**Pontibacter chinhatensis** sp. nov., isolated from pond sediment containing discarded hexachlorocyclohexane isomer waste

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A halotolerant, Gram-negative, rod-shaped and light-red-pigmented bacterium, designated LP51\(^T\), was isolated from pond sediment near a hexachlorocyclohexane dumpsite located at Chinhat, Lucknow, India. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain LP51\(^T\) formed a distinct phylectic clade along with the members of the genus **Pontibacter**. The 16S rRNA gene sequence similarity to members of the genus **Pontibacter** ranged from 94.2 to 99.4 %. The cells were motile, aerobic and catalase- and oxidase-positive. The major fatty acids were iso-C\(_{15}\) : 0 (17.8 %), iso-C\(_{15}\) : 0 3-OH (8.8 %), iso-C\(_{17}\) : 0 3-OH (5.7 %), summed feature 3 (C\(_{16}\) : 1\(\omega\)7c and/or C\(_{16}\) : 1\(\omega\)6c; 6.5 %) and summed feature 4 (iso-C\(_{17}\) : 1 I and/or anteiso-C\(_{17}\) : 1 B; 30.7 %). The polar lipid profile of strain LP51\(^T\) showed the presence of phosphatidylyethanolamine, an unidentified aminophospholipid, unknown aminolipids, unknown polar lipids and unknown glycolipids. DNA–DNA relatedness of strain LP51\(^T\) with respect to the most closely related type strain, **Pontibacter korlensis** X14-1\(^T\), was 47.2 %. On the basis of this information, it is proposed that the isolate be assigned to a novel species of the genus **Pontibacter**, for which the name **Pontibacter chinhatensis** sp. nov. is proposed. The type strain is LP51\(^T\) (=CCM 8436\(^T\)=MCC 2070\(^T\)).

The genus **Pontibacter**, which belongs to phylum **Bacteroidetes** and the family **Cytophagaceae**, was proposed by Nedashkovskaya et al. (2005). Species of the genus **Pontibacter** are distributed widely in nature, and have been isolated from marine actinians, desert soil, muddy water, sea water, forest soil, hexachlorocyclohexane- (HCH) contaminated soil and solar salterns (Nedashkovskaya et al., 2005; Suresh et al., 2006; Zhou et al., 2007; Dastager et al., 2010; Joung et al., 2011, 2013; Dwivedi et al., 2013; Kang et al., 2013; Singh et al., 2013; Zhang et al., 2013; Cao et al., 2014; Subhash et al., 2014). At the time of writing, the genus **Pontibacter** encompassed 21 species with effectively published names, the names of three of which have not been validly published: **Pontibacter actiniarum** (the type species; Nedashkovskaya et al., 2005), **P. akesuensis** (Zhou et al., 2007), **P. akumii** (Zhang et al., 2008), **P. niisaroi** (Dastager et al., 2010), **P. roseus** (Suresh et al., 2006; Wang et al., 2010), **P. xinjiangensis** (Wang et al., 2010), **P. salisaro** (Joung et al., 2011), **P. populi** (Xu et al., 2012), **P. lucknowensis** (Dwivedi et al., 2013), **P. saemangeumensis** (Kang et al., 2013), **P. rambachari** (Singh et al., 2013), **P. toksuensis** (Zhang et al., 2013), **P. odishensis** (Subhash et al., 2013), **P. jeungdoensis** (Joung et al., 2013), **P. ruber** and **P. deserti** (Subhash et al., 2014), **P. soli** (Dai et al., 2014), **P. humi** (Srinivasan et al., 2014), **P. yulensis** (Cao et al., 2014), **P. diazotrophicus** (Xu et al., 2014) and **P. indicus** (Singh et al., 2014). In the process of characterizing bacteria that exist in sediments of ponds that have been contaminated with HCH, a bacterial strain LP51\(^T\) was isolated from Chinhat, Lucknow, Uttar Pradesh, India (26° 53′ 8.903″ N 81° 20′ 22.495″ E). For this purpose, sediment samples were serially diluted with a sterile solution of 0.9 % NaCl and plated on nystatin- and streptomycin-amended marine agar (MA) plates (Vanbroekhoven et al., 2004). After incubation at 28 °C for 48 h, a pigmented colony was picked and cultured several times to get a pure culture. The colony was designated strain LP51\(^T\). A polyphasic approach (Vandamme et al., 1996; Prakash et al., 2007) was adopted for taxonomic classification of the novel bacterium LP51\(^T\).

16S rRNA gene sequence analysis of strain LP51\(^T\) was carried out as described by Lane (1991) using a 3100-Avant Genetic Analyzer 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

**Abbreviation:** HCH, hexachlorocyclohexane.
of 1435 bp of the 16S rRNA gene of strain LP51T was obtained, and this sequence was subjected to a similarity search using the Seqmatch tool of the RDP (http://rdp.cm.ccmu.edu/html/) and the BLAST program of the NCBI (http://www.ncbi.nlm.nih.gov). A non-redundant BLAST search of full-length sequences through GenBank (Altschul et al., 1990), RDP II (Maidak et al., 2001) and EzTaxon-e Server (http://www.ezbiocloud.net/eztaxon; Kim et al., 2012) identified its closest relatives. Nearly full-length 16S rRNA gene sequences that were closely related to that of P. korlensis X14-1T were retrieved from the GenBank nucleotide database for the reconstruction of a phylogenetic tree. The 16S rRNA gene sequence similarity between strain LP51T and the type strains of species of the genus *Pontibacter* with validly published names ranged from 99.4 to 94.2 %. Strain LP51T showed the highest sequence similarity to *P. niitensis* NII-0905T (99.4 %), followed by *P. korlensis* X14-1T (97.0 %). A phylogenetic tree was reconstructed using sequences of 48 closely related species selected from GenBank and EzTaxon-e Server. The 16S rRNA gene sequence of *Erythrobacter litoralis* DSM 8509T was used as an outgroup, and the selected sequences were aligned using the program CLUSTAL_X version 1.81b (Thompson et al., 1997). The alignment was checked manually for quality. Phylogenetic analysis was carried out using the MEGA software package version 5.2.2 (Tamura et al., 2011). The evolutionary distance matrix was calculated using the distance model of Jukes & Cantor (1969) and an evolution-distance model of Jukes & Cantor (1969) and an evolution-distance model of Jukes & Cantor (1969). The amount of bound probe DNA was calculated from the sequence of strain LP51T falls in a clade exclusively containing members belonging to the genus *Pontibacter* (Fig. 1).

DNA–DNA hybridization was carried out between LP51T and *P. korlensis* CCTCC AB 206081T as suggested, as the latter strain showed 97 % 16S rRNA gene sequence similarity to strain LP51T. Total genomic DNA of strain LP51T and *P. korlensis* X14-1T was extracted and purified and hybridization was done by following the protocol described by Kumari et al. (2008) and Tourouva & Antonov (1988). The amount of bound probe DNA was calculated by using a scintillation counter (1450 LSC and Luminescence counter Wallac Microbeta Trilux; PerkinElmer). All DNA–DNA hybridization values were below the threshold value of 70 % (Supplementary Table S1, available in the online Supplementary Material), as recommended for the delineation of bacterial species (Wayne et al., 1987), which confirms that strain LP51T represents a novel species of the genus *Pontibacter*.

Cell shape and morphology in exponentially growing cultures were examined using light (Eclipse E600; Nikon) and transmission electron (TEM 269D; Morgagni Fei) microscopy. Microscope study revealed that cells of strain LP51T were motile. Gliding motility of the organism was tested in a fresh Luria–Bertani (LB) broth culture using the hanging drop method (Bowman et al., 2003) along with motility agar (Farmer, 1999), and was found to be positive. Strain LP51T showed optimum growth on LB agar, nutrient agar, marine agar, tryptic soy yeast agar and brain heart infusion agar at 28 °C within 36 h of incubation, but limited growth occurred on R2A agar. Colonies of strain LP51T were light red, circular and smooth. The Gram staining test was performed using a Gram staining kit (HiMedia), and strain LP51T was found to stain Gram-negative. Antibiotic sensitivity tests were performed on Muller–Hinton II medium using ready-made antibiotic sensitivity discs (HiMedia) with varying amounts of antibiotics. Antibiotics tested were as follows (μg per disc in parentheses): 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). Oxidase activity was tested using oxidas disc from HiMedia and N,N,N’,N’-tetramethyl 1,4-phenylene-diamine reagent (bioMérieux). Catalase activity was examined by observing oxygen bubble production after the application of 3 % (v/v) hydrogen peroxide solution to colonies grown on LB agar (McCarthy & Cross, 1984). Production of acid from carbohydrates and degradation of xanthine and hypoxanthine were determined as described by Gordon et al. (1974). In order to determine growth at different temperatures, strain LP51T was streaked on LB agar plates and incubated at 4, 28, 37, 45 and 55 °C; additionally, cell growth was monitored by measuring the OD600 at different temperatures using LB broth as mentioned above. Growth at pH 3–11 (in increments of 1 pH unit) and 0–10 (w/v) NaCl (in increments of 1 %) was assessed using the protocol described by Arden Jones et al. (1979). The medium pH was maintained with buffers and was further checked after autoclaving the medium. 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 DNase agar (HiMedia). H2S production was tested using triple-sugar iron agar (HiMedia). The presence of flexirubin-type pigments was examined using 20 % (w/v) KOH (Bernardet et al., 2002; Bowman, 2000). Degradation of HCH isomers was assessed using the protocol described by Kumari et al. (2002). Although strain LP51T was isolated from HCH-contaminated soil sediment, it was unable to degrade HCH isomers. Assimilation of different carbohydrates was tested in basal medium (Gordon et al., 1974). Biochemical tests for hydrolysis of gelatin and aesculin and activities of urease and β-galactosidase were repeated using the API 20NE kit (bioMérieux) according to the manufacturer’s instructions. Differential results of biochemical tests of strain LP51T and its closest neighbour, *P. korlensis* X14-1T, are given in Table 1.
Fig. 1. Phylogenetic tree based on nearly complete 16S rRNA gene sequences showing the evolutionary relationships of strain LP51T and members of representative genera Pontibacter, Adhaeribacter and Hymenobacter. The tree was reconstructed by using the neighbour-joining method (Jukes & Cantor, 1969) in the MEGA 5.2.2 software, and rooting was done by using Erythrobacter litoralis DSM 8509T as an outgroup. Bar, 0.02 substitutions per nucleotide position. Bootstrap values >70 % based on 1000 replications are shown at branch points. GenBank accession numbers are shown in parentheses.
Table 1. Differential morphological and physiological characteristics of strain LP51T and related type strains

| Characteristic                  | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
|--------------------------------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| Colony colour*                 | LR | P | R | O | R | O | R | P | LP | P | B | R | P | R | P | P | P | P | P | P | P |
| Gliding motility               | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| Hydrolysis of:                 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Gelatin                       | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Casein                        | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Aesculin                      | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Starch                        | - | + | + | + | - | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Tween 20                      | + | + | NR | + | + | NR | NR | NR | NR | NR | NR | - | NR | + | - | NR | NR | NR | NR | NR | NR | NR |
| Tween 80                      | - | + | NR | - | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Urea                          | - | - | - | + | + | NR | - | NR | - | NR | + | - | NR | NR | NR | - | - | - | - | - | - | - |
| β-Galactosidase               | + | + | + | - | - | NR | - | NR | - | NR | - | - | - | + | + | + | + | + | + | + | W | - |
| Carbon source utilization     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| L-Arabinose                   | - | + | NR | + | + | NR | NR | NR | NR | NR | + | - | - | - | + | + | + | + | - | - | - | W |
| Sorbitol                      | + | - | NR | + | - | NR | NR | NR | NR | NR | + | - | - | - | - | + | + | + | - | + | NR | - |
| Xylitol                       | + | - | NR | NR | + | NR | NR | NR | NR | NR | - | - | - | - | + | - | - | - | - | - | - | NR |
| myo-Inositol                  | + | - | W | + | - | NR | NR | NR | NR | NR | + | - | - | - | - | + | - | NR | - | W | - | - |
| Rhamnose                      | + | - | W | + | - | NR | NR | NR | NR | NR | - | - | + | + | NR | - | + | NR | - | + | - | - |
| D-Ribose                     | - | + | NR | + | NR | NR | NR | NR | NR | NR | NR | NR | NR | NR | NR | NR | NR | NR | NR | NR | NR | + |
| Inulin                       | + | + | NR | NR | NR | NR | NR | NR | NR | NR | NR | NR | NR | NR | NR | NR | NR | NR | NR | NR | NR | - |
| Citric acid                   | - | - | - | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| DNA G+C content (mol%)        | 52.4 | 48.4 | 47.8 | 49.2 | 59.1 | 58.2 | 52.7 | 44.9 | 46.3 | 43.8 | 47.5 | 46 | 59.5 | 45.6 | 48.7 | 51.4 | 48.9 | 48.5 | 47.8 | 55.2 | 46.6 |

*+, Brown; LR, light red; O, orange; p, pink; r, red.
All tests were performed with the single reference strain \textit{P. korlensis} X14-1\textsuperscript{T} after taking the opinion of experts in the field. This is because efforts were made to obtain the type strain of \textit{P. niistensis} from the two culture collections where it was deposited (the NCIM in Pune, India, and the CCTCC in Wuhan, China) and from the authors who described the species (Dastager \textit{et al.}, 2010). The authors were unable to revive the strain, and the two culture collection centres confirmed that the strain was not available.

Biochemical differences between strain LP51\textsuperscript{T} and \textit{P. niistensis} NII-0905\textsuperscript{T} as originally described include their differential ability to utilize different carbon sources and to grow at different ranges of \textit{pH}, salinity and temperature. Growth of strain LP51\textsuperscript{T} occurred at 5–45 °C, 0–9 % (w/v) NaCl and pH 5.0–10, while \textit{P. niistensis} NII-0905\textsuperscript{T} was reported to grow at 15–42 °C, 0–10 % NaCl and pH 5–12 (Dastager \textit{et al.}, 2010). \textit{P. niistensis} NII-0905\textsuperscript{T} assimilated starch, arabinose and mannotol, while strain LP51\textsuperscript{T} assimilated inositol, trehalose, rhamnose and xylitol. \textit{β}-Galactosidase activity was detected in strain LP51\textsuperscript{T}, but this activity was absent from \textit{P. niistensis} NII-0905\textsuperscript{T}. Hydrolysis of gelatin was observed in strain LP51\textsuperscript{T} but not \textit{P. niistensis} NII-0905\textsuperscript{T}, while hydrolysis of Tween 80 and nitrate reduction were observed in \textit{P. niistensis} NII-0905\textsuperscript{T} but not in strain LP51\textsuperscript{T} (Dastager \textit{et al.}, 2010).

For fatty acid analysis, cells of strain LP51\textsuperscript{T} and \textit{P. korlensis} X14-1\textsuperscript{T} were harvested from LB agar plates after incubation at 28 °C for 2 days. Fatty acid methyl ester (FAME) analysis was carried out by Disha Life Sciences Ltd (Ahmedabad, India). The physiological age of the two strains was standardized by choice of sector from a quadrant streak on trypticase soy broth agar plates according to the MIDI protocol. FAMEs were analysed from two to four loops of inoculum from the third quadrant of a Petri dish that were subjected to saponification, methylation and extraction using the Sherlock Microbial Identification System (MIDI), and identification of fatty acids was achieved using the Aerobe TSBA 60 database version 6.0 B. The major fatty acids of strain LP51\textsuperscript{T} were iso-C\textsubscript{15}:0 (17.8 %), iso-C\textsubscript{15}:0 3-OH (8.8 %), iso-C\textsubscript{17}:0 3-OH (5.7 %), summed feature 3 (C\textsubscript{16}:1\textit{ω}7c and/or C\textsubscript{16}:1\textit{ω}6c) (6.5 %) and summed feature 4 (iso-C\textsubscript{17}:1 I and/or anteiso C\textsubscript{17}:1 B; 30.7 %). The fatty acid profile of strain LP51\textsuperscript{T} revealed qualitative and quantitative differences compared with the profile of closely related strain \textit{P. korlensis} X14-1\textsuperscript{T} (Table S2 and Fig. S1), further suggesting that LP51\textsuperscript{T} represents a novel species of the genus Pontibacter.

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 °C) at 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 loaded on a TLC plate (silica gel 60 F\textsubscript{254}, 20 × 20 cm; Merck art. no. 1.05554.0007) using petroleum ether (boiling point 60–80 °C) and diethyl ether (85 : 15, v/v). Purified menaquinone MK-7 was dissolved in diethyl ether and analysed by reversed-phase TLC according to Collins \textit{et al.} (1977).

Bacterial polyamines were extracted as described by Busse \& Auling (1988) and analysed by one-dimensional TLC. Ten microlitres of extracted sample was loaded on a TLC plate (silica gel 60 F\textsubscript{254}, 20 × 20 cm; Merck). Ethyl acetate/cyclohexane was used as the running solvent. For detection of polyamines, the TLC plate was allowed to air dry, after which it was visualized under UV light. Polyamines were identified by comparing \textit{Rf} values of commercially prepared standards obtained from Sigma Life Science. \textit{Syn}-Homospermidine was detected as the major polyamine. The DNA G + C content of strain LP51\textsuperscript{T} was calculated according to the method described by Gonzalez \& Saiz-Jimenez (2002) using an Applied Biosystems 7500 Real-Time PCR, and was found to be 52.4 mol%.

Polar lipid analysis of strain LP51\textsuperscript{T} was performed by two-dimensional TLC as described by Bligh \& Dyer (1959). Total polar lipids were detected by spraying with 10 % (w/v) molybdatophosphoric acid dissolved in ethanol (Merck) followed by drying at 120 °C for 15 min. Major polar lipids present in strain LP51\textsuperscript{T} were phosphatidylethanolamine, an unidentified aminophospholipid, four unknown aminolipids, six unknown polar lipids and three unknown glycolipids (Fig. S2).

On the basis of its morphological, physiological, genotypic and chemotaxonomic characteristics, strain LP51\textsuperscript{T} is considered to represent a novel species of the genus \textit{Pontibacter}, for which the name \textit{Pontibacter chinhatensis} sp. nov. is proposed.

**Description of \textit{Pontibacter chinhatensis} sp. nov**

\textit{Pontibacter chinhatensis} (chin.ha.ten’sis. N.L. masc. adj. chinhatensis of or belonging to Chinhat, where the type strain was isolated).

Cells are Gram-negative, aerobic, rod-shaped, motile by means of gliding, 1.1–1.5 μm long and 0.7–0.9 μm wide. Growth occurs on LB agar, nutrient agar, marine agar, tryptic soya yeast agar and brain heart infusion agar; limited growth occurs on R2A agar. Colonies are light red, entire, smooth, circular and convex and are visible after 36–48 h of incubation. Growth occurs at 5–45 °C (optimum 28 °C), in 0–9% NaCl and at pH 5.0–10, with optimal growth at pH 7.0–8.0. Catalase- and oxidase-positive but DNase-negative. Hydrolyses gelatin, Tween 20 and ascorbin but does not hydrolyse urea, Tween 80 and starch. Does not reduce nitrate. Production of indole and H\textsubscript{2}S is negative, but casein is decomposed. Flexirubin-type pigments are absent. Citrate, xanthine and hypoxanthine are not utilized. \textit{β}-Galactosidase activity is detected. Assimilates maltose, D-fructose, melibiose, sorbitol, D-glucose, raffinose, \textit{myo}-inositol, D-galactose, D-mannose, sucrose, lactose, xylitol, rhamnose, trehalose and inulin, but not D-mannitol, L-arabinose, D-ribose, sorbose, dulcitol or...
citric acid. Acid is produced with D-glucose, rhamnose and D-galactose. The type strain was isolated from an HCH-contaminated site, but it does not degrade HCH isomers. The polyamine pattern shows the presence of sym-homospermidine as the major polyamine. The major isoprenoid quinone is MK-7. Contains iso-C₁₅:₀, iso-C₁₅:₀ 3-OH, iso-C₁₇:₀ 3-OH, summed feature 3 (C₁₆:₁ω7c and/or C₁₆:₁ω6c) and summed feature 4 (iso-C₁₇:₁ 1 I and/or anteiso-C₁₇:₁ 1 B) as the major fatty acids. Major polar lipids are phosphatidylethanolamine, an unidentified aminophospholipid, four unknown aminolipids, six unknown polar lipids and three unknown glycolipids.

The type strain, LP51ᵀ (=CCM 8436ᵀ=MCC 2070ᵀ), was isolated from pond sediment containing discarded HCH waste in northern India, near Lucknow. The DNA G+C content of the type strain is 52.4 mol%.

Acknowledgements

The work was supported by grants from the Department of Biotechnology (DBT), Government of India, All India Network Project on Soil Biodiversity-Biofertilizer (ICAR), Department of Science and Technology, under project SR/SO/AS-24/2011, and a grant from the University of Delhi/Department of Science and Technology Promotion of University Research and Scientific Excellence (DU-DST PURSE). A. K. S. and N. G. gratefully acknowledge the Department of Biotechnology (DBT) and the Council for Scientific and Industrial Research (CSIR) for providing research fellowships. We thank SAIF-DST (Sophisticated Analytical Instrumentation Facility, Department of Science and Technology, Department of Anatomy, AIIMS) for providing the transmission electron microscope facility. We thank Professor J. P. Euzéby (Ecole Nationale Vétérinaire, Toulouse, France) for etymological advice.

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