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

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A hexachlorocyclohexane (HCH)-degrading bacterial strain (RL-3T) was isolated from an HCH dump site located in the northern part of India. Resting cell assays and analytical GC studies confirmed the ability of strain RL-3T to degrade HCH isomers. Southern blot hybridization studies revealed the presence of *lin* genes, which are involved in the HCH degradation pathway, in this bacterium. The 16S rRNA gene sequence of strain RL-3T showed that it was most closely related to *Sphingobium cloacae* JCM 10874T (97.3 %) and *Sphingobium fuliginis* MTCC 7295T (96.4 %). Phylogenetic analysis based on 16S rDNA gene sequences placed strain RL-3T between *S. cloacae* JCM 10874T and *S. fuliginis* MTCC 7295T. The DNA G+C content of strain RL-3T was 62 mol%. The DNA–DNA relatedness values of strain RL-3T with *S. cloacae* JCM 10874T and *S. fuliginis* CCM 7327T were 8.65 and 7.47 %, respectively. SGL1 was the major sphingolipid and spermidine was the major polyamine in strain RL-3T. The major fatty acids in strain RL-3T were C18:1ω7c (56.6 %), C16:0 (14 %) and C14:0 2-OH (7.4 %). Ubiquinone Q-10 was the major respiratory quinone. Phylogenetic distinctiveness, DNA–DNA relatedness values, biochemical and physiological characterization and unique phenotypic characteristics suggest that strain RL-3T represents a novel species of the genus *Sphingobium*, for which the name *Sphingobium ummariense* sp. nov. is proposed. The type strain is RL-3T (=MTCC 8599T=CCM 7431T).

Hexachlorocyclohexane (HCH) is a cyclic saturated chlorinated hydrocarbon insecticide that consists primarily of the isomers α- (60–70 %), β- (5–12 %), γ- (10–15 %) and δ-HCH (6–10 %) (Windholz *et al.*, 1976; Kutz *et al.*, 1991). Prior to the 1990s, two forms of HCH, technical HCH (a mixture of all isomers) and purified γ-HCH (the only isomer with insecticidal properties) were used extensively. Although the use of technical HCH and γ-HCH has been banned in many countries, residues of HCH isomers are still detected from almost all components of the environment (Willem & Wollent, 2005). Many HCH dump sites still exist worldwide and act as reservoirs for further contamination (Walker *et al.*, 1999; Willem & Wollent, 2005). An HCH dump site in the northern part of India was created during γ-HCH production between 1997 and 2006 (Kumar *et al.*, 2008). The characterization of strain RL-3T, which was isolated from this dump site, is reported in this study. The strain was found to degrade α-, β-, γ- and δ-HCH. Using a polyphasic taxonomic approach, it was revealed that strain RL-3T represents a novel species of the genus *Sphingobium*.

Strain RL-3T was isolated by an enrichment method (Sahu *et al.*, 1990). For this purpose, a soil sample was collected from the HCH dump site from a depth of 20 cm. A 1 g soil sample was inoculated into 9 ml minimal salt medium (MSM) supplemented with technical HCH (5 μg ml⁻¹). The MSM contained (l⁻¹): 0.5 g (NH₄)₂HPO₄, 0.2 g MgSO₄.7H₂O, 0.01 g FeSO₄.7H₂O, 0.1 g K₂HPO₄ and 0.01 g Ca(NO₃)₂. After 4 days, a 100 μl sample was plated on Luria–Bertani (LB) agar containing (l⁻¹): 10.0 g tryptone, 5.0 g yeast extract, 5.0 g NaCl, 1.0 g glucose and 15 g agar. Plates were incubated at 28 °C. A yellow-pigmented colony that appeared among many bacteria after 3 days incubation was picked up and restreaked on an LB agar plate. The isolate was named RL-3T and its ability to degrade α-, β-, γ- and δ-HCH was assessed by using a resting cell assay (Sharma *et al.*, 2006). *Sphingobium indicum* B90A (Pal *et al.*, 2005), which has been shown to have a high potential to degrade all HCH isomers (Kumari *et al.*, 2002; Raina *et al.*, 2007), was used as a...
reference strain. Strain RL-3T was able to degrade α-, β-, γ- and δ-HCH and, compared with Sphingobium indicum B90A\(^5\), its ability to degrade α- and δ-HCH was faster; γ-HCH was degraded at a comparable rate, but β-HCH was degraded more slowly (see Supplementary Fig. S1 in IJSEM Online). Southern blot hybridization experiments revealed the presence of \(\text{lin}\) genes (\(\text{linA, linB, linC, linD}\) and \(\text{linR}\)) and IS\(6100\) elements in the genome of strain RL-3T\(^5\) (data not shown). HCH-degrading \(\text{lin}\) genes have been reported previously from several sphingomonads that were found to degrade HCH isomers (Dogra et al., 2004). Whereas \(\text{lin}\) genes encode enzymes that are responsible for imparting the ability to degrade HCH to sphingomonads, IS\(6100\) elements have been implicated in the acquisition and spread of \(\text{lin}\) genes among bacteria (Lal et al., 2006).

PCR amplification and sequencing of the 16S rRNA gene were carried out according to Prakash & Lal (2006). The nearly complete 16S rRNA gene sequence (1419 bp) was obtained and used for similarity searches. The 16S rRNA gene sequences of species closely related to strain RL-3T\(^5\) were retrieved from NCBI (http://www.ncbi.nlm.nih.gov) and RDP (http://rdp.cme.msu.edu/html/). The 16S rRNA gene sequence of strain RL-3T\(^5\) showed 97.3 \% similarity to that of Sphingobium cloacae JCM 10874\(^7\) and 96.4 \% similarity to that of Sphingobium fuliginis MTCC 7295\(^7\).

For the construction of a phylogenetic tree, 16S rRNA gene sequences of recognized strains of the genera Sphingobium, Sphingomonas sensu stricto, Novosphingobium, Sphingopyxis and Sphingosinicella (Yabuuchi et al., 1990; Maruyama et al., 2006) that showed greatest similarity with strain RL-3T\(^5\) were used. Zymomonas mobilis ATCC 10988\(^T\) was used as an outgroup. Multiple sequence alignment of 16S rRNA gene sequences was performed by using CLUSTAL_X (Thompson et al., 1997). After multiple alignment, common gaps in the 16S rRNA gene sequences were removed and an evolutionary distance matrix was calculated using the distance model of Jukes & Cantor (1969). A phylogenetic tree was constructed by using the neighbour-joining and parsimony methods (Saitou & Nei, 1987; Felsenstein, 1993). In both cases, strain RL-3T\(^5\) clustered among species of the genus Sphingobium (Fig. 1).

Sphingobium cloacae JCM 10874\(^7\) and Sphingobium fuliginis MTCC 7295\(^7\), which showed 16S rRNA gene sequence similarities of 97.3 and 96.4 \%, respectively, with strain RL-3T\(^5\), were used for DNA–DNA hybridization studies.

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**Fig. 1.** Phylogenetic tree based on nearly complete 16S rRNA gene sequences showing the evolutionary relationship between strains RL-3\(^5\), Sphingobium cloacae JCM 10874\(^7\), Sphingobium fuliginis MTCC 7295\(^7\) and representative members of the genera Sphingomonas sensu stricto, Novosphingobium, Sphingopyxis and Sphingosinicella. Nucleotide substitution rates were calculated by the Jukes and Cantor model and the tree was constructed by using the neighbour-joining method. The tree was rooted using Zymomonas mobilis ATCC 10988\(^T\) as an outgroup. Bootstrap values (based on 100 resamplings) greater than 50 \% are shown at branch points. Bar, 0.01 nt substitutions per nt position.
The DNA of all three strains was extracted and purified (Sambrook et al., 1989). DNA–DNA hybridization between these strains was performed by using the membrane filter method (Touroua & Antonov, 1987) and the relatedness (%; mean of four replicates) was calculated. Strain RL-3T showed 8.65 and 7.47 % DNA–DNA relatedness with Sphingobium cloacae JCM 10874T and Sphingobium fuliginis MTCC 7295T, respectively. Both these values are far below the threshold value (70 %) recommended for the delineation of any species (Wayne et al., 1987). This indicated that strain RL-3T can be classified as a member of a novel species of the genus Sphingobium.

Fatty acid methyl ester (FAME) analysis was carried out at the Institute of Microbial Technology (IMTECH), Chandigarh, India. For this purpose, a culture was grown on trypticase soya broth agar medium at 28 °C. One loopful of grown culture was scraped from the plate and subjected to saponification, methylation and extraction (Miller, 1982; Kuykendall et al., 1988). The FAME mixtures were separated using the Sherlock Microbial Identification System (MIDI) by GC (Agilent 6890) fitted with a flame ionization detector. Identification and comparison were made using the Aerobe (TSBA50, version 5) database of the Sherlock Microbial Identification System. The major FAMEs in strain RL-3T were C18:1ω7c and C14:0 2-OH, which are generally present in sphingomonads (Busse et al., 1999). However, strain RL-3T showed minor qualitative and quantitative differences in FAME profiles when compared with Sphingobium cloacae JCM 10874T and Sphingobium fuliginis MTCC 7295T (see Supplementary Table S1 in IJSEM Online).

Polar lipid analysis was carried out by methods described by Tindall (1990a, b). For this purpose, 100 mg cell pellet was extracted with 10 ml solvent containing chloroform: methanol: 0.3 % aqueous NaCl (1 : 2 : 0.8). After extraction, the chloroform layer was separated and dried in a Buchi Rotavapour and redissolved in chloroform: methanol (2 : 1). Polar lipids were analysed by two-dimensional TLC (Silica 60; Merck) using the solvents chloroform: methanol: water (65 : 25 : 4, by vol.) and chloroform: methanol: acetic acid: water (80 : 12 : 15 : 4, by vol.) in the first and second directions, respectively. Finally, chromatograms were developed by spraying with different chemical-group-specific reagents. Results of TLC analysis indicated the presence of phosphatidylmonomethylthanolamine, phosphatidylethanolamine, phosphatidylglycerol, diphasphatidylglycerol, phosphatidylcholine and sphingoglycolipids in strain RL-3T (see Supplementary Fig. S2 in IJSEM Online).

The DNA G+C contents were determined as described by Gonzalez & Saiz-Jimenez (2002). Strain RL-3T had a G+C content of 62 mol%. Bacterial polyamines were extracted and analysed by HPLC at IMTECH, Chandigarh, India, according to Busse & Auling (1988) and Busse et al. (1997). Strain RL-3T contained spermine and spermidine, which are characteristic of the genus Sphingobium. Respiratory quinones were extracted and analysed according to Collins et al. (1977). Strain RL-3T contained only ubiquinone Q-10.

Morphological features (shape, size, colour, contour and pigment production) of the strain were studied on nutrient agar and LB plates; colonies of RL-3T were 1.5 and 2.0 mm in diameter, respectively, yellow-coloured, circular and smooth. Gram staining was performed using a Himedia kit. Motility of the organism was studied by the hanging drop method and in motility agar. Sensitivity to antibiotics was tested using Himedia Readymade Sensi-Discs on Mueller–Hinton II medium. Growth at different temperatures and catalase and oxidase tests were carried out as described by McCarthy & Cross (1984). Acid production from carbohydrates and degradation of xanthine and hypoxanthine were tested as described by Gordon et al. (1974). Hydrolysis of Tweens 20 and 80 and the ability of strain RL-3T to grow in the presence of NaCl were also tested (Arden-Jones et al., 1979). Urease activity was determined according to Christensen (1946). The other physiological tests and methods used were as described by Collins et al. (1989). The HCH-degrading activity of the strain was tested by GC (Dogra et al., 2004).

The phylogenetic tree based on 16S rRNA gene sequences clearly shows that strain RL-3T belongs to the genus Sphingobium (Fig. 1). Takeuchi et al. (2001) have shown that polyamine patterns and nitrate reduction are good diagnostic markers for differentiation of the Sphingobium and Sphingomonas sensu stricto. Polyamines and nitrate reduction data suggest that strain RL-3T should be classified in the genus Sphingobium. A comparison of the biochemical characteristics of strain RL-3T with the phylogenetically related species Sphingobium cloacae JCM 10874T and Sphingobium fuliginis MTCC 7295T clearly showed that strain RL-3T could be differentiated from them (Table 1). Low DNA–DNA hybridization values further confirmed that strain RL-3T represents a novel species of genus Sphingobium, for which the name Sphingobium ummariense sp. nov. is proposed.

**Description of Sphingobium ummariense sp. nov.**

*Sphingobium ummariense* (um.ma.ri.en’se. N.L. neut. adj. ummariense pertaining to Ummari, the HCH-contaminated site from where the type strain was isolated).

Gram-negative, aerobic, rod-shaped, mesophilic, non-spore-forming, non-motile bacterium. Colonies are small (1.5 mm on LB agar after 72 h incubation at 30 °C), yellow, smooth, entire and circular. Grows at 28 °C, but not at 4 or 42 °C. Optimum pH and NaCl concentration for growth are 7.5 and 1–2 %, respectively. Degrades α-, β-, γ- and δ-HCH isomers in liquid culture. Resistant to amoxicillin (10 μg), penicillin (10 μg), streptomycin (10 μg), erythromycin (10 μg) and ampicillin (10 μg), but sensitive to oxytetracycline (30 μg), kanamycin (30 μg), chloramphenicol (30 μg), nalidixic acid (30 μg), tetracycline (30 μg), vancomycin (30 μg), novobiocin...
Table 1. Comparative phenotypic and physiological characteristics of strain RL-3T and closely related species of the genus Sphingobium

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<th>Characteristic</th>
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<td>Colour</td>
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<td>Creamy white</td>
<td>Yellow</td>
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<td>Water soluble pigment</td>
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<td>δ-HCH</td>
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*Data from Prakash & Lal (2006).

(30 μg), chlorotetracycline (30 μg), rifamycin (30 μg), neomycin (30 μg) and gentamicin (10 μg). Positive for urease, but negative for catalase and oxidase. Acid is produced from glucose, xylose, arabinose, maltose and sorbitol, but not from adonitol, dulcitol, mannoitol, ribose or inositol. Tween 80 and starch are not degraded. Gelatin and aesculin are hydrolysed. Contains linA, linB, linC, linD and linR genes and IS6100. Sphingoglycolipid 1 is the major polar lipid. Major fatty acids are C18:1ω9tC, C16:0 and C14:0 2-OH. Spermidine is the major cellular polyamine. The major quinone is ubiquinone Q-10.

The type strain, RL-3T (=MTCC 8599T=CCM 7431T), was isolated from HCH-contaminated soil from Umhari village, Lucknow, India. The DNA G+C content of strain RL-3T is 62 mol%.

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References


