Sphingobium quisquiliarum sp. nov., a hexachlorocyclohexane (HCH)-degrading bacterium isolated from an HCH-contaminated soil

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A yellow-pigmented, hexachlorocyclohexane (HCH)-degrading bacterial strain, P25^T, was isolated from an HCH dump site located in the northern part of India. Phylogenetic analysis based on the 16S rRNA gene sequence showed that the strain belongs to the genus *Sphingobium*, as it showed highest sequence similarity to *Sphingobium amiense* IAM 15006^T (97.7 %). The 16S rRNA gene sequence similarity between strain P25^T and members of other species of the genus *Sphingobium* with validly published names ranged from 94.0 to 97.7 %. The DNA–DNA relatedness between strain P25^T and *Sphingobium amiense* IAM 15006^T and other related strains was found to be less than 30 %, confirming it to represent a novel species. The DNA G + C content of strain P25^T was 65 mol%. The polyamine profile showed the presence of spermidine. The predominant cellular fatty acids were summed feature 8 (18 : 1 ω 7c and/or 18 : 1 ω 6c; 48.3 %), 16 : 0 (13.7 %) and 14 : 0 2-OH (8.8 %). The polar lipid profile of strain P25^T also corresponded to those reported for sphingomonads (phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylidimethylethanolamine, phosphatidylglycerol, phosphatidylcholine, sphingoglycolipid), supporting its identification as a member of the family *Sphingomonadaceae*. The results obtained from DNA–DNA hybridization and biochemical and physiological tests clearly distinguished strain P25^T from closely related members of the genus *Sphingobium*. Thus, a novel species of the genus *Sphingobium* is proposed, *Sphingobium quisquiliarum* sp. nov. The type strain is P25^T (=MTCC 9472^T =CCM 7543^T).

Sphingomonads belong to the class *Alphaproteobacteria*, order *Sphingomonadales*. The order *Sphingomonadales* contains the genera *Sphingomonas*, *Sphingobium*, *Novosphingobium*, *Sphingopyxis* (Takeuchi *et al.*, 2001) and *Sphingosinicella* (Maruyama *et al.*, 2006). Previous studies of a hexachlorocyclohexane (HCH)-contaminated dump site revealed that sphingomonads were the predominant group of micro-organisms at such sites (Dadhwal *et al.*, 2009; Böltner *et al.*, 2005). These sphingomonads tolerate very high levels of HCH residues, and many could degrade HCH isomers (Dadhwal *et al.*, 2009).

A soil sample was collected from the HCH dump site, serially diluted and plated on nystatin/streptomycin-amended Luria–Bertani (LB) agar plates (Vanbroekhoven *et al.*, 2004; Dadhwal *et al.*, 2009). A yellow colony that

appeared within 36 h of incubation at 28 °C was picked and purified by repeated streaking on LB agar. The ability of the strain, designated P25^T, to degrade α -, β -, γ - and δ -HCH was determined by a resting cell assay as described previously (Sharma *et al.*, 2006). *Sphingobium indicum* B90A^T, which can degrade all HCH isomers, was used as a reference (Kumari *et al.*, 2002; Raina *et al.*, 2008) in the degradation assay (Supplementary Fig. S1, available in IJSEM Online). Strain P25^T was found to degrade all four HCH isomers, with activity decreasing in the order $\alpha > \gamma > \beta > \delta$. Moreover, strain P25^T degraded the α - and γ -HCH isomers faster than *Sphingobium indicum* B90A^T (Supplementary Fig. S1). Strain P25^T was characterized taxonomically by using a polyphasic approach (Kumar *et al.*, 2008; Singh & Lal, 2009; Jit *et al.*, 2008) and was found to represent a novel species of the genus *Sphingobium*.

16S rRNA gene sequencing of strain P25^T was carried out as described by Prakash & Lal (2006). The sequence obtained was assembled manually using Clone Manager software, version 5. A continuous stretch of 1381 bp of the 16S rRNA gene sequence of strain P25^T was subjected to similarity searches by using the SEQUENCE MATCH tool of the

Abbreviation: HCH, hexachlorocyclohexane.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain P25^T is EU781657.

Details of HCH degradation, details of the polar lipid profile and an electron micrograph of cells of strain P25^T and DNA–DNA hybridization results are available as supplementary material with the online version of this paper.

Ribosomal Database Project II (<http://rdp.cmc.msu.edu/html/>) and the BLAST program of National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>). Nearly full-length 16S rRNA gene sequences of strains closely related to P25^T were retrieved from GenBank for the reconstruction of a phylogenetic tree. The selected sequences were aligned using the CLUSTAL_X program, version 1.81b (Thompson *et al.*, 1997). The alignment was checked manually for quality and terminal nucleotides not common to all the 16S rRNA gene sequences were removed. Phylogenetic analysis was carried out using the PHYLIP package, version 3.5 (Felsenstein, 1993). The evolutionary tree was constructed using the maximum-likelihood method (Felsenstein, 1981) and analysis based on 100 resamplings, using the SEQBOOT, CONSENSE and DNAMLK program in the PHYLIP package (Fig. 1). The evolutionary distance matrix was calculated using the correction of Jukes & Cantor (1969). Similar tree topology was also found with parsimony and neighbour-joining methods (not shown). Strain P25^T showed highest sequence similarity with *Sphingobium amiense* IAM 15006^T (97.7 %) followed by *Sphingobium fuliginis* TKP^T (97.4 %), *Sphingobium chlorophenolicum* ATCC 33790^T (97.3 %), *Sphingobium herbicidovorans* MH^T (97.2 %) and *Sphingobium cloacae* S-3^T (97.0 %).

DNA–DNA hybridization was carried out between strain P25^T and five closely related type strains that showed more than 97 % 16S rRNA gene sequence similarity. Total

genomic DNA of the six strains was extracted and purified and hybridization was done by following the protocols described by Bala *et al.* (2004) and Tourova & Antonov (1987). The amount of bound probe DNA was calculated by using a scintillation counter (Wallac Microbeta Trilux 1450; Perkin Elmer). Percentage relatedness, calculated on the basis of data obtained (mean of the three replicates) by DNA–DNA hybridization, varied from 10.6 to 30 % (Supplementary Table S1). Pooled standard deviations of all the hybridization experiments were found to vary up to 14.6 %. All hybridization values were below 70 %, as is recommended for the delineation of species (Wayne *et al.*, 1987).

Fatty acid methyl ester analysis was carried out as described by Prakash *et al.* (2007). The fatty acid profile consisted of 14:0 (0.4 %), 16:0 (13.7 %), 18:0 (1.8 %), 16:1 ω 5c (1.8 %), summed feature 8 (18:1 ω 7c and/or 18:1 ω 6c; 48.3 %), 18:1 ω 5c (4.8 %), 14:0 2-OH (8.8 %), 15:0 2-OH (0.5 %), 16:0 2-OH (1.6 %), 18:1 iso 3-OH (0.8 %), 19:0 cyclo ω 8c (2.5 %), 11-methyl 18:1 ω 7c (6.6 %) and summed feature 3 (16:1 ω 7c and/or 15:0 iso 2-OH; 3.9 %) (Table 1). The presence of the 2-hydroxy fatty acids 14:0 2-OH and 16:0 2-OH and the absence of 3-hydroxy fatty acids are common features of sphingomonads (Busse *et al.*, 1999). Strain P25^T showed the major fatty acids summed feature 8 (18:1 ω 7c and/or 18:1 ω 6c), 16:0 and 14:0 2-OH, which are commonly found in members of the genus *Sphingobium* (Takeuchi *et al.*, 2001).

Fig. 1. Phylogenetic tree based on complete 16S rRNA gene sequences showing the relationships between strain P25^T and other members of the genus *Sphingobium*. The tree was constructed by the maximum-likelihood method and rooted by using *Zymomonas mobilis* ZM4 as an outgroup. Numbers at nodes represent bootstrap values (based on 100 resamplings). GenBank accession numbers are shown in parentheses. Bar, 0.1 substitutions per nucleotide position.

Table 1. Cellular fatty acid profiles of strain P25^T and the type strains of related *Sphingobium* species

Strains: 1, strain P25^T; 2, *Sphingobium amiense* IAM 15006^T; 3, *Sphingobium fuliginis* JCM 10874^T; 4, *Sphingobium herbicidovorans* DSM 6383^T; 5, *Sphingobium chlorophenolicum* ATCC 33790^T; 6, *Sphingobium cloacae* JCM 10874^T. All data were generated in this study; strains were grown on TSA at 28 °C for 48 h before fatty acid analysis. Values are percentages of total fatty acids; –, not detected.

Fatty acid	1	2	3	4	5	6
14:0	0.4	0.3	0.4	—	0.1	0.4
15:0	—	—	0.2	—	—	8.7
16:0	13.7	6.5	8.3	8.8	11.7	4.6
17:0	—	—	0.3	0.3	0.5	—
18:0	1.8	—	0.2	2.0	0.6	—
20:0	—	—	—	—	—	—
16:1 ω 5c	1.8	3.1	1.2	1.4	1.9	0.8
17:1 ω 6c	—	4.4	1.7	1.5	2.1	1.8
17:1 ω 8c	—	0.4	—	—	0.1	—
18:1 ω 5c	4.8	2.8	0.9	2.5	1.7	6.3
12:0 2-OH	—	—	—	—	0.2	0.2
14:0 2-OH	8.8	13.4	10.5	5.7	16.9	31.6
15:0 2-OH	0.5	0.9	0.4	—	0.3	0.8
16:0 2-OH	1.6	0.3	0.5	0.8	0.8	1.2
16:1 2-OH	—	0.3	—	0.4	—	0.6
18:1 2-OH	—	—	—	—	—	0.6
17:0 iso 3-OH	—	—	—	—	—	2.3
18:1 iso 3-OH	0.8	—	—	—	—	—
11-Methyl 18:1 ω 7c	6.6	2.2	—	6.9	2.6	2.6
10-Methyl 18:0	—	—	—	—	—	0.9
19:0 cyclo ω 8c	2.5	—	—	0.8	—	—
Summed feature 2*	—	—	—	—	13.8	0.8
Summed feature 3*	3.9	20.0	—	11.7	13.8	2.7
Summed feature 8*	48.3	44.8	65.8	56.5	45.9	41.2

*Summed features are groups of two or three fatty acids that cannot be separated with the MIDI System. Summed feature 2 contains 16:1 iso 1 and/or 14:0 3-OH; summed feature 3 contains 16:1 ω 7c and/or 15:0 iso 2-OH; summed feature 8 contains 18:1 ω 7c and/or 18:1 ω 6c.

The fatty acid profile of strain P25^T revealed qualitative and quantitative difference from the profiles of other closely related strains (Table 1).

Polar lipid analysis of strain P25^T was done by two-dimensional TLC as described by Gupta *et al.* (2009). The polar lipid profile of strain P25^T contained phosphatidylethanolamine, phosphatidylglycerol, phosphatidylmethylethanolamine, diphosphatidylglycerol, phosphatidylcholine and sphingoglycolipid (Supplementary Fig. S2 and Supplementary Table S1). These are common lipids reported for sphingomonads (Busse *et al.*, 1999). From comparison of the polar lipid profiles of strain P25^T and phylogenetically close relatives in the genus *Sphingobium*, it is evident that the sphingoglycolipid of strain P25^T corresponds to SGL-1 of *Sphingobium fuliginis* MTCC 7295^T (Prakash & Lal, 2006) and SGL of *Sphingobium*

chlorophenolicum ATCC 33790^T (Busse *et al.*, 1999). The presence of sphingoglycolipid confirms that strain P25^T is a member of the family *Sphingomonadaceae* (Yabuuchi *et al.*, 1990; Busse *et al.*, 1999; Takeuchi *et al.*, 2001). However, phosphatidylmonomethylethanolamine, present in small amounts in other species of the genus *Sphingobium*, was absent from the polar lipid profile of strain P25^T.

Polyamines were extracted as described by Busse & Auling (1988) and analysed by one-dimensional TLC (silica gel 60 F₂₅₄, 20 × 20 cm; Merck). Spermidine was identified as the polyamine in strain P25^T, which is a characteristic feature of the genus *Sphingobium* (Busse *et al.*, 1999; Takeuchi *et al.*, 2001). The G+C content of strain P25^T calculated by the method of Gonzalez & Saiz-Jimenez (2002) was 65 mol%.

Cell morphology was examined using light and electron microscopy (Morgagni Fei 269D transmission electron microscope). Cells were negatively stained with 0.5 % uranyl acetate followed by preparation and examination of carbon-coated grids for transmission electron microscopy (Supplementary Fig. S3). Microscope study revealed that strain P25^T is non-flagellated. Motility of the organism was also tested on motility agar. Colonies of strain P25^T showed maximum growth on LB agar, nutrient agar and tryptic soya agar (TSA) at 28 °C within 24–48 h of incubation. The maximum colony diameter was 0.5–1.5 mm. Colonies of strain P25^T were yellow, circular and smooth. Gram-staining was carried out by using a Gram stain kit (HiMedia). Susceptibility towards antibiotics was determined on Mueller–Hinton agar using ready-made susceptibility test discs (HiMedia). Antibiotics tested were as follows (µg antibiotic per disc): amikacin (30), ampicillin (10), chloramphenicol (30), ciprofloxacin (5), gentamicin (10), kanamycin (30), oxytetracycline (30), polymyxin B (300), rifampicin (5), tetracycline (30) and vancomycin (30). Further characterization of strain P25^T was carried out using several biochemical and physiological tests and the results were compared with closely related members of the genus *Sphingobium* (Table 2). All biochemical tests were carried out at 28 °C. Production of acids from carbohydrates and degradation of xanthine and hypoxanthine were determined by Gordon *et al.* (1974). Growth at 4, 10, 20, 28, 37, 40 and 45 °C, at pH 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 and at NaCl concentrations of 0, 1, 2, 3, 4 and 5 % (w/v) and hydrolysis of Tween 20 were tested according to Arden-Jones *et al.* (1979). Urease activity was tested as described by Christensen (1946). Hydrolysis of casein, gelatin, starch and aesculin was tested as described by Cowan & Steel (1965). β -Galactosidase activity was assessed using HiMedia ONPG discs. Catalase and oxidase production was tested as described by McCarthy & Cross (1984). DNase activity was tested using HiMedia DNase agar. Nitrate reduction was tested by the method described by Prakash & Lal (2006).

The inference drawn from DNA–DNA hybridization and comparison of 16S rRNA gene sequences, fatty acid and polar lipid profiles and morphological, biochemical

Table 2. Differential morphological and physiological characteristics of strain P25^T and phylogenetically closely related members of the genus *Sphingobium*

Strains: 1, strain P25^T; 2, *Sphingobium amiense* IAM 15006^T; 3, *Sphingobium fuliginis* TKP^T; 4, *Sphingobium herbicidovorans* DSM 11019^T; 5, *Sphingobium chlorophenolicum* ATCC 11390^T; 6, *Sphingobium cloacae* JCM 10874^T. All data were generated in this study. All strains were negative for acid production from lactose, mannose, *myo*-inositol, adonitol, malonate and ribose and for utilization of gelatin, casein, Tween 80, citrate, starch, xanthine and hypoxanthine. All strains were positive for oxidase and catalase.

Characteristic	1	2	3	4	5	6
Colony colour*	Y	CY	Y	Y	Y	CY
Motility	–	+	–	+	–	–
Hydrolysis of:						
Aesculin	+	–	+	+	+	+
Tween 20	+	+	–	+	+	–
Urease	–	–	–	–	+	–
β -Galactosidase	–	–	–	+	–	–
Indole test	+	–	–	–	–	–
DNase	–	–	+	+	–	–
Nitrate reduction	–	–	+	+	+	–
Acid production from:						
Glucose	+	+	+	+	+	–
Galactose	+	+	+	+	+	–
Xylose	+	+	+	+	+	–
Fructose	+	+	+	–	–	+
Sucrose	+	+	–	+	–	+
Cellobiose	+	–	–	+	+	–
Mannitol	–	+	–	–	–	–
Raffinose	+	–	+	+	+	+
Sorbitol	+	+	–	–	–	–
Trehalose	+	–	+	+	+	–

*CY, Creamy yellow; Y, yellow.

and physiological properties with closely related members of the genus *Sphingobium* is that strain P25^T represents a novel species of the genus *Sphingobium*, for which the name *Sphingobium quisquiliarum* sp. nov. is proposed.

Description of *Sphingobium quisquiliarum* sp. nov.

Sphingobium quisquiliarum (quis.qui.li.a'rum. L. gen. pl. n. *quisquiliarum* of refuse, rubbish, trash, referring to the isolation of the type strain from an HCH dump site).

Gram-negative, aerobic, non-flagellated, non-motile, non-spore-forming, rod-shaped bacterium (2.0–2.3 μ m long and 0.5–1.5 μ m in diameter). Colonies are yellow, small (diameter 0.1–1.5 mm on LB agar after 48 h of incubation at 28 °C), entire, smooth, circular, convex and opaque. The optimum temperature, pH and NaCl concentration for growth are 28 °C, pH 7 and 1% (w/v). Catalase- and oxidase-positive. Nitrate is not reduced to nitrite. Can hydrolyse aesculin and Tween 20 but not Tween 80, gelatin,

starch or casein. No β -galactosidase activity is detected. Indole production is positive. Acid is produced from D-glucose, galactose, xylose, fructose, sucrose, cellobiose, raffinose, sorbitol and trehalose (after a long period of incubation) but not from lactose, mannose, *myo*-inositol, adonitol or malonate. Sensitive to amikacin, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, oxytetracycline, polymyxin B, rifampicin, tetracycline and vancomycin but resistant to ampicillin. Degrades α -, β -, γ - and δ -HCH isomers in liquid culture. Spermidine is present. Major polar lipids are phosphatidylglycerol, diphosphatidylglycerol and sphingoglycolipid. The major fatty acids are summed feature 8 (18:1 ω 7c and/or 18:1 ω 6c), 16:0 and 14:0 2-OH.

The type strain, P25^T (=MTCC 9472^T =CCM 7543^T), was isolated from HCH-contaminated soil at Ummari village, Lucknow, India.

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