Pontibacter lucknowensis sp. nov., isolated from a hexachlorocyclohexane dump site

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The genus Pontibacter belongs to the phylum Bacteroidetes and was first described by Nedashkovskaya et al. (2005). At the time of writing, there were seven species with validly published names belonging to this genus, Pontibacter actiniarum (Nedashkovskaya et al., 2005), P. akesuensis (Zhou et al., 2007), P. korlensis (Zhang et al., 2008), P. nistensis (Dastager et al., 2010), P. roseus (Suresh et al., 2006), P. xinjiangensis (Wang et al., 2010) and P. populi (Xu et al., 2012). In addition, ‘Pontibacter salisaro’ has also been described (Joung et al., 2011), but the name is not yet validly published. The type strain of the recognized species of the genus are Gram-negative, rod-shaped, aerobic, heterotrophic and pigmented. Various species of the genus Pontibacter have been isolated from marine actinians, desert soil, muddy water and forest soil (Nedashkovskaya et al., 2005; Zhou et al., 2007; Suresh et al., 2006; Dastager et al., 2010). In our ongoing effort (Kaur et al., 2011) to identify the cultivable and unculturable diversity at a hexachlorocyclohexane (HCH) dump site, Lucknow, Uttar Pradesh in North India (27°00′ N and 81°09′ E), we describe here a further species of the genus Pontibacter. For this purpose, soil samples from the HCH dump site were collected, serially diluted and plated on Luria–Bertani (LB) agar plates. An orange-coloured colony, designated DM9T, appeared within 36 h of incubation at 28°C which was picked and purified by streaking on LB agar. A polyphasic approach (Prakash et al., 2007) was adopted for taxonomic classification of strain DM9T.

16S rRNA gene sequence determines the phylogenetic relationship among bacteria based on its universal distribution, highly conserved nature, the fundamental role of the ribosome in protein synthesis, no horizontal transfer and its rate of evolution, which represents an appropriate level of variation between organisms. The 16S rRNA gene sequence analysis of strain DM9T was carried out as described by Prakash et al. (2007) 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 1400 bp of the 16S rRNA gene of the strain DM9T was obtained and this sequence was subjected to similarity search 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 BLASTN search of full sequences through GenBank (Altschul et al., 1990), RDP II (Maidak et al., 2001) and EzTaxon Server version 2.1 (Chun et al., 2007) identified its closest relatives. 16S rRNA gene sequence analysis (1400 bp) revealed that strain DM9T showed highest similarity to P. korlensis X14-1T (96.21%). The
nearly full-length 16S rRNA gene sequences of all members of the genus *Pontibacter* were retrieved for the construction of a phylogenetic tree. The 16S rRNA gene sequence similarity between strain DM9<sup>T</sup> and member of the genus *Pontibacter* ranged from 93.92 to 96.21%. Strain DM9<sup>T</sup> showed highest similarity to *P. korlensis* X14-1<sup>T</sup> (96.21%) followed by *P. salisaro* HMC5104 (95.89%), *P. actiniarum* KMM 6156<sup>T</sup> (95.35%), *P. niistensis* NII-0905<sup>T</sup> (95.06%), *P. roseus* SRC-1<sup>T</sup> (94.43%), *P. populii* HYL7-15<sup>T</sup> (94.06%), *P. akesuensis* AKS 1<sup>T</sup> (94.01%) and *P. xinjiangensis* 311-10<sup>T</sup> (93.92%). A phylogenetic tree was constructed using sequences of closely related species selected from GenBank (93.92%). A phylogenetic tree was constructed using the program CLUSTAL_X version 1.81b (Thompson et al., 1997). Trimming of terminal nucleotides that were not common to all sequences was carried out manually. Phylogenetic analysis was carried out using the 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 TREECON software (Fig. 1). Strain DM9<sup>T</sup>, falls in the clade containing members exclusively belonging to the genus *Pontibacter*.

Cell morphology was examined using light (Eclipse E 600; Nikon) and electron microscopes (transmission electron microscope, TEM 269D; Morgagni, FEI) (see Fig. S1 available in IJSEM Online). Microscopic analysis revealed that strain DM9<sup>T</sup> is non-flagellated. Gliding motility of the organism was tested on a fresh LB broth culture using the hanging-drop method (Bowman et al., 2003) and was found to be positive. Colonies of strain DM9<sup>T</sup> showed optimum growth on LB agar and marine agar at 28 °C within 36 h of incubation. DM9<sup>T</sup> colonies were orange-coloured, circular and smooth. The Gram staining test was performed using a Gram staining kit (HiMedia); the strain was found to be Gram-negative. Antibiotic sensitivity tests were performed on Muller-Hinton II medium using Readymade Sensi-discs (HiMedia) with varying amounts of antibiotics.

**Fig. 1.** Phylogenetic tree based on nearly complete 16S rRNA gene sequence data showing the evolutionary relationship of strain DM9<sup>T</sup> and representative members of the 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 *Reichenbachiella agariperforans* KMM 3525<sup>T</sup> as the outgroup. Bar, 0.02 nt substitutions per 1000 nt positions. The GenBank accession numbers for the 16S rRNA gene sequences of each strain are shown in parentheses.
of antibiotics. Antibiotics tested were as follows (μg antibiotic 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 oxidase discs from HiMedia. Catalase activity was tested by adding 3% (v/v) hydrogen peroxide solution to colonies grown on LB (McCarthy & Cross, 1984). Production of acid from carbohydrate and degradation of xanthine and hypoxanthine was determined using the method of Gordon et al. (1974). Growth at different temperatures (4, 28, 37, 45 and 55 °C), pH (3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0) and salt concentrations [0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10% (w/v) NaCl] was carried out as described by Arden-Jones et al. (1979). Hydrolysis of Tweens 20 and 80 was tested according to the method of 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 the protocol of Christensen (1946). Indole production was tested as described by Smibert & Krieg (1994). Citrate utilization was tested using a HiMedia kit. The nitrate reduction test was performed as described by Smibert & Krieg (1994). DNase activity was tested using HiMedia DNase agar plates. Degradation of HCH isomers was assessed as described by Kumari et al. (2002). Though isolated from the HCH dump site, strain DM9\(^T\) was not found to degrade HCH. Assimilation of different carbohydrates was done in basal media (Gordon et al., 1974). There were marked differences in several biochemical features between strain DM9\(^T\) and P. korlensis X14-1\(^T\). For instance, strain DM9\(^T\) was found to be positive for urease and assimilation of D-mannitol while P. korlensis X14-1\(^T\) was negative for these tests. Similarly, while strain DM9\(^T\) was found to be negative for utilization and assimilation of fructose, D-galactose D-mannose and sucrose and for hydrolysis of gelatin and starch but P. korlensis X14-1\(^T\) was positive for these tests. Differential results of biochemical tests of strain DM9\(^T\) and its closest relatives are given in Table 1.

For fatty acid analysis, cells of strain DM9\(^T\) and P. korlensis X14-1\(^T\) were harvested from LB agar plates after incubation.

### Table 1. Differential morphological and physiological characteristics of strain DM9\(^T\) and other species of the genus Pontibacter

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>1</th>
<th>2</th>
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<tr>
<td>Isolation source</td>
<td>Soil</td>
<td>Soil</td>
<td>Saltern</td>
<td>Marine</td>
<td>Soil</td>
<td>Muddy water</td>
<td>Soil</td>
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<tr>
<td>Colony colour</td>
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<td>Red</td>
<td>Pink</td>
<td>Light pink</td>
<td>Dark pink</td>
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<td>Temperature (°C)</td>
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<td>7–45</td>
<td>20–37</td>
<td>6–43</td>
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<td>0–8</td>
<td>0–10</td>
<td>0–10</td>
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<td>0–4</td>
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<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<td>NR</td>
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<tr>
<td>Aesculin</td>
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<td>+</td>
<td>+</td>
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<td>+</td>
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<td>+</td>
<td>+</td>
<td>–</td>
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<td>+</td>
<td>NR</td>
<td>+</td>
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<tr>
<td>d-Glucose</td>
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<td>+</td>
<td>NR</td>
<td>+</td>
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<td>–</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Lactose</td>
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<td>+</td>
<td>NR</td>
<td>–</td>
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<td>NR</td>
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<tr>
<td>Sucrose</td>
<td>–</td>
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<td>Citric acid</td>
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<td>NR</td>
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<td>–</td>
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<tr>
<td>DNA G+C content (mol%)</td>
<td>49.2</td>
<td>48.2</td>
<td>46</td>
<td>48.7</td>
<td>51.4</td>
<td>59.5</td>
<td>51.4</td>
<td>44.9</td>
<td>47.8</td>
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</table>
at 28 °C for 2 days. Fatty acid methyl ester analysis was carried out at Royal Life Sciences. Fatty acid methyl esters were analysed from two to four loops of inoculum of culture nearly at the same phase of growth. The inoculum was scraped from a Petri dish and subjected to saponification, methylation and extraction using the method of Miller (1982) and Kuykendall et al. (1988). The fatty acid methyl ester mixtures were separated using the Sherlock Microbial Identification System (MIDI), and identification of the fatty acids was made using the Aerobe TSA, version 6.0 B database. The predominant fatty acids of strain DM9T were summed feature 4 [comprising anteiso-C17:1 B and/or iso-C17:1 I (32.3 %)], iso-C15:0 (21.54 %) and iso-C17:03-OH (6.00 %). The cellular fatty acid profile of strain DM9T was similar to those of *P. korlensis* X-14-1T and other closely related species. However, the fatty acid profile of strain DM9T revealed qualitative and quantitative differences as compared with the profile of other closely related strains (Table S1) further suggesting that DM9T 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 (60–80 °C boiling point) 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 F254, 20 × 20 cm, Merck, 1.055±0.0007) 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 reverse-phase TLC according to the protocol of Collins et al. (1977).

For polyamine analysis, cells were cultivated in LB agar medium at 28 °C. Bacterial polyamines were extracted as described by Busse & Auling (1988) and analysed by one-dimensional TLC. A 10 μl volume of extracted sample was loaded on a TLC plate (Silica gel 60 F254, 20 × 20 cm; Merck, 1.055±4.0007) using petroleum ether (boiling point 60–80 °C) and diethyl ether (85:15, v/v). Purified homospermidine was detected as the major polyamine. The DNA G+C content of strain DM9T, calculated by the method described by Gonzalez & Saiz-Jimenez (2002) using the Applied Biosystems 7500 Real-Time PCR instrument, was 49.2 mol%, which is in accordance with that of other members of the genus *Pontibacter*.

Polar lipid analysis of strain DM9T was performed by two-dimensional TLC as described by Bligh & Dyer (1959). Total polar lipids were detected by spraying with 5 % (w/v) molybdophosphoric acid dissolved in ethanol (Merck) followed by drying at 120 °C for 15 min. Major polar lipids present in strain DM9T were phosphatidylethanolamine, an unidentified aminophospholipid (APL1), unknown aminolipids (AL1, AL2) and unknown polar lipids (L1–4) (Fig. S2).

**Description of Pontibacter lucknowensis sp. nov.**

*Pontibacter lucknowensis* (luck.now.en’sis, N.L. masc. adj. lucknowensis of or belonging to Lucknow).

Cells are Gram-negative, motile by gliding, 0.6–0.7 μm in diameter and 1.3–3.1 μm in length. Luxurious growth occurs on LB. Colonies are orange in colour, entire, smooth, circular, convex and appear after 36 h of incubation. Growth occurs at 6–45 °C; optimum growth is observed at 28 °C. Growth occurs in 0–4 % NaCl (optimum 2 %) and at pH 6.0–9.0 (optimum pH 7.0–8.0). Pigment can be extracted with organic solvents. Oxidase- and catalase-positive. Hydrolyses aesculin, urea, Tweens 20 and 80, and casein but does not hydrolyse gelatin and starch. Nitrate reduction and indole production is negative. Assimilates D-glucose, maltose, D-mannitol, lactose, cellobiose, inositol, D-arabinose, ribose, rhamnose and sorbitol but not D-fructose, D-galactose, D-mannose or sucrose. Citrate is not utilized. Sensitive to tetracycline, gentamicin, rifampicin, ciprofloxacin, streptomycin, oxytetracycline, amikacin and kanamycin but resistant to ampicillin, vancomycin and chloramphenicol. Although isolated from an HCH-contaminated site, DM9T was unable to degrade any of the HCH isomers. MK-7 is the major respiratory quinone. Major fatty acids are iso-C17:0 3-OH, C15:0 and summed feature 4 (comprising anteiso-C17:1 B and/or iso-C17:1 I). *sym*-Homospermidine is the major polyamine. Major polar lipids are phosphatidylethanolamine, an unidentified aminophospholipid, two unknown aminolipids and four unknown polar lipids.

The type strain is DM9T (＝CCM 7955T＝MTCC 11079T), isolated from soil of an open HCH dump site situated in north India. The DNA G+C content of the type strain is 49.2 mol%.

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