**Pontibacter indicus** sp. nov., isolated from hexachlorocyclohexane-contaminated soil

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An orange-pigmented bacterial strain, designated LP100T, was isolated from hexachlorocyclohexane-contaminated soil (Lucknow, India). A neighbour-joining tree based on 16S rRNA gene sequences showed that strain LP100T occupied a distinct phylogenetic position in the *Pontibacter* species cluster, showing highest similarity with *Pontibacter lucknowensis* DM9T (97.4%). Levels of similarity to strains of other *Pontibacter* species ranged between 94.0 and 96.8%. Strain LP100T contained MK-7 as the predominant menaquinone and sym-homospermidine was the major polyamine in the cell. The major cellular fatty acids of strain LP100T were anteiso-C17:0 A, iso-C15:0 and iso-C18:1 H. The polar lipid profile of strain LP100T showed the presence of phosphatidylethanolamine, an unidentified aminophospholipid, three unknown aminolipids and two unknown polar lipids. The G+C content of strain LP100T was 58.2 mol%. The results of DNA–DNA hybridization, biochemical and physiological tests clearly distinguish the novel strain from closely related species of the genus *Pontibacter*. Therefore, strain LP100T represents a novel species of the genus *Pontibacter* for which the name *Pontibacter indicus* is proposed. The type strain is LP100T (≡CCM8435≡MCC2027).

**Introduction**

The genus *Pontibacter*, a member of the family Cytophagaceae, phylum Bacteroidetes, was first described by Nedashkovskaya et al. (2005). The genus *Pontibacter* comprises species that are Gram-negative, rod-shaped, aerobic and heterotrophic, and contain menaquinone MK-7 as the main respiratory quinone. At the time of writing, the genus *Pontibacter* comprised 11 species with validly published names: *Pontibacter actiniarum* (Nedashkovskaya et al., 2005), *Pontibacter akesuensis* (Zhou et al., 2007), *Pontibacter korlensis* (Zhang et al., 2008), *Pontibacter niitensis* (Dastager et al., 2010), *Pontibacter roseus* (Suresh et al., 2006; Wang et al., 2010), *Pontibacter xinjiangensis* (Wang et al., 2010), 'Pontibacter salisaro' (Joung et al., 2011), *Pontibacter papuli* (Xu et al., 2012), *Pontibacter lucknowensis* (Dwivedi et al., 2012), *Pontibacter saeman-gumensis* (Kang et al., 2012) and *Pontibacter rambhanderi* (Singh et al., 2013). Species of the genus *Pontibacter* have been isolated from diverse environments, including marine actinians, desert soil, muddy water, seawater, forest soil and hexachlorocyclohexane (HCH)-contaminated soil (Nedashkovskaya et al., 2005; Zhou et al., 2007; Suresh et al., 2006; Kang et al., 2012; Dastager et al., 2010; Dwivedi et al., 2012; Singh et al., 2013). Here, we have isolated micro-organisms from a pond primarily contaminated with HCH. We have previously characterized several bacterial strains from the HCH dump site (Lal et al., 2010). Soil samples from a pond (Lucknow, Uttar Pradesh, India: 26°34’N 81°59’E) were collected, suspended in 0.9% NaCl, serially diluted and plated on nystatin- and streptomycin-amended Luria Bertani (LB) agar plates (Vanbroekhoven et al., 2004). After incubation at 28°C for 48 h a pigmented colony was picked and subcultured several times to get a pure culture. The colony was designated strain LP100T. A polyphasic approach (Prakash et al., 2007) was adopted for taxonomic classification of this novel bacterium.

16S rRNA gene sequencing of strain LP100T was carried using the universal bacterial primer set 8F, 341F, 786F and 1542R (Lane, 1991) using a 3100-Avant Genetic Analyzer sequencer at the Department of Zoology, University of Delhi, India. The sequence thus obtained was assembled manually using Sequencing Analysis, version 5.1.1, and Clone Manager software, version 5. A continuous stretch of 1426 bp of the 16S rRNA gene of strain LP100T was obtained and this sequence was subjected to similarity searches using the Seqmatch tool of the Ribosomal Database Project (http://rdp.cme.msu.edu/) and BLAST program of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov). A non-redundant

**Abbreviation:** HCH, hexachlorocyclohexane.

The GenBank/EMBL/DDJB accession number for the 16S rRNA gene sequence of strain LP100T is KC469980.

Two supplementary figures and two supplementary tables are available with the online version of this paper.

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BLASTN search of full-length sequences through GenBank (Altschul et al., 1990), RDP II (Maidak et al., 2001) and EzTaxon-e Server (Kim et al., 2012) identified its closest relatives. The nearly full-length 16S rRNA gene sequences that were closely related to strain LP100T were retrieved from GenBank for reconstruction of the phylogenetic tree. Levels of 16S rRNA gene sequence similarity between strain LP100T and the type strains of recognized species of the genus Pontibacter ranged from 93.8 to 97.4%. Strain LP100T showed highest sequence similarity to \textit{P. lucknowensis} DSM9\(^T\) (97.4%). Levels of similarity to the other \textit{Pontibacter} species were 93.4–96.4%. A phylogenetic tree was constructed using sequences of closely related species selected from GenBank and EzTaxon Server version 2.1. The 16S rRNA gene sequence of \textit{Erythrobacter litoralis} DSM 8509\(^T\) was used as an outgroup and the selected sequences were aligned using the program \textsc{Clustal X} version 1.81b (Thompson et al., 1997). The alignment was checked manually for quality. Phylogenetic analysis was carried out using the \textsc{Treeconw} software package version 1.3b (Van de Peer & De Wachter, 1994). The evolutionary distance matrix was calculated using the distance model of Jukes & Cantor (1969) and an evolutionary tree was constructed using the neighbour-joining method of Saitou & Nei (1987). Statistical evaluation of the tree topology based on 1000 resamplings was done using the bootstrap option in the \textsc{Treeconw} software (Fig. 1). Strain LP100T\(^T\) fell in the clade containing members exclusively belonging to the genus \textit{Pontibacter}.

DNA–DNA hybridization was carried out between strain LP100T and \textit{P. lucknowensis} DSM9\(^T\), which showed more than 97% 16S rRNA gene sequence similarity. Total genomic DNA of both strain LP100T and \textit{P. lucknowensis} DSM9\(^T\) was extracted and purified, and hybridization was done following the protocol described by Kumar et al. (2008) and Tourova & Antonov (1988). The amount of bound probe DNA was calculated by using a scintillation counter (1450 LSC & Luminescence counter Wallac, PerkinElmer). All the DNA–DNA hybridization values were below the threshold value of 70% (Table S1, available in IJSEM Online) recommended for the delineation of bacterial species (Wayne et al., 1987), which confirms that strain LP100T\(^T\) represents a novel species of the genus \textit{Pontibacter}.

For fatty acid analysis, cells of strain LP100T were harvested from an LB agar plate after incubation at 28 \(^\circ\)C for 2 days. Analysis of fatty acid methyl esters was carried out at Royal Life Sciences, Secundarabad, India. The physiological age of strain LP100T\(^T\) was standardized by choice of the sector from a quadrant streak on triplicate soy broth agar plates according to the MIDI protocol. Fatty acid methyl esters were analysed from 2–4 loops of inocula from the third quadrant of a Petri dish subjected to saponification, methylation and extraction according to the methods of Miller (1982) and Kuykendall \textit{et al.} (1988). The fatty acid methyl ester mixtures were prepared and separated using the Sherlock Microbial Identification System (MIDI) and identification of the fatty acids was made using the Aerobe RTSBA database, version 6.0 B (Sasser, 1990). The major fatty acids of strain LP100T\(^T\) were anteiso-C\(_{17:0}\) A (31.7%), iso-C\(_{15:0}\) (12.5%) and iso-C\(_{18:1}\) H (11.4%). The fatty acid profile of strain LP100T\(^T\) showed qualitative and quantitative differences from that of \textit{P. lucknowensis} DM9\(^T\) (Table S2), further suggesting that LP100T\(^T\) represents a novel species of the genus \textit{Pontibacter}.

Bacterial polyamines were extracted as described by Busse & Auling (1988) and analysed by one-dimensional TLC. Ten microlitres of extracted sample was loaded on the TLC plate (Silica gel 60 F254, 20 × 20 cm; Merck) with ethyl acetate/ cyclohexane as the running solvent. For the detection of polyamines the TLC plate was allowed to air dry after which it was visualized under UV light. Polyamines were identified by comparing \(R_f\) values by using commercially prepared standards obtained from Sigma Life Science. \textit{Sym}-Homospermidine was detected as the major polyamine. The DNA G+C content of strain LP100T\(^T\) was calculated according to the method described by Gonzalez & Saiz-Jimenez (2002) using an Applied Biosystems 7500 Real-Time PCR system and was found to be 58.2 mol%.

Quinones were extracted from 200 mg dry cell mass with a 10% aqueous solution of 0.3% (w/v) NaCl in methanol and petroleum ether (boiling point 60–80 \(^\circ\)C) at a ratio of 1:1. The upper phase was collected and dried in a rotary evaporator (Büchi). The residue was dissolved in 100 \(\mu\)l acetonitrile. The extract was loaded on a TLC plate (Silica gel 60 F254, 20 × 20 cm; Merck) with petroleum ether (boiling point 60–80 \(^\circ\)C) and diethyl ether (85:15, v/v). Purified menaquinones were dissolved in diethyl ether and analysed by reversed-phase TLC according to Collins \textit{et al.} (1977). The major respiratory quinone was MK-7, a characteristic feature of the genus \textit{Pontibacter}.

Polar lipid analysis of strain LP100T\(^T\) was performed by two-dimensional TLC as described by Bligh & Dyer (1959). Total polar lipids were detected by spraying with 5% (w/v) molybdotrichlorophosphoric acid dissolved in ethanol (Merck) followed by drying at 120 \(^\circ\)C for 15 min. Major polar lipids present in strain LP100T\(^T\) were phosphatidylethanolamine (PE), an unidentified aminophospholipid (APL1), unknown aminolipids (AL1–3) and unknown polar lipids (L1 and 2) (Fig. S1).

Cell morphology was examined using a light microscope (Olympus) and transmission electron microscope (TEM 269D; Morgagni, Fei) (Fig. S2). Microscopic study revealed that strain LP100T\(^T\) was motile. Gliding motility of the organism was tested on fresh LB broth culture using the hanging drop method (Bowman \textit{et al.}, 2003) as well as motility agar, which were found to be positive. Colonies of strain LP100T\(^T\) showed optimum growth on LB agar, nutrient agar, marine agar, triplicate soya yeast agar and brain heart infusion agar at 28 \(^\circ\)C within 36 h of incubation; only slight growth occurred on R2A agar. Colonies were orange, circular and smooth. The Gram–stain reaction was performed using a Gram-stain kit (HiMedia) which confirmed strain LP100T\(^T\) as a Gram-negative bacterium. Antibiotic sensitivity tests were
performed on Muller-Hinton II medium using readymade antibiotic sensitivity-discs (HiMedia) containing (μg per disc): amikacin (30), ampicillin (10), chloramphenicol (30), ciprofloxacin (5), gentamicin (10), kanamycin (30), nalidixic acid (30), penicillin-G (10), rifampicin (5), tetracycline (30) and vancomycin (30). After 3 days, signs of growth inhibition were assumed to indicate sensitivity to nalidixic acid (30), penicillin-G (10), rifampicin (5), ciprofloxacin (5), gentamicin (10), kanamycin (30), tetracycline (30) and vancomycin (30). Following incubation, antibiotic sensitivity was determined by oxidation of hydrogen peroxide solution to colonies grown on LB based on bubble production after the application of 3 % (v/v)  H₂O₂. Growth at different temperatures, strain LP100T was streaked on LB agar plates and incubated at 4, 28, 37, 45 and 55 °C. Growth at pH 3–11 (increments of 1 pH unit) and with 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 (w/v) NaCl was determined as described by Arden-Jones et al. (1979). pH testing is important in cases where there is a decline in pH of the media after autoclaving. This is the case with media at pH 10 or more which undergo a drop by 0.5 units. Thus, to report authentic data regarding the growth of an organism at pH 10 or more it should always be tested so the pH can be properly maintained. Hydrolysis of Tween 20 and 80 was tested according to Arden-Jones et al. (1979). Hydrolysis of gelatin, casein, aesculin and starch was determined as described by Cowan & Steel (1965). Urease activity was tested in accordance with Christensen (1946). Indole production was tested as described by Smibert & Krieg (1994). Citrate utilization was tested using Simmons citrate agar (HiMedia). The nitrate reduction test was performed as described by Smibert & Krieg (1994). DNase activity was tested using

![Fig. 1. Phylogenetic tree based on nearly complete 16S rRNA gene sequence data showing the evolutionary relationship of strain LP100T and members of representative genera Pontibacter, Adhaeribacter and Hymenobacter. The tree was constructed by using the neighbour-joining (Jukes & Cantor, 1969) method of TREECONW software and the rooting was done by using Erthrobacter litoralis DSM 8509T as an outgroup. Bootstrap values >50 % based on 1000 replications are shown at branch points. The GenBank accession number for the 16S rRNA gene sequence of each strain is shown in parentheses. Bar, 0.02 substitutions per nucleotide position.](image-url)
DNase agar (HiMedia). H₂S production was tested using triple-sugar iron agar (HiMedia). The presence of flexirubin-type pigments was examined using a 20% (w/v) KOH solution (Bernardet et al., 2002; Bowman, 2000). Degradation of HCH isomers was carried out using the protocol described by Kumari et al. (2002). Strain LP100ᵀ was isolated from HCH-contaminated soil sediment but it was unable to degrade any of the HCH isomers. Assimilation of different carbohydrates was tested in basal media (Gordon et al., 1974). Differential results of biochemical tests of strain LP100ᵀ with its closest neighbour, P. lucknowensis DM9ᵀ, are given in Table 1.

Based on the combination of phenotypic, chemotaxonomic and phylogenetic data, strain LP100ᵀ can be differentiated from P. lucknowensis DM9ᵀ, and it is suggested to represent a novel species of the genus Pontibacter, for which the name Pontibacter indicus sp. nov. is proposed.

**Description of Pontibacter indicus sp. nov.**

*Pontibacter indicus* (in’di.cus. L. masc.adj. indicus Indian, referring to the isolation of the type strain from India.)

Cells are aerobic, Gram-negative, rod-shaped, 0.5–0.9 μm in diameter and 1.1–1.7 μm in length and motile. Growth occurs on LB agar, nutrient agar, marine agar, trypticase soy yeast agar, brain heart infusion agar and R2A agar. Colonies are orange, entire, smooth, translucent, circular and convex and appear after 36 h of incubation. Growth occurs at 5–40 °C (optimum 28 °C). Growth occurs in 0–4% NaCl and at pH 6.0–10.0 with optimal growth at pH 7.0–8.0. Flexirubin-type pigments are absent. Catalase-positive, but oxidase- and DNase-negative. Hydrolyses gelatin, urea, Tween 20 and Tween 80 but not aesculin or starch. Indole and H₂S production are negative. Citrate, xanthine and hypoxanthine are not utilized. β-Galactosidase activity is not detected. Assimilates D-glucose, maltose, D-mannitol, sucrose, D-arabinose, sorbitol, D-fructose and xylitol but not cellobiose, lactose, D-mannose, myo-inositol, rhamnose, D-ribose or D-galactose. Forms acid with the following sugars: maltose, D-glucose, D-mannitol, sucrose, D-arabinose, D-fructose and sorbitol. Sensitive to chloramphenicol, nalidixic acid, vancomycin, penicillin, polymyxin B, oxytetracycline, tetracycline, rifampicin and ciprofloxacin but resistant to streptomycin, ampicillin, kanamycin, amikacin and gentamicin. Although isolated from HCH-contaminated soil sediment, unable to degrade HCH isomers. The major isoprenoid quinone is MK-7. Major fatty acids are anteiso-C₁₇:₀ A, iso-C₁₅:₀ and iso-C₁₈:₁ H. The polyamine pattern showed the presence of sym-homospermidine as the major polyamine. Major polar lipids are phosphatidylethanolamine (PE), an unidentified aminophospholipid (APL1), unknown aminolipids (AL1–3) and unknown polar lipids (L1 and 2).

The type strain, LP100ᵀ (=CCM8435ᵀ=MC2027ᵀ), was isolated from HCH-contaminated soil sediment situated around a lindane production unit in northern India. The DNA G+C content of the type strain is 58.2 mol%.

**Table 1.** Differential morphological and physiological characteristics between strain LP100ᵀ and *Pontibacter lucknowensis* DM9ᵀ

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>LP100ᵀ</th>
<th>P. lucknowensis DM9ᵀ</th>
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<tr>
<td>Temperature growth range (°C)</td>
<td>5–40</td>
<td>6–45</td>
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<tr>
<td>Hydrolysis of:</td>
<td></td>
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<tr>
<td>Gelatin</td>
<td>+</td>
<td>−</td>
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<tr>
<td>Aesculin</td>
<td>−</td>
<td>+</td>
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<td>Carbon source utilization</td>
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<tr>
<td>Cellobiose</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>D-Fructose</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>α-Lactose</td>
<td>−</td>
<td>+</td>
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<td>Sucrose</td>
<td>+</td>
<td>−</td>
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<tr>
<td>D-Ribose</td>
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<tr>
<td>DNA G+C content (mol%)</td>
<td>58.2</td>
<td>49.2</td>
</tr>
</tbody>
</table>

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**References**


