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

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A yellow-pigmented, hexachlorocyclohexane (HCH)-degrading bacterium, designated F2T, was isolated from an HCH dumpsite at Ummari village in Lucknow, India. Phylogenetic analysis based on 16S rRNA gene sequences showed that the isolate belonged to the genus Sphingobium. Its closest neighbour was Sphingobium japonicum UT26T (99.2% 16S rRNA gene sequence similarity). The DNA G+C content was 65.7 mol%. The polyamine profile showed the presence of spermidine. The respiratory pigment was ubiquinone Q-10. The predominant cellular fatty acids were C16:0 (12.5%), C14:0 2-OH (8.1%), summed feature 3 (consisting of C16:1v7c and/or C18:1v6c; 5.8%) and summed feature 8 (consisting of C18:1v7c and/or C18:1v6c; 53.1%). The major polar lipids of strain F2T were phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol and sphingoglycolipid. DNA–DNA relatedness and biochemical and physiological characters clearly distinguished the isolate from its closest phylogenetic neighbours. Thus, strain F2T represents a novel species of the genus Sphingobium, for which the name Sphingobium lucknowense sp. nov. is proposed. The type strain is strain F2T (=MTCC 9456T =CCM 7544T).

The genus Sphingobium of the order Sphingomonadales was proposed by Takeuchi et al. (2001) after the subdivision of the genus Sphingomonas. Presently, the sphingomonads belong to five genera: Sphingomonas, Sphingobium, Novosphingobium, Sphingopyxis (Takeuchi et al., 2001) and Sphingosinicella (Maruyama et al., 2006). The sphingomonads are known for their role in biodegradation of pollutants and bioremediation of polluted environments (Basta et al., 2005; Lal et al., 2010). They are known to degrade a wide range of xenobiotic compounds (Basta et al., 2005), one of which is hexachlorocyclohexane (HCH). HCH is a cyclic, saturated, chlorinated hydrocarbon that has been used extensively for the control of agricultural pests and in health programmes since the 1950s. Two forms of HCH have been used: technical HCH [a mixture of four HCH isomers: a (60–70%), β (5–12%), γ (10–15%) and δ (6–10%)] and lindane (γ-HCH; the only isomer with insecticidal properties). Since the purification of γ-HCH from technical HCH is expensive, both technical HCH and lindane have been used. The purification of 1 tonne of γ-HCH results in the generation of 10–12 tonnes of waste, containing a-, β- and δ-HCH (Walker et al., 1999), which have been discarded in open dumpsites. Although the use of both technical HCH and lindane is banned in many countries, the problem of HCH contamination continues to exist because of the dumpsites (Lal et al., 2010). Sphingomonads have been found to be the predominant bacteria at HCH-contaminated dumpsites (Böttner et al., 2005; Dadhwal et al., 2009).

A dumpsite created in 1997 in India (Jit et al., 2011) was found to contain several sphingomonads capable of HCH degradation (Dadhwal et al., 2009). This dumpsite has been the focus of our studies for the past 5 years and we have reported several HCH-degrading sphingomonads (Singh & Lal, 2009; Bala et al., 2010) and HCH-tolerant bacteria (Sharma et al., 2010) from here. It is interesting to note that bacteria with better abilities to degrade HCH isomers have been found at this site, which contains 35 000–40 000 tonnes of HCH waste (Jit et al., 2011). In the present investigation, we isolated from the dumpsite a sphingomonad that was found to degrade all four HCH isomers faster than other sphingomonads isolated at the same site (Dadhwal et al., 2009).

The soil sample was serially diluted and plated on Luria–Bertani (LB) agar supplemented with nystatin and streptomycin (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 isolate, strain F2T, was characterized taxonomically.
using a polyphasic approach described by Kumar et al. (2008) and Singh & Lal (2009).

Amplification and sequencing of the 16S rRNA gene was carried out as described by Prakash et al. (2007). The sequence thus obtained was assembled manually using Clone Manager version 5. A continuous stretch (1436 bp) of the 16S rRNA gene sequence of strain F2\textsuperscript{T} was obtained and subjected to similarity searches using the sequence matching tool of the Ribosomal Database Project II (http://rdp.cme.msu.edu/) and the NCBI BLAST program (http://blast.ncbi.nlm.nih.gov/). The nearly full-length 16S rRNA gene sequences of strains closely related to strain F2\textsuperscript{T} were retrieved from GenBank and aligned using CLUSTAL X version 1.81b (Thompson et al., 1997). The alignment was checked manually for quality and terminal nucleotides not common to all sequences were removed. Phylogenetic analysis was carried out using PHYLIP version 3.5 (Felsenstein, 1993). A phylogenetic tree was constructed with the maximum-likelihood method (Felsenstein, 1981) and bootstrap analysis was based on 1000 resamplings, using SEQBOOT, CONSENSE and DNAMLK in the PHYLIP package. The evolutionary distance matrix was calculated using the correction of Jukes & Cantor (1969). Strain F2\textsuperscript{T} showed high 16S rRNA gene sequence similarity to Sphingobium japonicum UT26\textsuperscript{T} (99.2 %), S. indicum B90A\textsuperscript{T} (99.0 %), S. francense Sp\textsubscript{+}\textsuperscript{T} (98.8 %), S. chlorophenolicum ATCC 33790\textsuperscript{T} (98.6 %), S. chinhatense IP26\textsuperscript{T} (98.5 %), S. rhizovicinum CC-FH12-1\textsuperscript{T} (97.6 %), S. changbukense DJ77\textsuperscript{T} (97.6 %), S. vermicomposti VC-230\textsuperscript{T} (97.4 %), S. amiense YT\textsuperscript{T} (97.2 %) and S. quisquiliarum P25\textsuperscript{T} (97.2 %). In the maximum-likelihood phylogenetic tree, the isolate formed a distinctly separate lineage within the genus Sphingobium (Fig. 1). The topologies of trees generated with parsimony, neighbouring-joining and maximum-likelihood methods were found to be similar.

DNA–DNA hybridization was carried out between strain F2\textsuperscript{T} and the ten most closely related strains (>97 % 16S rRNA gene sequence similarity). Total genomic DNA was extracted, purified and hybridized as described by Kumar et al. (2008) and Tourova & Antonov (1987). The amount of bound probe DNA was calculated using a liquid scintillation counter (Wallac 1450 MicroBeta Trilux; PerkinElmer). The maximum standard deviation found in all experiments was 5.8 %. Strain F2\textsuperscript{T} showed low DNA–DNA relatedness (3.1–56.4 %) with all of the reference strains (Supplementary Table S1, available in IJSEM Online). As the threshold value of 70 % DNA–DNA similarity was not met, the species was described as Sphingobium lucknowense sp. nov.

**Fig. 1.** Maximum-likelihood phylogenetic tree based on complete 16S rRNA gene sequences showing the relationships between strain F2\textsuperscript{T} and other members of the genus Sphingobium. Bootstrap values (>500) based on 1000 resamplings are shown at branch nodes. Brevundimonas diminuta DSM 1635 was used as an outgroup. Bar, 1 substitution per 1000 nucleotide positions.
relatedness is recommended for the delineation of bacterial species (Wayne et al., 1987), the results confirmed that strain F2T represented a novel species of the genus Sphingobium.

Fatty acid methyl ester analysis was carried out at the Disha Institute of Biotechnology, Ahmadabad, India. Cultures were grown on trypticase soy broth agar (TSBA) for 24 h and two to four loops of inoculum scraped from the third sector of a quadrant streak were saponified, methylated and extracted using the methods of Miller (1982) and Kuykendall et al. (1988). The fatty acid methyl esters were separated using the Sherlock Microbial Identification System (MIDI, USA) and identified using the Aerobe version TSBA 50 database. The fatty acid composition consisted of C14:0 (0.5 %), C16:0 (12.5 %), C17:0 (0.7 %), C18:0 (0.9 %), C12:0 2-0H (0.1 %), C14:0 2-OH (8.1 %), C15:0 2-OH (0.4 %), C16:0 2-OH (1.3 %), C16:1o5c (3.8 %), C17:1o8c (0.2 %), C17:1o6c (3.5 %), C18:1o5c (4.1 %), C20:1o7c (0.4 %), iso-C20:0 (0.3 %), 11-methyl C18:1o7c (2.3 %), C19:0 cyclo 08c (1.3 %), summed feature 3 (C16:1o7c and/or C16:1o6c; 5.8 %) and summed feature 8 (C18:1o7c and/or C18:1o6c; 53.1 %) (Table 1). A common feature of sphingomonads is the presence of 2-hydroxy fatty acids and the absence of 3-hydroxy fatty acids (Busse et al., 1999). The major fatty acids of strain F2T were C16:0, C14:0 2-OH, summed feature 3 and summed feature 8, which are commonly found in the genus Sphingobium (Takeuchi et al., 2001). However, there were both qualitative and quantitative differences between the fatty acid composition of the isolate and those of the reference strains (Table 1).

Quinones were extracted from 200 mg dry cell mass with a 10 % aqueous solution of 0.3 % (w/v) NaCl in methanol/petroleum ether (boiling point 60–80 °C) in 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) using petroleum ether/diethyl ether (85:15, v/v). Purified ubiquinones were dissolved in 2-propanol and analysed by reversed-phase TLC according to Collins & Jones (1980). The isolate contained ubiquinone Q-10. Polar lipid analysis of strain F2T was carried out by two-dimensional TLC as described by Gupta et al. (2009). Aqueous (1 %) premulin was used for the detection of lipids under UV light. Polar lipid analysis of strain F2T exhibited the presence of phosphatidylethanolamine, phosphatidylmethylethanolamine, phosphatidylglycerol, diposphatidylglycerol, sphingoglycolipid and unknown phospholipids (Supplementary Fig. S1). Polyamines were extracted as described by Busse & Auling (1988) and analysed by one-dimensional TLC, using 10 μl of the extracted sample on a silica gel plate and ethyl acetate/cyclohexane (2:3, v/v) as the solvent. Strain F2T contained spermidine, which is a characteristic feature of the genus Sphingobium (Busse et al., 1999; Takeuchi et al., 2001). The G+C content of strain F2T was calculated by the method described by Gonzalez & Saiz-Jimenez (2002) using a 7500

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*Summed features represent groups of two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 3 consisted of C16:1o7c and/or C18:1o6c; summed feature 7 consisted of C19:0o7c and/or C19:0o6c; summed feature 8 consisted of C18:1o7c and/or C18:1o6c.

Real-Time PCR system (Applied Biosystems) and was found to be 65.7 mol%.

Cell morphology was examined using transmission electron microscopy (TEM 269D; Morgagni Fei) (Supplementary Fig. S2). Strain F2T was non-flagellated, and the non-motility of the organism was confirmed using motility agar. The growth characteristics of strain F2T were studied on LB agar, NA and tryptic soy agar (TSA). Strain F2T grew well under aerobic conditions on the media tested (28 °C, 24–48 h). Colonies were yellow, circular and smooth and varied in diameter.
from 0.9 to 1.5 mm. Gram-staining was performed using a Gram-stain kit (HiMedia). All phenotypic tests were carried out at 28 °C unless otherwise stated. Susceptibility to antibiotics was determined on Mueller–Hinton agar using ready-made susceptibility test discs (HiMedia), containing (µg 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 F2T was carried out using biochemical and physiological tests. Growth at 4, 10, 20, 28, 37, 40 and 45 °C, at pH 4–10 (at intervals of 1 pH unit) and with 0–9 % NaCl (w/v) (at intervals of 1 % NaCl) was determined as described by Arden-Jones et al. (1979). Acid production from carbohydrates and degradation of xanthine and hypoxanthine was determined by Gordon et al. (1974). Hydrolysis of Tweens 20 and 80 was 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 tested using ONPG discs (HiMedia). Catalase and oxidase production was tested as described by McCarthy & Cross (1984). Nitrate reduction was tested as described by Prakash et al. (2007). Characteristics that differentiate the isolate from closely related members of the genus Sphingobium are given in Table 2.

On the basis of the phenotypic, chemotaxonomic and phylogenetic analysis, strain F2T represents a novel species of the genus Sphingobium, for which the name Sphingobium lucknowense sp. nov. is proposed.

Description of Sphingobium lucknowense sp. nov.

*Sphingobium lucknowense* (luck.no.wen’se. N.L. neut. adj. lucknowense of or belonging to Lucknow).

Cells are Gram-negative, aerobic, non-flagellated, non-motile, non-spore-forming rods (1.5 µm long and 0.6–0.7 µm wide). Colonies are yellow, small (diameter 0.9–1.5 mm on LB agar after 48 h of incubation at 28 °C), entire, smooth, circular, convex and opaque. The optimum conditions for growth are 28 °C, pH 7 and 2% NaCl. Catalase-negative and oxidase-positive. Nitrate is not reduced to nitrite. Hydrolyses casein, but not Tweens 20 or 80, gelatin, starch or aesculin. No β-galactosidase activity is detected. Indole production is negative. Produces acid from D-glucose, galactose, xylose, lactose, mannose, maltose, rhamnose, trehalose and L-arabinose, but not from fructose, sucrose, cellobiose, mannitol, raffinose, ribose, sorbitol or myo-inositol. Sensitive to amikacin, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, oxytetracycline, polymyxin B, rifampicin, tetracycline and vancomycin, but resistant to ampicillin. Degradation of α-, β-, γ- and δ-HCH isomers in liquid culture. Spermidine is present. The major polar lipids are phosphatidylethanolamine, phosphatidyl-methylethanolamine, phosphatidylglycerol, diphasatidylglycerol and sphingoglycolipid. The respiratory pigment ubiquinone Q-10 is present. The major fatty acids are C16:0, C14:0 2-OH, summed feature 3 (C16:1ω7c and/or C16:1ω6c) and summed feature 8 (C18:1ω7c and/or C18:1ω6c). The G+C content of the type strain is 65.7 mol% (real-time PCR).

The type strain, F2T (=MTCC 9456T=CCM 7544T), was isolated from HCH-contaminated soil at Ummari village, Lucknow, India.

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