Sphingopyxis indica sp. nov., isolated from a high dose point hexachlorocyclohexane (HCH)-contaminated dumpsite

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A Gram-stain-negative, aerobic, non-motile, non-spore-forming, rod-shaped and light-yellow-pigmented bacterium, designated DS15T, was isolated from a soil sample collected from a hexachlorocyclohexane dumpsite in Lucknow, Uttar Pradesh, India. Strain DS15T showed highest 16S rRNA gene sequence similarity to Sphingopyxis panaciterrulae DCY34T (98.7%) and Sphingopyxis soli BL03T (98.0%). The 16S rRNA gene sequence similarity between strain DS15T and species of genus Sphingopyxis with validly published names ranged from 92.5% to 98.7%. The DNA G+C content of strain DS15T was 67.5 mol%. The chemotaxonomic markers in strain DS15T were consistent with its classification in the genus Sphingopyxis, i.e. Q-10 as the major ubiquinone and summed feature 8 (C18:1ω7c/C18:1ω6c), C17:1ω6c, summed feature 3 (C16:1ω7c/C16:1ω6c), C14:0 2-0H, C15:0 2-0H, C16:0 and C17:1ω8c as the predominant fatty acids. The major polar lipids of strain DS15T were phosphatidylethanolamine (PE), diphasatidylglycerol (DPG), phosphatidylcholine (PC), phosphatidylglycerol (PG) and sphingoglycolipids (SGL) and spermidine was detected as the major polyamine. Phylogenetic analysis, DNA–DNA hybridization, and chemotaxonomic and phenotypic analysis support the conclusion that strain DS15T represents a novel species within the genus Sphingopyxis, for which the name Sphingopyxis indica is proposed. The type strain is DS15T (=MTCC 9455T = CCM 7542T = MCC 2023T).

The genus Sphingopyxis was proposed by Takeuchi et al. (2001) and belongs to class Alphaproteobacteria, order Sphingomonadales and family Sphingomonadaceae. At the time of writing, 17 species of the genus Sphingopyxis have been taxonomically classified. Several species of this genus have been isolated from different ecological niches including soil (Lee et al., 2008a; Lee et al., 2008b; Srinivasan et al., 2010), landfill soil (Choi et al., 2010), hexachlorocyclohexane-contaminated soil (Sharma et al., 2010), sludge blanket (Kim et al., 2005), wastewater treatment plant (Kämpfer et al., 2002), river subsurface (Godoy et al., 2003), mineral water source (Lee et al., 2001 & Pal et al., 2006), hydrocarbon-contaminated soil (Zhang et al., 2010) and seawater (Yoon & Oh, 2005; Yoon et al., 2005; Kim et al., 2008; Vancanneyt et al., 2001). Members of the genus Sphingopyxis are distinctly yellow to brown-pigmented, Gram-negative, aerobic, non-spore-forming and non-fermenting, with a DNA G+C content in the range of 58–69.2 mol%. In the present study, we describe the phenotypic, chemotypic and genotypic properties of strain DS15T, a novel species of the genus Sphingopyxis to determine its taxonomic position.

A yellow-pigmented bacterium, designated strain DS15T, was isolated by standard dilution plating from a soil sample collected from a hexachlorocyclohexane-contaminated dumpsite, Lucknow, Uttar Pradesh in North India (27° 00‘ N and 81° 09‘ E). For isolation, 5 g soil was suspended in 45 ml sterile phosphate buffered saline (1×) [pH 7.2, containing NaCl (8.5 g l⁻¹), Na₂HPO₄·2H₂O (1.91 g l⁻¹) and KH₂PO₄ (0.38 g l⁻¹)] and incubated on a shaker maintained at 28 °C for 18 h. It was then serially diluted and plated on Luria–Bertani (LB) agar (HiMedia; 1 % tryptone, 0.5 % yeast extract, 0.5 % NaCl, 1.5 % agar) and incubated at 28 °C for 2 days. Colonies were then picked and cultured on LB agar. The culture was preserved in 20 % (v/v) glycerol at −80 °C for further study. Since strain DS15T showed very good growth on LB agar/broth at 28 °C, routine cultivation was done on LB agar/broth at 28 °C and all the morphological, physiological and biochemical characteristics of strain DS15T were investigated at this temperature.

Sequence analysis of the 16S rRNA gene of strain DS15T was carried out using a 3100 Avant Genetic Analyzer.

†These authors contributed equally to this work.

Abbreviations: RDP, Ribosomal Database Project; TEM, transmission electron microscopy.

The GenBank/EMBL/DBJ accession number for 16S rRNA gene sequence of strain DS15T is EF494193.

Two supplementary figures and a supplementary table are available with the online version of this paper.
(Applied Biosystems) at the Department of Zoology, University of Delhi, India. The 16S rRNA gene sequence obtained was checked both for quality and gaps using Sequencing Analysis v 5.1.1 software. A continuous stretch of 1401 bp of 16S rRNA gene sequence of strain DS15\textsuperscript{T} was assembled using Clone Manager software (version 5) and similarity searches were performed using the BLAST program of the National Center for Biotechnological Information (NCBI) (http://www.ncbi.nlm.nih.gov), the Sequence Match tool of the Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp) and the EzTaxon server (http://www.eztaxon.org/; Chun et al., 2007). The sequence similarity search revealed that the nearest neighbours of the strain DS15\textsuperscript{T} were Sphingopyxis panaciterrae JCM 14844\textsuperscript{T} (98.7\% 16S rRNA gene sequence similarity), Sphingopyxis soli JCM 15910\textsuperscript{T} (98.0\%), Sphingopyxis unnariensis CCM 7428\textsuperscript{T} (97.8\%), Sphingopyxis panaciterrae LMG 24003\textsuperscript{T} (97.6\%), Sphingopyxis granuli NBRC 100800\textsuperscript{T} (97.6\%), Sphingopyxis witflariensis DSM 14551\textsuperscript{T} (97.3\%), Sphingopyxis ginsengisoli LMG 23390\textsuperscript{T} (97.3\%), Sphingopyxis chilensis LMG 20986\textsuperscript{T} (97.1\%) Sphingopyxis macrogotaibida JCM 10192\textsuperscript{T} (97.0\%) and Sphingopyxis taeonensis KCTC 2884\textsuperscript{T} (97.0\%). Phylogenetic analysis was done by retrieving 16S rRNA gene sequences of the closely related species from the NCBI, RDP and EzTaxon databases. The 16S rRNA gene sequences of all closely related species along with Rhodanobacter lindanii clasticus PR5557\textsuperscript{T} (outgroup) were aligned using the CLUSTAL_X version 1.81 program (Thompson et al., 1997). The evolutionary distance matrix was generated using the distance model of Jukes and Cantor (1969) and the evolutionary tree was constructed using the maximum-likelihood method (Felsenstein, 1981) of the PHYLIP 3.69 software package (Felsenstein, 1993). The tree topology was evaluated by bootstrap analysis (Felsenstein, 1985) based on 100 replications (Fig. 1). Strain DS15\textsuperscript{T} formed a distinct cluster with Sphingopyxis panaciterrae DCY34\textsuperscript{T} and Sphingopyxis soli BL03\textsuperscript{T}.

The signature nucleotides found in the 16S rRNA gene sequence of strain DS15\textsuperscript{T} were shared in all positions based on Escherichia coli numbering (Brosius et al., 1978) with the pattern characteristic for the genus Sphingomonas sensu stricto (Takeuchi et al., 2001). These nucleotides were C:G at position 52:359, G at position 134, U at position 593 and G:C at position 987:1218. This further supported the placement of strain DS15\textsuperscript{T} within the genus Sphingopyxis.

Cell morphology and motility were observed with a light microscope (Nikon; 400 × magnification) using the hanging drop method with cells grown in LB broth for 3 days at 28 °C. The presence or absence of flagella was examined by transmission electron microscopy (TEM, Morgagni 269D TEM; Fei) using cells from an exponentially growing culture by negatively staining with 0.5% uranyl acetate followed by air drying and examination of the grids under TEM.

Growth at different temperatures (4, 10, 22, 28, 37, 42 and 55 °C) was tested on LB agar. The ability of strain DS15\textsuperscript{T} to grow at different salt concentrations (1–12%, w/v) and pH values (4.0 to 13.0, adjusted with 1.0 M HCl or 1.0 M NaOH, at intervals of 1 pH unit) was examined in LB broth at 28 °C as described by Arden-Jones et al. (1979). Gram and spore-staining were performed by using HiMedia’s Gram and spore staining kit. Colony morphology (shape, size, colour and pigmentation) was studied on LB agar after 72 h incubation at 28 °C. Catalase and oxidase production was tested as described by McCarthy & Cross (1984).

Hydrolysis of casein, gelatin, aesculin, starch and Tween 20 was determined as described by Cowan & Steel (1965). Substrate utilization as sole carbon source was tested by using the HiMedia’s kit for biochemical tests according to the manufacturer’s guidelines. Tests for indole production and nitrate reduction were performed according to the protocol of Smibert & Krieg (1994). Table 1 lists the differential morphological and physiological characteristics of strain DS15\textsuperscript{T} that differentiate it from related members of the genus Sphingopyxis.

Susceptibility to antibiotics was tested on Mueller–Hinton Agar (HiMedia) plates using antibiotic discs containing the following compounds (µg antibiotic per disc): chloramphenicol (30), gentamicin (10), kanamycin (30), oxytetracycline (30), rifampicin (5), tetracycline (30), vancomycin (30), polymyxin B (300), ciprofloxacin (5), amikacin (30), nalidixic acid (30) and penicillin (10).

Cell biomass for analysis of polar lipids, quinones and polyamines was obtained from growing cultures grown in LB broth at 28 °C by shaking at 200 r.p.m. followed by freeze drying. Polar lipid analysis of strain DS15\textsuperscript{T} was carried out using 100 mg lyophilized cell culture as described by Bligh & Dyer (1959) and separated by two-dimensional TLC using 9 x 9 cm silica plates (Merck). The solvent system chloroform : methanol : water [65 : 24 : 4 (by volume)] was used in first dimension and chloroform : methanol : acetic acid : water [8 : 12 : 15 : 4 (by volume)] in second dimension. The polar lipids profile was detected using a spray of 1% aqueous primuline solution viewed under UV light (Kumari et al., 2011). Polyamines were extracted as described by Busse & Auling, (1988) and analysed by one-dimensional TLC. Briefly, 15 µl of the extracted sample was applied to a TLC plate (Silica gel 60 F254, 20 × 20 cm, Merck) and the running solvent used was ethylacetate : cyclohexane (2 : 3, v/v). Quinones were extracted from 200 mg dry cell mass with a 10% aqueous solution of 0.3% NaCl (w/v) 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. 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 (boiling point 60–80 °C) and diethyl ether (85 : 15, v/v). Purified ubiquinones were dissolved in 2-propanol and analysed by reverse-phase TLC according to the protocol of Collins & Jones (1980).

For fatty acid methyl ester (FAME) analysis, cells were harvested from LB agar plates after incubation at 28 °C for...
3 days. Fatty acid methyl esters were analysed from two to four loops of inoculum of culture at almost the same phase of growth scraped from a Petri dish and subjected to saponification, methylation and extraction using the method of Miller (1982) and Kuykendall et al. (1988). The mixtures were separated using the Sherlock Microbial Identification System (MIDI) and identification of the fatty acids was made using the Aerobe database (RTSBA, version 121 6.0B). Summed feature 8 (C18:1ω7c/C18:1ω9c) (32.6 %), C17:1ω6c (21.2 %), summed feature 3 (C16:1ω7c/C16:1ω6c) (15.7 %), C14:0 2-OH (6.5 %), C15:0 2-OH (6.1 %), C16:0 (6.0 %) and C17:1ω8c (2.6 %) were the predominant fatty acids detected (Table 2). As reported by Takeuchi et al. (2001) the major fatty acids of genus Sphingopyxis are C18:1, saturated C16:0 and C16:1, C14:0 2-OH, C15:0 2-OH and/or C16:0 2-OH. Hence the profile obtained for strain DS15T is in accordance with that of the genus Sphingopyxis.

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 machine at the Department of Zoology, University of Delhi, India.
DNA–DNA hybridization was carried out between strain DS15T and ten reference strains, which showed more than 97 % 16S rRNA gene sequence similarity with strain DS15T. Total genomic DNA of all strains was extracted and purified and hybridization was done following the protocol as described by Kumar et al. (2008) and Tourova & Antonov (1988). The amount of bound probe DNA was calculated by using scintillation counter (1450 LSC & Luminescence Wallac Microbeta Trilux; PerkinElmer). Percentage relatedness calculated on the basis of the data obtained (mean of three replicates) by DNA–DNA hybridization varied from 8.4 % to 44.5 % (Table S1, available in IJSEM Online). All hybridization values were well below the 70 % cut-off point for species classification, as recommended by Wayne et al. (1987), thus supporting the view that the isolated strain DS15T represented a novel species of the genus Sphingopyxis.

### Table 1. Differential morphological and physiological characteristics of strain DS15T and other members of the genus Sphingopyxis

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
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<th>8</th>
<th>9</th>
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<th>11</th>
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<tbody>
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<td>Colony colour</td>
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<td>Pale yellow</td>
<td>Yellow</td>
<td>Yellow</td>
<td>White</td>
<td>Yellow</td>
<td>Yellow to beige</td>
<td>Whitish-brown or yellow</td>
<td>Yellow</td>
<td>Pale yellow</td>
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<td>+</td>
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<td>+</td>
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<td>−</td>
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<td>(+)</td>
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<td>(+)</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(+)</td>
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</table>

DNA–DNA hybridization was carried out between strain DS15T and ten reference strains, which showed more than 97 % 16S rRNA gene sequence similarity with strain DS15T. Total genomic DNA of all strains was extracted and purified and hybridization was done following the protocol as described by Kumar et al. (2008) and Tourova & Antonov (1988). The amount of bound probe DNA was calculated by using scintillation counter Wallac Microbeta Trilux; PerkinElmer). Percentage relatedness calculated on the basis of the data obtained (mean of three replicates) by DNA–DNA hybridization varied from 8.4 % to 44.5 % (Table S1, available in IJSEM Online). All hybridization values were well below the 70 % cut-off point for species classification, as recommended by Wayne et al. (1987), thus supporting the view that the isolated strain DS15T represented a novel species of the genus Sphingopyxis.

### Table 2. Cellular fatty acid profile of strain DS15T and the type strains of related species of the genus Sphingopyxis

<table>
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<tr>
<th>Fatty acids</th>
<th>1</th>
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<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
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<tr>
<td>C16:0</td>
<td>6.0</td>
<td>8.9</td>
<td>3.9</td>
<td>14.4</td>
<td>13.6</td>
<td>11.6</td>
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<td>1.8</td>
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<td>4.5</td>
<td>18.0</td>
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<td>7.3</td>
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<td>ND</td>
<td>ND</td>
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<td>11-Methyl C18:1ω7c</td>
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<td>3.5</td>
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<td>4.1</td>
<td>8.2</td>
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<td>3.0</td>
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<tr>
<td>C15:0 2-OH</td>
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<td>1.5</td>
<td>8.4</td>
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<tr>
<td>C16:0 2-OH</td>
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<td>3.2</td>
<td>2.5</td>
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<tr>
<td>Summed feature*</td>
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<td>ND</td>
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<td>28.7</td>
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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 consists of C16:1ω7c and/or iso-C15:0 2-OH; summed feature 8 consists of one or more of C18:1ω7c and C18:1ω9c.
The major polar lipids detected in strain DS15\(^T\) were phosphatidylethanolamine (PE), diphosphatidylglycerol (DPG), phosphatidylcholine (PC), phosphatidylglycerol (PG) and sphingoglycolipids (SGL) (Fig. S1). The quinone detected was ubiquinone Q-10 and the major polyamine was spermidine along with traces of putrescine and spermine. Thus, the chemotaxonomic markers in strain DS15\(^T\) were consistent with its classification in the genus Sphingopyxis (Takeuchi et al., 2001). The G+C content of the genomic DNA was 67.5 mol\%. Based on the phylogenetic analysis using 16S rRNA gene sequence, DNA–DNA hybridization relationships with most closely related members of the genus Sphingopyxis, morphological and physiological properties, biochemical features and chemotaxonomic studies, it can be concluded that strain DS15\(^T\) is a novel species of genus Sphingopyxis, for which the name Sphingopyxis indica sp. nov. is proposed.

**Description of Sphingopyxis indica sp. nov.**

*Sphingopyxis indica* (in’di.ca. N.L. fem. adj. *indica* of India, place of isolation of the type strain).

Cells are Gram-stain-negative, aerobic, non-flagellated, non-motile, non-spoore-forming and rod-shaped (1.51 μm long and 0.52 μm diameter) (Fig. S2). Colonies are pale-yellow-pigmented, small (diameter 0.5–1 mm on LB agar after 72 h of incubation at 28 °C), convex, dry, opaque with round configuration and smooth and entire margin. Optimal growth occurs at 28 °C, pH 7.0 and 0.5 % (w/v) NaCl. Positive for oxidase production, but negative for catalase activity. Nitrate is not reduced to nitrite, and indole production is absent. Hydrolyses aesculin and Tween 20, but not starch, casein or gelatin. Assimilates fructose, lactose, xylose and L-arabinose, but not D-mannitol, sorbitol or ribose. The type strain is sensitive to chloramphenicol, gentamicin, oxytetracycline, rifampicin, tetracycline, vancomycin, polymyxin B, ciprofloxacin, kanamycin, neomycin, amikacin, butrescine and amikacin, but resistant to nalidixic acid, ampicillin and penicillin. Major polar lipids are phosphatidylethanolamine (PE), diphosphatidylglycerol (DPG), phosphatidylcholine (PC), phosphatidylglycerol (PG) and sphingoglycolipids (SGL) (Fig. S1). The quinone detected was ubiquinone Q-10 and the major polyamine detected was spermidine along with traces of putrescine and spermine. Thus, the chemotaxonomic markers in strain DS15\(^T\) were consistent with its classification in the genus Sphingopyxis (Takeuchi et al., 2001). The G+C content of the genomic DNA was 67.5 mol\%.

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**References**


Gonzalez, J. M. & Saiz-Jimenez, C. (2002). *Sphingopyxis witflariensis* sp. nov., isolated from an activated dumpsite in Lucknow, Uttar Pradesh, India. The DNA G+C content of the type strain is 67.5 mol%.


