>T</sup> from these species and indicated that strain TKP<sup>T</sup> represents a novel species of *Sphingobium*, for which the name *Sphingobium fuliginis* sp. nov. is proposed.

During the classification of strain TKP<sup>T</sup>, it was found that *Sphingomonas cloacae* JCM 10874<sup>T</sup> clustered with members of *Sphingobium* and not with *Sphingomonas*. It also showed the highest 16S rRNA gene sequence similarity (95–97%) to...
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), chlorotetracycline (30 μg), rifampicin (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

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

<table>
<thead>
<tr>
<th>Character</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
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<tbody>
<tr>
<td>Colour</td>
<td>Yellow</td>
<td>Creamy white</td>
<td>Yellow</td>
<td>Light yellow</td>
<td>Creamy yellow</td>
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<tr>
<td>Motility</td>
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<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Cell size (μm)</td>
<td>0.5 × 1.5</td>
<td>1.1 × 3.1</td>
<td>0.4 × 2.1</td>
<td>0.7 × 3.5</td>
<td>1.1 × 1.7</td>
</tr>
<tr>
<td>Water-soluble pigment</td>
<td>Yellow</td>
<td>ND</td>
<td>Brown</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Optimum growth temperature (°C)</td>
<td>37</td>
<td>25</td>
<td>28</td>
<td>28</td>
<td>27</td>
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<tr>
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<td>ND</td>
<td>ND</td>
<td>+</td>
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<td>Hydrolysis of:</td>
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<td>+</td>
<td>−</td>
<td>−</td>
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<tr>
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<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
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<td>Nitrate reductase</td>
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<td>−</td>
<td>−</td>
<td>−</td>
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<tr>
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<td>−</td>
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<td>−</td>
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</table>

*Data from this study.*
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%), 14:2 2-OH (10·51%), 16:0 2-OH (0·52%), 16:1 ω5c (1·29%), 15:0 2-0H (0·44%), 17:1 ω8c (0·28%), 17:1 ω6c (1·70%), 18:1 ω7c (65·80%), 18:1 ω5c (0·97%) and 11-methyl 18:1 ω7c (1·16%).

The type strain, strain TKP<sup>T</sup> (=MTCC 7295<sup>T</sup> = CCM 7327<sup>T</sup>), 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. cloaca of a sewer, the source of the type strain).


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

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**References**


Christensen, W. B. (1946). Urea decomposition as a means of differentiating *Proteus* and paracolon cultures from each other and from *Salmonella* and *Shigella* types. *J Bacteriol* 52, 461–466.


