Sphingobium lactosutens sp. nov., isolated from a hexachlorocyclohexane dump site and *Sphingobium abikonense* sp. nov., isolated from oil-contaminated soil

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The taxonomic position of a yellow-pigmented bacterial strain, designated DS20^T, isolated from a hexachlorocyclohexane dump site at Lucknow, India was determined based on a polyphasic taxonomic characterization. A neighbour-joining tree based on 16S rRNA gene sequences showed that strain DS20^T occupied a distinct phylogenetic position in the *Sphingobium* cluster, showing highest similarity with '*Pseudomonas abikonensis*' IAM 12404 (98.8%), followed by *Sphingobium rhizovicinum* CC-FH12-1^T (97.4%) and *Sphingobium olei* IMMIB HF-1^T (97.2%). Therefore, the taxonomic characterization of '*P. abikonensis*' NBRC 16140 was also undertaken. Phylogenetic, chemotaxonomic and morphological analyses, based on signature sequences, DNA–DNA hybridizations, fatty acid profiles, physiological characterizations and polar lipid profiles confirmed that both strains DS20^T and '*P. abikonensis*' NBRC 16140 represent two distinct species of the genus *Sphingobium*. Therefore, two novel *Sphingobium* species are proposed, *Sphingobium lactosutens* sp. nov. (type strain, DS20^T=CCM 7540^T=MTCC 9471^T) and *Sphingobium abikonense* sp. nov. (type strain, NBRC 16140^T=IAM 12404^T=KCTC 2864^T).

While investigating microbial diversity of a hexachlorocyclohexane (HCH) dumpsite in Ummari village, Lucknow, India, with HCH levels between 1.3×10^4 and 3.9×10^5 ppm, a yellow-pigmented bacterial strain, DS20^T, was isolated using the method as described by Singh & Lal (2009). Even though isolated from such a highly contaminated site, strain DS20^T was found to not degrade HCH.

The phylogenetic tree based on 16S rRNA gene sequences showed that strain DS20^T belonged to the *Sphingobium* cluster, showing closest similarity to '*Pseudomonas abikonensis*' IAM 12404 (98.8%). '*Pseudomonas abikonensis*' IAM 12404 was isolated from an oil-contaminated soil and is able to metabolize dibenzothiophene to sulfur-containing organic acid compounds (Anzai *et al.*, 2000; Yamada *et al.*, 1968). Thus, the taxonomic position of '*P.*

abikonensis' NBRC 16140 was also reassessed. In the past, many incompletely characterized, polar-flagellated, Gram-negative, rod-shaped, aerobic bacteria have been clustered into the genus *Pseudomonas* (Anzai *et al.*, 2000) and most of them have been subsequently reclassified and transferred to other genera (Vandamme *et al.*, 1997; Viallard *et al.*, 1998; Grimes *et al.*, 1997; Spröer *et al.*, 1998; Nohynek *et al.*, 1996; Kim *et al.*, 2000). '*P. abikonensis*' IAM 12404 was, in fact, included in the *Sphingomonas* cluster on the basis of 16S rRNA gene sequence comparisons by Anzai *et al.* (2000) and the need for further taxonomic characterization was suggested (Anzai *et al.*, 2000; Lee *et al.*, 2005). The polyphasic approach used in this study suggested that strain DS20^T and '*P. abikonensis*' NBRC 16140 represent two distinct species of the genus *Sphingobium*.

The 16S rRNA gene sequence analysis of strain DS20^T was performed as described by Gupta *et al.* (2008) using a 3100-Avant Genetic Analyzer sequencer (Applied Biosystems) at the Department of Zoology, University of Delhi. A continuous stretch of 1334 bp for the 16S rRNA gene sequence of strain DS20^T was checked for quality and gaps. Similarity searches were performed using the sequence match program of the Ribosomal Database Project (<http://rdp.cme.msu.edu/>) and BLAST program of NCBI (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>). For

Abbreviations: FAME, fatty acid methyl ester; HCH, hexachlorocyclohexane.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Sphingobium lactosutens* DS20^T and *Sphingobium abikonense* IAM 12404^T are EU675846 and AB021416, respectively.

Tables of fatty acid compositions and signature sequences and figures showing polar lipid patterns and transmission electron micrographs for *Sphingobium lactosutens* DS20^T and *Sphingobium abikonense* NBRC 16140^T are available as supplementary material with the online version of this paper.

the construction of the phylogenetic tree, nearly complete 16S rRNA gene sequences of all 15 *Sphingobium* species with validly published names were retrieved from GenBank. The selected sequences were aligned using CLUSTAL X (Thompson *et al.*, 1997), terminal nucleotides not present in all of the sequences were removed manually from the alignment file and phylogenetic analysis was carried out using PHYLIP version 3.5 (Felsenstein, 1993) and TREECON (Van de Peer & De Wachter, 1994). The evolutionary distance matrix was calculated according to the model of Jukes & Cantor (1969) and clustering with neighbour joining (Saitou & Nei, 1987) was performed. Phylogenetic analysis was also done with the parsimony method (Fitch, 1971) using DNAPARS. Calculation of bootstrap values were based on 1000 resamplings.

The topology of the phylogenetic tree (Fig. 1) revealed that strain DS20^T and '*P. abikonensis*' IAM 12404 formed a monophyletic clade with species of the genus *Sphingobium*. Strain DS20^T shared 98.8 % sequence similarity with '*P. abikonensis*' IAM 12404, 97.4 % with *Sphingobium rhizovicinum* CC-FH12-1^T, 97.2 % with *Sphingobium olei* IMMIB HF-1^T, 96.7 % with *Sphingobium amiense* YT^T and 96.5 % with *Sphingobium cloacae* S-3^T. '*P. abikonensis*' IAM 12404 shared 96.8 % similarity with both *S. rhizovicinum* CC-FH12-1^T and *S. olei* IMMIB HF-1^T, 96.3 % sequence similarity with *S. amiense* YT^T and 96.1 % with *S. cloacae*

S-3^T. Strain DS20^T and '*P. abikonensis*' IAM 12404 showed sequence similarities of 93–96 % with other members of the genus *Sphingobium*. Additionally, the 16S rRNA gene sequences of strain DS20^T and '*P. abikonensis*' IAM 12404 were found to contain the nucleotide-specific signatures for the genus *Sphingobium* cluster II (Takeuchi *et al.*, 2001) (Supplementary Table S1, available in IJSEM Online). This indicated that both strains belonged to the genus *Sphingobium* and further analyses were done to confirm their phylogenetic positions.

To further clarify the taxonomic status of strain DS20^T and '*P. abikonensis*' NBRC 16140, DNA–DNA hybridization studies were carried out between strain DS20^T, '*P. abikonensis*' NBRC 16140, *S. olei* IMMIB HF-1^T, *S. rhizovicinum* CC-FH12-1^T, *S. amiense* YT^T, *S. cloacae* S-3^T and *Sphingobium xenophagum* DSM 6383^T as described by Pal *et al.* (2005). The amount of bound probe DNA was estimated using a scintillation counter (Beckman Instruments, USA) and hybridization values were expressed as percentages of bound probe. The DNA–DNA hybridization data showed 42.3 % DNA–DNA relatedness between strain DS20^T and '*P. abikonensis*' NBRC 16140 and less than 50 % DNA–DNA relatedness between either strain DS20^T or '*P. abikonensis*' NBRC 16140 and other related *Sphingobium* strains, including *S. olei* IMMIB HF-1^T, *S. rhizovicinum* CC-FH12-1^T,

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences showing the phylogenetic relationships of strains DS20^T and IAM 12404^T with related species, constructed using the neighbour-joining method. *Zymomonas mobilis* ATCC 10988^T was used as the outgroup. Bootstrap values based on 1000 resamplings are shown at branch nodes. Bar, 0.02 substitutions per nucleotide position.

S. amiense YT^T, *S. cloacae* S-3^T and *S. xenophagum* DSM 6383^T. The pooled standard deviations of all of the hybridization experiments were between 3 and 7%. The hybridization results were below the threshold of 70% set by Wayne *et al.* (1987) for the delineation of bacterial species and clearly indicated that strain DS20^T and '*P. abikonensis*' NBRC 16140 are representatives of two distinct novel species of the genus *Sphingobium*.

Fatty acid methyl ester (FAME) analysis was carried out at the Institute of Microbial Technology (IMTECH), Chandigarh, India. Bacterial cultures were harvested by centrifugation and the pellets were subjected to saponification, methylation and extraction, using the methods described by Miller (1982) and Kuykendall *et al.* (1988). The analysis was done using the Sherlock Microbial Identification System (MIDI, USA). The FAME analysis of both strain DS20^T and '*P. abikonensis*' NBRC 16140 showed the presence of 2-hydroxy fatty acids and the absence of 3-hydroxy fatty acids, which are characteristic features of the family *Sphingomonadaceae* (Busse *et al.*, 1999). Further analysis revealed that strain DS20^T and '*P. abikonensis*' NBRC 16140 showed FAME profiles that are characteristic of the genus *Sphingobium* (Takeuchi *et al.*, 2001) with the predominance of 18:1 ω 7c (59.5 and 67.6%, respectively), 16:0 (5.9 and 5.7%, respectively), 14:0 2-OH (10.4 and 7.2%, respectively) and summed feature 3 (16:1 ω 7c and/or 16:1 ω 6c; 10.8 and 10.4%, respectively). However, the quantitative differences in the fatty acid profiles indicated that strain DS20^T and '*P. abikonensis*' NBRC 16140 represent distinct species of the genus *Sphingobium* (Supplementary Table S2, in IJSEM Online).

Polar lipid extraction was performed as described by Gupta *et al.* (2009). Polar lipids were identified by comparing *R_f* values with commercially prepared standards (Sigma) and lipids extracted from reference type strains. The examination of total polar lipids of strain DS20^T and '*P. abikonensis*' NBRC 16140 revealed the presence of polar lipids that are commonly found in *Sphingobium* species. Comparison with the standards identified phosphatidylmonomethylethanolamine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidyltrimethylethanolamine, diphosphatidylglycerol, sphingoglycolipid, phosphatidylcholine and an unidentified polar lipid. Although the major lipids were found in both strain DS20^T and '*P. abikonensis*' NBRC 16140, there were differences: phosphatidylcholine was detected in strain DS20^T but not in '*P. abikonensis*' NBRC 16140 (Supplementary Fig. S1, in IJSEM Online).

Cultural features of strain DS20^T and '*P. abikonensis*' NBRC 16140 were studied on Luria–Bertani (LB) agar plates after incubation at 28 °C for 48–72 h. Both strains produced yellow-pigmented circular colonies. Gram-staining was performed using a kit from Himedia (Mumbai, India). Cell morphology was studied using transmission electron micrographs taken at All India Institute of Medical

Sciences (Delhi, India): a loopful of grown culture was suspended in Karnovsky's fixative for 2 h, washed with phosphate buffer and stained with 1% phosphotungstic acid for observation with a transmission electron microscope (model 269D; Morgagni). Motility was also checked on motility agar (Farmer, 1999). Whereas cells of strain DS20^T were rod-shaped and non-motile without flagella, cells of '*P. abikonensis*' NBRC 16140 were rod-shaped with a single polar flagellum (Supplementary Fig. S2, in IJSEM Online).

Growth at different temperatures (4–50 °C) was examined. Growth at different pH values (4–12) and salt concentrations (1–10%) were examined at 28 °C. Acid production from carbohydrates and degradation of xanthine and hypoxanthine were tested as described by Gordon *et al.* (1974). Catalase test and growth at different temperatures was carried out as described by McCarthy & Cross (1984). Hydrolysis of aesculin and Tween 20 and the ability of the strains to grow in the presence of NaCl were tested as described by Arden-Jones *et al.* (1979). Hydrolysis of gelatin and starch was carried out as described by Cowan & Steel (1965). Urease activity was detected as described by Christensen (1946). Nitrate reduction was tested according to Smibert & Krieg (1994). Other physiological methods were used as described by Collins *et al.* (1989). Antibiotic sensitivity was checked on Mueller–Hinton II medium (Himedia; India) using ready-made discs (Himedia) with varying amount of antibiotics, from 5 to 30 µg/disc. The results that differentiate the strains from 13 species of the genus *Sphingobium* are listed in Table 1.

To conclude, the results of the phylogenetic, chemotaxonomic and morphological analyses clearly place strain DS20^T and '*P. abikonensis*' NBRC 16140 in the genus *Sphingobium* as representing two novel species, for which the names *Sphingobium lactosutens* sp. nov. and *Sphingobium abikonense* sp. nov., are proposed.

Description of *Sphingobium lactosutens* sp. nov.

Sphingobium lactosutens (lac.to.su'tens. N.L. n. *lactosum* lactose; L. v. *utor* to use, make use of, employ; N.L. part. adj. *lactosutens* using lactose, assimilating lactose).

Cells are Gram-negative, aerobic, non-spore-forming, non-motile and rod-shaped (2.6 µm × 1.3 µm). Produces yellow-coloured, small, smooth and circular colonies. Grows at 20–37 °C (optimum, 28 °C) and pH 6–10 (optimum, pH 7). Does not grow in >5% NaCl. Produces acid from D-glucose, D-galactose, L-arabinose, D-xylose, lactose, cellobiose, maltose and trehalose but not from D-mannose, D-fructose, sucrose, D-ribose, D-mannitol, raffinose, rhamnose, melibiose, *meso*-inositol, adonitol or sorbitol. Positive for aesculin hydrolysis, citrate utilization and nitrate reduction. Negative for urease, catalase, gelatinase, xanthine and hypoxanthine hydrolysis, DNase and starch hydrolysis. Sensitive to (µg per disc): ciprofloxacin (5), rifampicin (5), kanamycin (30), vancomycin (30), gentamicin (10), tetracycline (30), chloramphenicol (30),

Table 1. Differential physiological characteristics of strains DS20^T, NBRC 16140^T and other *Sphingobium* species

Strains: 1, DS20^T (*Sphingobium lactosutens* sp. nov.; data from this study); 2, NBRC 16140^T (*Sphingobium abikonense* sp. nov.; this study); 3, *S. olei* IMMIB HF-1^T (Young *et al.*, 2007); 4, *S. rhizovicinum* CC-FH12-1^T (Young *et al.*, 2008); 5, *S. xenophagum* DSM 6383^T (Stolz *et al.*, 2000); 6, *S. francense* Sp +^T (Pal *et al.*, 2005); 7, *S. japonicum* UT26^T (Pal *et al.*, 2005); 8, *S. indicum* B90A^T (Pal *et al.*, 2005); 9, *S. chungbukensis* DJ77^T (Kim *et al.*, 2000); 10, *S. chlorophenolicum* ATCC 37097^T (Ushiba *et al.*, 2003); 11, *S. herbicidovorans* MH^T (Ushiba *et al.*, 2003); 12, *S. amiense* YT^T (Ushiba *et al.*, 2003); 13, *S. yanoikuyae* ATCC 51230^T (Ushiba *et al.*, 2003); 14, *S. cloacae* S-3^T (Ushiba *et al.*, 2003); 15, *S. fuliginis* TKP^T (Prakash & Lal, 2006). +, Positive; −, negative; w, weakly positive; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Acid from:															
L-Arabinose	+	+	+	+	+	+	+	+	+	−	+	−	+	−	+
D-Galactose	+	+	ND	+	−	+	+	+	+	ND	ND	ND	+	ND	ND
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+
Maltose	+	+	+	+	+	ND	ND	ND	+	−	+	+	+	−	+
D-Mannose	−	+	−	+	−	−	−	−	+	−	−	−	−	−	ND
D-Xylose	+	+	+	ND	+	+	+	+	+	ND	ND	ND	ND	ND	+
Catalase	−	−	+	−	+	+	+	+	+	ND	ND	+	ND	ND	+
Urease	−	+	ND	ND	−	ND	ND	ND	−	+	−	−	−	−	−
Citrate utilization	+	+	ND	ND	−	−	−	−	−	−	−	−	+	−	ND
Aesculin hydrolysis	+	+	+	+	+	+	+	−	+	+	+	−	+	−	−
Nitrate reduction	w	−	−	+	−	ND	ND	ND	−	−	−	−	−	−	w

*Data were from this study.

amikacin (30), polymyxin B (30), neomycin (30) and oxytetracycline (30) and resistant to ampicillin (10) and penicillin (10). Major fatty acids are 18:1 ω 7c, 14:0 2-OH, 16:0 and summed feature 3 (16:1 ω 7c and/or 16:1 ω 6c). The polar lipid profile contains phosphatidylmonomethylethanolamine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidyltrimethylethanolamine, diphosphatidylglycerol, sphingoglycolipid, phosphatidylcholine and an unidentified polar lipid.

The type strain, DS20^T (=CCM 7540^T=MTCC 9471^T), was isolated from a HCH dump site in Ummari village at Lucknow, India.

Description of *Sphingobium abikonense* sp. nov.

Sphingobium abikonense (a.bi.ko.nen'se. N.L. neut. adj. *abikonense* pertaining to Abiko, where the type strain was isolated).

Cells are Gram-negative, aerobic, non-spore-forming, rod-shaped (1.5 μ m $\times$ 0.4 μ m) and motile by means of a single polar flagellum (>5.2 μ m). Produces yellow-coloured, small, smooth and circular colonies. Grows at 20–37 °C (optimum, 28 °C) and pH 6–10 (optimum, pH 7). Does not grow in >5 % NaCl. Produces acid from D-glucose, D-galactose, L-arabinose, D-xylose, cellobiose, trehalose, maltose, rhamnose and D-mannose but not from lactose, D-fructose, sucrose, D-ribose, D-mannitol, raffinose, melibiose, meso-inositol, adonitol or sorbitol. Positive for aesculin hydrolysis, citrate utilization and urease activity but negative for nitrate reduction, gelatinase, xanthine and

hypoxanthine hydrolysis, DNase and catalase. Sensitive to (μ g per disc): ciprofloxacin (5), rifampicin (5), kanamycin (30), vancomycin (30), gentamicin (10), tetracycline (30), chloramphenicol (30), amikacin (30), polymyxin B (30), neomycin (30), oxytetracycline (30) and resistant to ampicillin (10) and penicillin (10). Major fatty acids are 18:1 ω 7c, 14:0 2-OH, 16:0 and summed feature 3 (16:1 ω 7c and/or 16:1 ω 6c). The polar lipid profile contains phosphatidylmonomethylethanolamine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidyltrimethylethanolamine, diphosphatidylglycerol, sphingoglycolipid and an unidentified polar lipid.

The type strain, NBRC 16140^T (=IAM 12404^T=KCTC 2864^T), was isolated from oil-contaminated soil.

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