Sphingobium chinhatense sp. nov., a hexachlorocyclohexane (HCH)-degrading bacterium isolated from an HCH dumpsite

Mandeep Dadhwal, Simran Jit, Hansi Kumari and Rup Lal

Molecular Biology Laboratory, Department of Zoology, University of Delhi, Delhi – 110007, India

A yellow-pigmented, hexachlorocyclohexane (HCH)-degrading bacterium, strain IP26T, was isolated from an HCH dumpsite and subjected to a polyphasic analysis in order to determine its taxonomic position. Strain IP26T showed maximum 16S rRNA gene sequence similarity with Sphingobium francense Sp+T (98.5 %), Sphingobium japonicum UT26T (98.4 %) and Sphingobium indicum B90AT (98.2 %). Phylogenetic analysis based on 16S rRNA gene sequences also showed that strain IP26T formed a cluster with these three HCH-degrading strains. Chemotaxonomic data (major polyamine, spermidine; major quinone, ubiquinone with ten isoprene units; major polar lipids, phosphatidylmonomethylethanolamine, phosphatidylethanolamine, diposphatidylglycerol, phosphatidylcholine; and presence of 2-hydroxy fatty acid) supported inclusion of strain IP26T in the genus Sphingobium. However, the results of DNA–DNA hybridization and morphological and biochemical tests clearly allowed phenotypic and genotypic differentiation of strain IP26T from recognized species of the genus Sphingobium. Strain IP26T thus represents a novel species of the genus Sphingobium for which the name Sphingobium chinhatense sp. nov. is proposed. The type strain is IP26T (=MTCC8598T =CCM 7432T).

Hexachlorocyclohexane (HCH) contamination can occur as a result of the unusual production process of this compound (Lal et al., 2006). HCH is prepared by the chlorination of benzene in the presence of UV. This generally leads to the production of four main HCH isomers, α-, β-, γ- and δ-, in the ratio 60–70, 5–12, 10–12 and 6–10 %, respectively (Willett et al., 1998). Among these, only γ-HCH, also called lindane, has insecticidal activity. During the purification process, the remaining isomers (one tonne of lindane produces nine tonnes of HCH waste) are discarded in the open, thus creating an HCH-contaminated dumpsite. We discovered one such dumpsite in the vicinity of an industry that has been producing lindane for the past 10 years (Dadhwal et al., 2009). Around 24 bacterial strains were isolated from these sites and their potential to degrade HCH isomers was evaluated (Dadhwal et al., 2009). Some of these bacterial isolates have been characterized by using a polyphasic approach (Kumar et al., 2008; Singh & Lal, 2009).

Strain IP26T was isolated from highly HCH-contaminated soil by culturing on LB agar using a serial dilution method (Dadhwal et al., 2009). The strain was found to degrade α-, β-, γ- and δ-HCH isomers faster than Sphingobium indicum B90AT (Dadhwal et al., 2009) (Supplementary Fig. S1, available in IJSEM Online). Strain IP26T was characterized taxonomically and found to represent a novel species of Sphingobium.

Colony size, shape and colour were studied on LB agar plates incubated at 28 °C. Gram-staining and spore-staining were performed using HiMedia kits. Motility of strain IP26T was assessed by the hanging drop method as well as on motility agar medium. Catalase and oxidase tests were carried out as described by McCarthy & Cross (1984). Hydrolysis of aesculin and Tween 80 and the ability of this strain to grow in the presence of NaCl were tested as described by Arden-Jones et al. (1979). Acid production from carbohydrates and degradation of xanthine and hypoxanthine were tested as described by Gordon et al. (1974). Other physiological properties were examined as described by Collins et al. (1989). Strain IP26T was cultivated on LB at 4, 10, 20, 28, 37, 40 and 45 °C to determine growth at different temperatures. Growth at pH 3–10 was evaluated in LB adjusted with HCl or NaOH. Antibiotic sensitivity was checked on Mueller–Hinton II medium using ready-made discs (HiMedia) with varying

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

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain IP26T is EF190507.

Four supplementary figures and two supplementary tables are available with the online version of this paper.
amounts of erythromycin, kanamycin, tetracycline and ampicillin.

Strain IP26<sup>T</sup> is Gram-stain-negative, non-motile, rod-shaped and non-spore-forming. It formed yellow-pigmented, convex, circular colonies after 48 h of incubation. The temperature range for growth of strain IP26<sup>T</sup> was 20–40 °C. No growth occurred at 45 °C. The pH range for growth was pH 6–9. Strain IP26<sup>T</sup> was positive for oxidase and catalase activity. Other morphological and biochemical characteristics of strain IP26<sup>T</sup> are given in the species description and those characteristics that differentiate strain IP26<sup>T</sup> from related members of the genus *Sphingobium* are listed in Table 1.

The 16S rRNA gene of strain IP26<sup>T</sup> was amplified by colony PCR as described previously (Kumar *et al.*, 2008). The amplified product was purified and used for cycle sequencing with BigDye terminator chemistry and a 3100 Avant Genetic Analyzer (Applied Biosystems). A 16S rRNA gene sequence similarity search was conducted using the Ribosomal Database Project (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp) and the BLAST program of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Sequence similarity analysis indicated that the nearest relatives of strain IP26<sup>T</sup> are *Sphingobium francense* Sp<sup>T</sup> (98.5 %), *Sphingobium japonicum* UT262<sup>T</sup> (98.4 %) and *Sphingobium indicum* B90A<sup>T</sup> (98.2 %).

A phylogenetic tree was reconstructed as described by Prakash & Lal (2006). All sequences were aligned using the CLUSTAL_X program (Thompson *et al.*, 1997), gaps common to all sequences were removed and the alignment was checked manually. Evolutionary trees were inferred using maximum-parsimony (Fitch, 1971), neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) algorithms. The evolutionary distance matrix was calculated using the correction of Jukes & Cantor (1969). The topology of the resultant tree was evaluated using bootstrap analysis (Felsenstein, 1985) of the neighbour-joining method based on 100 resamplings. The tree indicated that strain IP26<sup>T</sup> branched with its

### Table 1. Physiological characteristics of strain IP26<sup>T</sup> compared with other physiologically related members of the genus *Sphingobium*

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closest neighbours, *Sphingobium francense* Sp+T, *Sphingobium japonicum* UT26T and *Sphingobium indicum* B90A3T (Fig. 1).

Fatty acid methyl ester (FAME) analysis was carried out at the Disha Institute of Biotechnology, Nagpur, India, using the following procedure. Fatty acid methyl esters were analysed from 2–4 loops of inoculum scraped from a Petri dish and subjected to saponification, methylilation and extraction using the methods of Miller (1982) and Kuykendall *et al.* (1988). The FAME mixtures were separated using the Sherlock Microbial Identification System (MIDI), and fatty acids were identified using the Aerobe (TSBA version 50) database. Polyamines were extracted as described by Busse & Auling (1988) and analysed by TLC at IMTECH, Chandigarh, India. A 10 μl sample was applied to a TLC plate (Silica gel 60, 20 × 20 cm; Merck 105554) and ethylacetate/cyclohexane (2 : 3) was used as the running solvent. Fluorescence was observed at 310 nm. Quinones were extracted from 200 mg dry cell mass with a 10 % aqueous solution of 0.3 % (w/v) NaCl in methanol and petroleum ether (60–80 °C boiling point) at a ratio of 1 : 1. The upper phase was collected and dried in a rotavapor (Buchi). The residue was dissolved in 100 μl acetone. The extract was developed on a TLC plate (Silica gel 60 F254, 20 × 20 cm; Merck 105554) using petroleum ether (boiling point 60–80 °C) and diethyl ether (85 : 15, v/v). Purified ubiquinones were dissolved in 2-propanol and analysed by reversed-phase TLC according to Collins & Jones (1980). Polar lipid analysis was carried out according to the methods described by Tindall (1990a, b). Lyophilized culture (100 mg) was extracted with 10 ml solvent containing chloroform/methanol/0.3 % aqueous NaCl (1 : 2 : 0.8). After extraction, the chloroform layer was dried in a rotavapor and redissolved in 250 μl chloroform/methanol (2 : 1). Polar lipids were analysed by two-dimensional TLC (Silica gel 60 F254, 20 × 20 cm; Merck 105554) using chloroform/methanol/acetic acid (65 : 25 : 4) for the first dimension and chloroform/methanol/acetic acid/water (80 : 12 : 15 : 4) for the second dimension. Individual lipids were detected by using the following spraying reagents: ninhydrin for lipids containing free amino groups (Consden & Gordon, 1948); Zinzadze reagent for lipids containing phosphate esters (Dittmer & Lester, 1964); Dragendorff reagent for lipids containing quaternary nitrogen compounds (Wagner *et al.*, 1961; Beiss, 1964); and *α*-naphthol for carbohydrate-containing lipids (Jacin & Mishkin, 1965). Molybdatophosphoric acid was used to detect total lipids (Gunstone & Jacobsberg, 1972). An aqueous premulic solution (1 %) was also used to detect total lipids (Gupta *et al.*, 2009).

The similarity index of the isolate from the Aerobe TSBA 50 library showed that the profile of strain IP26T is closest to those of members of the genus *Sphingobium*. The fatty acid profile of strain IP26T is shown in Supplementary Table S1. The major fatty acids were C18 : 1v7c (49.1 %), C14 : 02-OH (13.95 %) and C16 : 0 (13.2 %), a common feature of the genus *Sphingobium* (Takeuchi *et al.*, 2001). The presence of C14 : 02-OH, and sometimes other 2-hydroxy fatty acids, and a lack of 3-hydroxy fatty acids are

**Fig. 1.** Phylogenetic tree based on 16S rRNA gene sequences showing the relationship between strain IP26T and other *Sphingobium* species. The tree was reconstructed by using the maximum-likelihood method and rooted by using *Zymomonas mobilis* ATCC 10988T as the outgroup. Nineteen species were used to reconstruct the phylogenetic tree. Numbers at nodes represent bootstrap values (based on 100 resamplings). GenBank accession numbers for 16S rRNA gene sequences are shown in parentheses. Bar, 0.1 substitution per nucleotide position.
common features of the sphingomonads (Busse et al., 1999). The fatty acid profile of strain IP26\textsuperscript{T} showed minor qualitative and quantitative differences from S. francense Sp\textsuperscript{+\textsuperscript{T}}, S. japonicum UT26\textsuperscript{T} and S. indicum B90A\textsuperscript{T}, but major differences from other phylogenetically distinct species (Supplementary Table S1). Both spermine and spermidine were identified as polyamines in strain IP26\textsuperscript{T}. Spermidine was the major polyamine, which is a characteristic feature of the genus Sphingobium (Busse et al., 1999; Takeuchi et al., 2001). Only ubiquinone Q-10 was found in strain IP26\textsuperscript{T}. Ubiquinone Q-10 is found in all members of the Alphaproteobacteria and is also an important feature of the sphingomonads. All members of the genus Sphingobium have been shown to contain Q-10 (Busse et al., 1999). The polar lipid profile of strain IP26\textsuperscript{T} comprised phosphatidylmonomethyllethanolamine, phosphatidylphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylcholine, phosphoglycolipids, an unknown aminolipid, an unknown glycolipid and unknown phospholipids (Supplementary Fig. S4). These are the common polar lipids reported for sphingomonads (Busse et al., 1999). The polar lipid profile of strain IP26\textsuperscript{T} was not identical to those of other phylogenetically close members of the genus Sphingobium, S. francense Sp\textsuperscript{+\textsuperscript{T}}, S. japonicum UT26\textsuperscript{T} and S. indicum B90A\textsuperscript{T}. Phosphatidylphosphatidylglycerol was absent from the polar lipid profile of S. francense Sp\textsuperscript{+\textsuperscript{T}}, S. japonicum UT26\textsuperscript{T} and S. indicum B90A\textsuperscript{T} but present in strain IP26\textsuperscript{T} (Pal et al., 2005). However, phosphatidylphosphatidylglycerol was present in the lipid profiles of other species of the genus Sphingobium (Busse et al., 1999). Similarly, strain IP26\textsuperscript{T} contained a very small amount of phosphatidylmonomethyllethanolamine compared with S. francense Sp\textsuperscript{+\textsuperscript{T}}, S. japonicum UT26\textsuperscript{T} and S. indicum B90A\textsuperscript{T} (Pal et al., 2005).

We have reported previously that the HCH-degrading sphingomonads S. francense Sp\textsuperscript{+\textsuperscript{T}}, S. japonicum UT26\textsuperscript{T} and S. indicum B90A\textsuperscript{T} contain lin genes responsible for the degradation of HCH, which are associated with IS6100 elements (Dogra et al., 2004). Southern blot hybridization was performed to determine the copy number of lin genes and IS6100 elements in strain IP26\textsuperscript{T} (Dadhwal et al., 2009). Two copies of linA (Supplementary Fig. S2) and one copy of linB, linC and linDER were found in strain IP26\textsuperscript{T} (data not shown). However, the positions of the lin genes in the genome of strain IP26\textsuperscript{T} were not the same as those reported for S. francense Sp\textsuperscript{+\textsuperscript{T}}, S. japonicum UT26\textsuperscript{T} and S. indicum B90A\textsuperscript{T} (Dadhwal et al., 2009). We also detected more than four copies of the IS6100 element in strain IP26\textsuperscript{T} (Supplementary Fig. S3).

DNA–DNA hybridization of strain IP26\textsuperscript{T} was carried out according to Kumar et al. (2008) with closely related Sphingobium strains. The DNA–DNA hybridization values of strain IP26\textsuperscript{T} with S. francense Sp\textsuperscript{+\textsuperscript{T}}, S. japonicum UT26\textsuperscript{T}, S. indicum B90A\textsuperscript{T}, S. herbicidovorans DSM 11019\textsuperscript{T}, S. chungbukense IMSNU 11152\textsuperscript{T} and S. chlorophenolicum ATCC 33790\textsuperscript{T} were 54, 39, 30, 10, 16.3 and 12 %, respectively (Supplementary Table S2). All DNA–DNA hybridization values were below the threshold value (70 %) that has been suggested for delineating a bacterial species (Wayne et al., 1987). The results of DNA–DNA hybridization show that strain IP26\textsuperscript{T} represents a novel species of the genus Sphingobium.

It is concluded from the above phenotypic and phylogenetic data that, in spite of the high 16S rRNA gene sequence similarity, strain IP26\textsuperscript{T} represents a novel species within the genus Sphingobium, for which the name Sphingobium chinhatense sp. nov. is proposed.

**Description of Sphingobium chinhatense sp. nov.**

*Sphingobium chinhatense* (chin.hat.en’se. N.L. neut. adj. chinhatense pertaining to Chinhat, from where the type strain was isolated).

Cells are Gram-stain-negative, non-motile, strictly aerobic, rod-shaped and non-spore-forming. Colonies are yellow, circular, smooth and pinpoint after 5 days of growth on LB media. Degrades α-, β-, δ- and γ- isomers of HCH. Positive for oxidase and catalase activity, but negative for indole production and starch, casein and gelatin hydrolysis. Sensitive to 5 % NaCl but tolerant of 3 % NaCl. Does not grow at pH 10. Aesculin is hydrolysed but no hydrolysis of xanthine, hypoxanthine or Tewens 20 or 80 is observed. Assimilates L-serine, N-acetylglucosamine, salicin, caprate, sodium benzoate and sodium pyruvate but does not assimilate lactose, D-xylose, D-ribose, adonitol, D-mannitol, D-sorbitol, L-histidine, L-sorbos, dulcitol or raffinose. Acid is produced from D-glucose, D-galactose, D-arabinose, D-xylose, D-fructose, D-ribose, trehalose, L-proline and myo-inositol but no acid is produced from cellobiose, sucrose or mellibiose. Sensitive to erythromycin (15 µg), kanamycin (30 µg) and tetracycline (30 µg) and resistant to ampicillin (10 µg). A detailed fatty acid profile is presented in Supplementary Table S1. Major fatty acids present are C\textsubscript{18:1}\textasciitilde, C\textsubscript{16:0} and C\textsubscript{14:0} 2-OH. Major polar lipids are phosphatidylmonomethyllethanolamine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylcholine, phosphoglycolipid, phosphatidylphosphatidylglycerol and unknown aminolipid, unknown glycolipid and five unidentified phospholipids. Major polyamine is spermidine. The respiratory quinone consists of ubiquinone with ten isoprene units (Q-10).

The type strain, IP26\textsuperscript{T} (=MTCC8598\textsuperscript{T} = CCM 7432\textsuperscript{T}), was isolated from an HCH dumpsite of the lindane manufacturing industry at Chinhat, Lucknow, India.

**Acknowledgements**

This work was supported by grants from the Department of Biotechnology (DBT) and National Bureau of Agriculturally Important Micro-organisms (NBAIM), Government of India. M. D., S. J. and H. K. acknowledge CSIR, Government of India, for providing research fellowships. We would also like to thank Dr Rakesh Jain for polyamine analysis and Dr J. P. Euzéby for etymological advice.

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