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 F2^T, 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* UT26^T (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 C_{16:0} (12.5 %), C_{14:0} 2-OH (8.1 %), summed feature 3 (consisting of C_{16:1}ω7c and/or C_{16:1}ω6c; 5.8 %) and summed feature 8 (consisting of C_{18:1}ω7c and/or C_{18:1}ω6c; 53.1 %). The major polar lipids of strain F2^T were phosphatidylethanolamine, phosphatidylmethylethanolamine, phosphatidylglycerol, diphosphatidylglycerol and sphingoglycolipid. DNA–DNA relatedness and biochemical and physiological characters clearly distinguished the isolate from its closest phylogenetic neighbours. Thus, strain F2^T represents a novel species of the genus *Sphingobium*, for which the name *Sphingobium lucknowense* sp. nov. is proposed. The type strain is strain F2^T (=MTCC 9456^T =CCM 7544^T).

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: α (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 α-, 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 (Böltner *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 F2^T, was characterized taxonomically

Abbreviation: HCH, hexachlorocyclohexane.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain F2^T is EF534725.

Two supplementary figures and a supplementary table are available with the online version of this paper.

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^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^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^T showed high 16S rRNA gene sequence

similarity to *Sphingobium japonicum* UT26^T (99.2%), *S. indicum* B90A^T (99.0%), *S. francense* Sp+^T (98.8%), *S. chlorophenolicum* ATCC 33790^T (98.6%), *S. chinhatense* IP26^T (98.5%), *S. rhizovicinum* CC-FH12-1^T (97.6%), *S. chungbukense* DJ77^T (97.6%), *S. vermicomposti* VC-230^T (97.4%), *S. amiense* YT^T (97.2%) and *S. quisquiliarum* P25^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^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^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

Fig. 1. Maximum-likelihood phylogenetic tree based on complete 16S rRNA gene sequences showing the relationships between strain F2^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 F2^T 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 C_{14:0} (0.5 %), C_{16:0} (12.5 %), C_{17:0} (0.7 %), C_{18:0} (0.9 %), C_{12:0} 2-OH (0.1 %), C_{14:0} 2-OH (8.1 %), C_{15:0} 2-OH (0.4 %), C_{16:0} 2-OH (1.3 %), C_{16:1}ω5c (3.8 %), C_{17:1}ω8c (0.2 %), C_{17:1}ω6c (3.3 %), C_{18:1}ω5c (4.1 %), C_{20:1}ω7c (0.4 %), iso-C_{20:0} (0.3 %), 11-methyl C_{18:1}ω7c (2.3 %), C_{19:0} cyclo ω8c (1.3 %), summed feature 3 (C_{16:1}ω7c and/or C_{16:1}ω6c; 5.8 %) and summed feature 8 (C_{18:1}ω7c and/or C_{18:1}ω6c; 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 F2^T were C_{16:0}, C_{14:0} 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 F2^T 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 F2^T exhibited the presence of phosphatidylethanolamine, phosphatidylmethylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, 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 F2^T 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 F2^T was calculated by the method described by Gonzalez & Saiz-Jimenez (2002) using a 7500

Table 1. Cellular fatty acid compositions of strain F2^T and type strains of closely related species of the genus *Sphingobium*

Strains: 1, *Sphingobium lucknowense* sp. nov. F2^T; 2, *S. japonicum* UT26^T; 3, *S. indicum* B90A^T; 4, *S. francense* Sp+^T; 5, *S. chlorophenolicum* ATCC 33790^T; 6, *S. chinhatense* IP26^T; 7, *S. rhizovicinum* CC-FH12-1^T; 8, *S. chungbukense* DJ77^T; 9, *S. vermicomposti* VC-230^T; 10, *S. amiense* YT^T; 11, *S. quisquiliarum* P25^T. All data were obtained in this study using cells cultured on LB agar at 28 °C for 48 h. Values are percentages of total fatty acids; –, not detected.

Fatty acid	1	2	3	4	5	6	7	8	9	10	11
C _{14:0}	0.5	0.2	0.2	0.2	0.4	0.2	0.2	0.7	0.8	0.2	0.5
C _{16:0}	12.5	7.8	10.7	10.4	8.5	8.2	7.3	3.7	6.6	4.6	7.8
C _{17:0}	0.7	0.8	1.4	2.2	1.1	–	–	–	1.5	0.9	0.3
C _{18:0}	0.9	0.9	–	2.7	–	1.4	2.2	–	–	–	–
C _{19:0}	–	–	–	0.1	–	–	–	0.1	0.1	–	0.1
C _{16:1} ω5c	3.8	3.9	2.6	2.1	4.0	3.0	2.6	2.4	3.9	1.7	3.9
C _{16:1} ω7c	–	0.1	–	0.3	–	–	–	–	0.4	–	–
C _{17:1} ω6c	3.3	3.4	3.8	5.2	3.8	5.4	5.7	2.9	6.1	14.7	3.5
C _{17:1} ω8c	0.2	0.3	0.2	0.2	0.2	0.9	0.2	0.1	1.0	1.8	0.5
C _{18:1} ω5c	4.1	4.5	4.9	5.3	5.8	5.2	5.3	7.4	5.0	3.7	11.0
C _{20:1} ω7c	0.4	0.2	0.3	0.3	0.2	0.4	0.5	–	0.4	0.4	0.3
C _{12:0} 2-OH	0.1	0.2	0.2	0.2	0.3	0.2	0.2	0.2	0.1	–	0.1
C _{14:0} 2-OH	8.1	7.7	11.6	11.5	14.7	9.0	7.8	12.1	10.0	7.3	2.9
C _{15:0} 2-OH	0.4	0.3	0.4	0.4	0.5	0.2	0.3	0.2	1.3	1.6	0.2
C _{16:0} 2-OH	1.3	1.2	1.5	2.3	1.8	1.3	1.0	0.8	2.3	0.7	0.3
C _{17:0} 2-OH	–	0.1	–	–	0.3	0.3	0.1	0.3	0.1	–	0.1
iso-C _{17:0}	–	–	–	–	–	0.1	0.2	–	1.3	0.2	0.2
iso-C _{19:0}	–	0.4	–	–	–	–	1.5	–	–	–	0.6
iso-C _{20:0}	0.3	–	0.1	–	–	0.2	0.3	–	–	–	0.1
11-Methyl C _{18:1} ω7c	2.3	–	3.7	3.7	1.8	2.7	2.4	4.8	2.7	2.1	4.2
C _{19:0} cyclo ω8c	1.3	0.1	0.2	0.2	0.3	0.2	0.7	1.1	0.8	–	2.6
Summed features*											
3	5.8	6.8	6.8	3.3	8.6	5.7	4.5	4.3	13.8	8.1	5.3
7	–	–	–	–	–	0.1	0.2	0.2	0.1	0.2	–
8	53.1	59.7	50.4	45.8	46.9	54.6	53.0	57.1	34.7	49.1	53.4

*Summed features represent groups of two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 3 consisted of C_{16:1}ω7c and/or C_{16:1}ω6c; summed feature 7 consisted of C_{19:1}ω7c and/or C_{19:1}ω6c; summed feature 8 consisted of C_{18:1}ω7c and/or C_{18:1}ω6c.

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 F2^T was non-flagellated, and the non-motility of the organism was confirmed using motility agar. The growth characteristics of strain F2^T were studied on LB agar, NA and tryptic soy agar (TSA). Strain F2^T 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 F2^T 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 F2^T 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. Degrades α -, β -, γ - and δ -HCH isomers in liquid culture. Spermidine is present. The major polar lipids are phosphatidylethanolamine, phosphatidylmethylethanolamine, phosphatidylglycerol, diphosphatidylglycerol and sphingoglycolipid. The respiratory pigment

Table 2. Differential morphological and physiological characteristics of strain F2^T and type strains of closely related species of the genus *Sphingobium*

Strains: 1, *S. lucknowense* sp. nov. F2^T; 2, *S. japonicum* UT26^T; 3, *S. indicum* B90A^T; 4, *S. francense* Sp + ^T; 5, *S. chlorophenolicum* ATCC 33790^T; 6, *S. chinhatense* IP26^T; 7, *S. rhizovicinum* CC-FH12-1^T; 8, *S. chungbukense* DJ77^T; 9, *S. vermicomposti* VC-230^T; 10, *S. amiense* YT^T; 11, *S. quisquiliarum* P25^T. All data were obtained in this study. All strains formed yellow colonies and were positive for acid production from glucose, galactose and rhamnose. All strains were negative for xanthine and hypoxanthine degradation and H₂S production.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Motility	+	–	–	–	–	–	+	–	+	+	–
Hydrolysis of:											
Aesculin	–	+	–	+	+	–	+	+	+	–	+
Tween 20	–	+	+	+	+	–	+	+	+	–	+
Tween 80	–	+	–	–	–	–	–	–	–	–	–
Casein	+	–	–	–	–	–	+	–	+	–	–
Gelatin	–	–	–	–	–	–	+	–	–	–	–
Starch	–	–	+	–	–	–	–	–	–	–	–
Nitrate reduction	–	–	–	–	–	+	+	–	–	–	–
Production of:											
Indole	–	–	–	–	–	–	–	–	–	–	+
Citrate	–	–	–	–	–	–	+	–	–	–	–
Oxidase	+	+	+	+	+	+	–	+	+	+	+
Catalase	–	+	+	+	+	+	–	+	+	+	+
Urease	–	+	+	+	+	+	+	–	–	–	–
β -Galactosidase	–	–	–	–	–	–	+	–	+	–	–
Acid production from:											
Xylose	+	+	+	+	+	+	–	+	+	+	+
Fructose	–	+	+	–	–	+	+	+	+	+	+
Sucrose	–	+	–	–	–	–	–	–	–	+	+
Cellobiose	–	+	–	+	+	–	–	–	–	–	+
Mannitol	–	–	–	–	–	–	–	–	–	+	–
Raffinose	–	–	–	+	+	+	+	–	–	–	+
Sorbitol	–	–	–	–	–	–	–	–	–	+	+
Trehalose	+	+	+	–	+	+	+	+	+	–	+
Maltose	+	–	+	+	–	+	+	+	–	–	+
Ribose	–	+	+	+	–	+	–	+	–	–	–
Lactose	+	–	–	+	–	+	+	+	+	–	–
<i>myo</i> -Inositol	–	–	–	–	–	+	–	–	–	–	–
L-Arabinose	+	+	+	+	+	+	+	–	+	–	+

ubiquinone Q-10 is present. The major fatty acids are C_{16:0}; C_{14:0} 2-OH, summed feature 3 (C_{16:1}ω7c and/or C_{16:1}ω6c) and summed feature 8 (C_{18:1}ω7c and/or C_{18:1}ω6c). The G + C content of the type strain is 65.7 mol% (real-time PCR).

The type strain, F2^T (=MTCC 9456^T =CCM 7544^T), was isolated from HCH-contaminated soil at Ummari village, Lucknow, India.

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