Flavobacterium lindanitolerans sp. nov., isolated from hexachlorocyclohexane-contaminated soil

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A Gram-negative, non-spore-forming, cream–yellow-pigmented bacterial strain, IP-10^T, was isolated from soil samples from a waste site highly contaminated with hexachlorocyclohexane in Ummari village, India. The organism showed the highest 16S rRNA gene sequence similarity of 92.7% with *Flavobacterium soli* KCTC 12542^T and levels of 87–92% with the type strains of other recognized species of the genus *Flavobacterium*. The DNA G + C content of strain IP-10^T was 31 mol%. The predominant fatty acids were iso-C_{15:0} (22.1%), iso-C_{17:0} 3-OH (18.5%) and summed feature 3 (comprising C_{16:1ω7c} and/or iso-C_{15:0} 2-OH; 13.2%). Strain IP-10^T could be differentiated from recognized species of the genus *Flavobacterium* based on a number of phenotypic features. Strain IP-10^T is therefore considered to represent a novel species of the genus *Flavobacterium*, for which the name *Flavobacterium lindanitolerans* sp. nov. is proposed. The type strain is IP-10^T (=MTCC 8597^T=CCM 7424^T).

The genus *Flavobacterium*, the type genus of the family *Flavobacteriaceae*, was proposed by Bergey *et al.* (1923) and the description was considerably emended by Bernardet *et al.* (1996). It belongs to the phylum *Bacteroidetes* (Bernardet & Bowman, 2006) (formerly the *Flavobacterium–Flexibacter–Bacteroides* group; Woese *et al.*, 1990). Members of the genus are widely distributed in nature and are routinely isolated from soil (Yoon *et al.*, 2006), sediments, freshwater and marine water ecosystems, microbial mats (Van Trappen *et al.*, 2003, 2004), sea ice (Humphry *et al.*, 2001) and infected fish. The genus *Flavobacterium* comprises Gram-negative, non-spore-forming, strictly aerobic, predominantly gliding, pigmented bacteria with menaquinone-6 as the major respiratory quinone and a DNA G + C content of 30–41 mol% (Bernardet & Bowman, 2006; Park *et al.*, 2006). *Flavobacterium aquatile* is the type species of the genus.

The present study deals with the taxonomic characterization of a novel species of the genus *Flavobacterium* isolated from soil from a recently created waste site for a lindane manufacturing unit situated in Ummari village, Lucknow city, in the state of Uttar Pradesh, India. Lindane, γ -hexachlorocyclohexane (HCH), is the only HCH isomer that has insecticidal properties. Strain IP-10^T was isolated after 7 days incubation at 28 °C by plating serially diluted

soil samples on Luria–Bertani agar plates (10 g tryptone, 5 g yeast extract, 5 g NaCl, 1 g D-glucose, 15 g agar per litre) containing nystatin (30 µg ml⁻¹) and streptomycin (200 µg ml⁻¹), used to isolate HCH-degrading sphingomonads (Vanbroekhoven *et al.*, 2004). 16S rRNA gene sequence analysis, fatty acid profiling and other phenotypic characteristics revealed that the new isolate belonged to the genus *Flavobacterium* but exhibited sufficient significant differences from other closely related members of the genus to be considered as representing a novel species.

16S rRNA gene sequence analysis was carried out as described by Prakash *et al.* (2007). Briefly, a single colony of strain IP-10^T was picked with a sterile toothpick, suspended in 50 µl of sterile deionized water and heated at 95 °C for 10 min. Following centrifugation at 9000 g for 5 min and ten-fold dilution with sterile deionized water, the 16S rRNA gene sequence was amplified by using the universal primer pair 27F (5'-AGAGTTTGATCCTGG-CTCAG-3') and 1492R (5'-TACGTTACCTTGTTA-CGACTT-3'). The amplified product was purified and used for cycle sequencing with BigDye terminator chemistry and run on a 3100 Avant Genetic Analyzer (Applied Biosystems).

The stretch of the 16S rRNA gene sequence obtained (1359 bp) was checked manually for quality and gaps. Similarity searches were conducted by using the Sequence Match tool of 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>). For construction of phylogenetic trees, sequences of the 19 most closely

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

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain IP-10^T is EF424395.

A phylogenetic tree obtained by using the EzTaxon server is available with the online version of this paper.

related species of the genus *Flavobacterium* were selected from the RDP and NCBI databases. The 16S rRNA gene sequence of the type strain of *Cellulophaga lytica* was used as an outgroup and all the selected sequences were aligned via the CLUSTAL_X program (Thompson *et al.*, 1997). Terminal nucleotides not common to all sequences were removed. Phylogenetic analysis was carried out by using the PHYLIP package, version 3.5c (Felsenstein, 1993), and TREECON (Van de Peer & De Wachter, 1994). The evolutionary distance matrix was calculated according to the distance model of Jukes & Cantor (1969) and an evolutionary tree was constructed by using the neighbour-joining method of Saitou & Nei (1987). The topology of the phylogenetic tree was statistically evaluated based on 100 resamplings by using the SEQBOOT and CONSENSE programs in the PHYLIP package and the bootstrap option in the TREECON program. Phylogenetic analysis was also performed with the parsimony method by using DNAPARS, including bootstrap analysis of 100 resamplings.

Strain IP-10^T showed highest 16S rRNA gene sequence similarity of 92.7% with *Flavobacterium soli* KCTC 12542^T, and showed levels of similarity of 87–92% with the type strains of other recognized species of the genus *Flavobacterium*. Strain IP-10^T occupied a separate position in the neighbour-joining tree (Fig. 1) and formed a distinct cluster with *Flavobacterium columnare* ATCC 23463^T,

Flavobacterium terrae DSM 18829^T, *Flavobacterium saliperosum* CGMCC 1.3801^T, *Flavobacterium suncheonense* KACC 11423^T, *Flavobacterium cucumis* DSM 18830^T and *Flavobacterium croceum* EMB47^T. This grouping was clearer when the EzTaxon server (<http://www.eztaxon.org/>; Chun *et al.*, 2007) was used to construct the tree (see Supplementary Fig. S1 in IJSEM Online). This topology was confirmed in the maximum-parsimony tree. Based on low levels of 16S rRNA gene sequence similarity and phylogenetic analysis, it was concluded that strain IP-10^T represents a novel species of the genus *Flavobacterium*.

Fatty acid methyl ester (FAME) analysis was carried out at the Microbial Type Culture Collection (MTCC) division of the Institute of Microbial Technology, Chandigarh, India. For this purpose, strain IP-10^T was grown on trypticase soy agar (TSA) at 37 °C for 22–24 h. Extraction, saponification and methylation of fatty acids were carried out as described by Miller (1982) and Kuykendall *et al.* (1988). The mixture of FAMES was separated on a gas chromatograph (Agilent 6890) equipped with flame-ionization detector. Identification of FAMES was accomplished by using the Aerobe (TSBA50, version 5) database of the Sherlock Microbial Identification System (MIDI Inc.).

The major fatty acids of strain IP-10^T were iso-C_{15:0} (22.0%), iso-C_{17:0} 3-OH (18.5%) and summed feature 3

Fig. 1. Phylogenetic tree based on nearly-complete 16S rRNA gene sequences showing the relationships between strain IP-10^T and a selection of species of the genus *Flavobacterium*. The tree was constructed by the neighbour-joining method and rooted by using *Cellulophaga lytica* ATCC 23178^T as an outgroup. Numbers at nodes represent bootstrap percentages (based on 100 resamplings; only values >50% are shown). Bar, 0.05 substitutions per nucleotide position.

(comprising C_{16:1ω7c} and/or iso-C_{15:0} 2-OH; 13.2 %). A predominance of iso-C_{15:0} and iso-C_{17:0} 3-OH is found in most members of the family *Flavobacteriaceae*. Overall, the FAME profile of strain IP-10^T matched that of other members of the genus *Flavobacterium* (Bernardet *et al.*, 1996; Bernardet & Bowman, 2006), but minor differences in the respective proportions of some components might have resulted from the different growth conditions used.

Growth of strain IP-10^T was tested on nutrient agar, Luria–Bertani agar, TSA, Anacker and Ordal's agar (Anacker & Ordal, 1955) and MacConkey agar. As strain IP-10^T showed luxuriant growth on TSA and in the corresponding broth (TSB) at 37 °C, most phenotypic tests were performed at this temperature. Gram and spore staining were performed by using HiMedia's Gram and spore staining kits, respectively. Colony morphology (shape, size, colour and pigmentation) was studied on TSA after 72 h incubation at 37 °C. For observation of cell morphology, a suspension of strain IP-10^T was placed on a carbon-coated copper grid, negatively stained with uranyl acetate (0.5 %) and observed by using a transmission electron microscope (model 269D; Morgagni). Gliding motility was investigated by using phase-contrast microscopy of a hanging-drop preparation from a TSB culture (Bernardet *et al.*, 2002). Growth at different pH and salt concentrations was tested in TSB adjusted to pH 4, 5, 6, 7, 7.5, 8, 9 and 10 with 1.0 M HCl or 1.0 M NaOH or supplemented with 1, 2, 3, 4 or 5 % (w/v) NaCl as described by Arden-Jones *et al.* (1979). Growth in TSB was also tested at 10, 25, 37 and 40 °C. Antibiotic resistance was tested on Muller–Hinton agar by using commercial discs (HiMedia); plates were incubated at 37 °C for 72 h. Catalase and oxidase production was tested as described by McCarthy & Cross (1984). Tests for hydrolysis of casein, gelatin, starch, tyrosine, aesculin and Tween 80 were performed as described by Cowan & Steel (1965). Hydrolysis of chitin, pectin and carboxymethyl-cellulose was tested on Czapek–Dox agar (2 g NaNO₃, 1 g K₂HPO₄, 0.5 g MgSO₄, 0.5 g KCl, 0.01 g FeSO₄ and 15 g agar per litre) containing 0.5 % (w/v) of each substrate. Urease activity was tested according to Christensen (1946). Tests for the production of indole, the reduction of nitrate and the Voges–Proskauer reaction were performed according to Smibert & Krieg (1994). Production of H₂S was tested on stabs of triple-sugar iron agar (15 g peptone, 5 g proteose-peptone, 3 g beef extract, 3 g yeast extract, 1 g lactose, 1 g sucrose, 1 g glucose, 5 g NaCl, 0.2 g FeSO₄, 0.3 g Na₂S₂O₃, 0.024 g phenol red and 12 g agar per litre). Production of a precipitate on egg yolk was tested on TSA containing 30 % egg yolk emulsion (HiMedia). Production of β-galactosidase was determined by using commercial discs (HiMedia) following the manufacturer's instructions. DNase activity was tested by using DNase agar (HiMedia) supplemented with 0.005 % methyl green. The presence of flexirubin-type pigments and Congo red absorption were determined according to Reichenbach (1992) and Bernardet *et al.* (2002). Utilization of glucose, sucrose and lactose was tested as described by Hugh & Leifson

(1953) under aerobic as well as anaerobic conditions (created by overlaying sterile liquid paraffin). Importantly, this medium contained peptone (0.2 %) in which anaerobic growth of some members of the genus *Flavobacterium* has been reported (Bernardet & Bowman, 2006). The ability to degrade HCH was tested according to Sharma *et al.* (2006). The G+C content of the genomic DNA was determined as described by Gonzalez & Saiz-Jimenez (2002) by using an Applied Biosystems 7500 real-time PCR at the Department of Zoology, University of Delhi, India.

The morphological, physiological and biochemical features of strain IP-10^T are given in the species description below, in Table 1 and in Fig. 2. Although allocated to the genus *Flavobacterium* based on 16S rRNA gene sequence data, strain IP-10^T could be differentiated from all related species of the genus *Flavobacterium* based on a number of phenotypic characteristics (Table 1). DNA–DNA hybridization experiments were not performed as 16S rRNA gene sequence similarity with other strains was well below the recommended limit of 97 % (Wayne *et al.*, 1987). Strain

Table 1. Differential phenotypic characteristics between strain IP-10^T and phylogenetically related species of the genus *Flavobacterium*

Strains: 1, IP-10^T; 2, *F. terrae* DSM 18829^T (data from Weon *et al.*, 2007); 3, *F. cucumis* DSM 18830^T (Weon *et al.*, 2007); 4, *F. aquatile* DSM 1132^T; 5, *F. columnare* LMG 13035^T (Bernardet *et al.*, 1996); 6, *F. croceum* EMB47^T (Park *et al.*, 2006); 7, *F. indicum* DSM 17447^T (Saha & Chakrabarti, 2006); 8, *F. saliperosum* JCM 13331^T (Wang *et al.*, 2006); 9, *F. suncheonense* KACC 11423^T (Kim *et al.*, 2006). +, Positive; –, negative; (+), weakly positive; ++, strongly positive; v, variable among the data sources; ND, data not available.

Characteristic	1	2	3	4	5	6	7	8	9
Growth on:									
Nutrient agar	+	+	+	–	–	–	(+)	+	+
TSA	++	(+)	(+)	+	–	(+)	–	+	(+)
Gliding motility	+	–	+	+	+	–	–	–	–
Production of:									
Oxidase	–	+	+	+	+	+	+	–	+
Catalase	+	–	+	+	+	–	(+)	+	+
Flexirubin-type pigment	–	+	–	–	+	–	–	+	–
Nitrate reductase	–	–	–	v	v	–	–	–	–
β-Galactosidase	–	–	–	+	–	ND	ND	ND	–
Hydrolysis of:									
Aesculin	+	–	+	v	–	–	–	–	–
Gelatin	–	+	+	v	+	+	+	+	+
Starch	–	+	+	v	–	–	+	–	–
Tyrosine	–	–	+	v	–	–	–	+	+
Absorption of	–	–	–	–	+	–	–	–	–
Congo red									
DNA G+C content (mol%)	31	34	38	33	32	41	31	41	39

Fig. 2. Transmission electron micrographs of negatively stained cells of strain IP-10^T showing a group of parallel rods (a) and a chain of rods (b). Bars, 2 μm.

IP-10^T is thus considered to represent a novel species of the genus *Flavobacterium*, for which the name *Flavobacterium lindanitolerans* sp. nov. is proposed.

Description of *Flavobacterium lindanitolerans* sp. nov.

Flavobacterium lindanitolerans (lin.da.ni.to.le'rans. N.L. n. *lindanum* lindane; L. part. adj. *tolerans* tolerating; N.L. part. adj. *lindanitolerans* lindane-tolerating).

Cells are Gram-negative, non-sporulating, aerobic, non-flagellated rods that are motile by gliding, 0.5 μm in diameter and 1.8 μm in length, occurring singly, in groups of parallel cells or in short chains. Predominantly aerobic. Weak anaerobic growth occurs in peptone-containing media. Growth occurs on Luria–Bertani, nutrient, Anacker and Ordal's and MacConkey agars. Colonies on TSA are flat, cream–yellow with entire margins and 3.5 mm in diameter after 72 h incubation. Growth occurs at 10–37 °C (optimum, 37 °C), at pH 6–9 (optimum, 7.5) and in the presence of 1–2% NaCl (optimum, 1%). Catalase activity is present, but oxidase, urease and nitrate reductase activities are absent. Aesculin is hydrolysed, but

gelatin, casein, DNA, starch, carboxymethylcellulose, pectin, chitin, tyrosine and Tween 80 are not. Although isolated from a highly HCH-contaminated environment, the organism is unable to degrade HCH. Congo red is not absorbed by the colonies. Flexirubin-type pigments are not produced (KOH test-negative). No pigment is produced on tyrosine agar. Indole and H₂S are not produced. No precipitate is formed on egg yolk agar. Methyl red and Voges–Proskauer reactions are negative. No acid is produced from glucose, sucrose or lactose. Sensitive to nalidixic acid (30 μg), tetracycline (30 μg), vancomycin (30 μg), amoxicillin (10 μg), penicillin (10 μg), neomycin (30 μg), novobiocin (30 μg), chlortetracycline (30 μg) and neomycin (30 μg), but resistant to streptomycin (10 μg), kanamycin (30 μg), chloramphenicol (30 μg), rifamycin (5 μg), erythromycin (15 μg), ampicillin (10 μg) and gentamicin (10 μg). Major fatty acids are C_{15:0} (22.1%), iso-C_{17:0} 3-OH (18.5%), summed feature 3 (comprising iso-C_{15:0} 2-OH and/or C_{16:1}ω7c; 13.2%), iso-C_{15:0} 3-OH (8.8%), iso-C_{15:1} G (6.7%), iso-C_{16:0} (5.2%), C_{18:0} (4.6%), C_{15:0} 3-OH (3.5%), C_{15:0} 2-OH (3.1%), iso-C_{16:0} 3-OH (2.3%), iso-C_{17:1}ω9c (1.7%), C_{17:0} 3-OH (1.2%) and anteiso-C_{15:0} (1.1%). Traces (<1%) of the following fatty acids are also present: iso-C_{17:0}, C_{17:1}ω6c and C_{18:1}ω9c. The G+C content of the genomic DNA as determined by real-time PCR is 31 mol%.

The type strain, IP-10^T (=MTCC 8597^T=CCM 7424^T), was isolated from soil that was highly contaminated by HCH from a waste site at Ummari village, Lucknow city, Uttar Pradesh, India.

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