**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<sup>T</sup>, 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<sup>T</sup> formed a distinct phyletic 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<sub>15</sub>:0 (17.8%), iso-C<sub>15</sub>:0 3-OH (8.8%), iso-C<sub>17</sub>:0 3-OH (5.7%), summed feature 3 (C<sub>16</sub>:1ω7c and/or C<sub>16</sub>:1ω6c; 6.5%) and summed feature 4 (iso-C<sub>17</sub>:1 I and/or anteiso-C<sub>17</sub>:1 B; 30.7%). The polar lipid profile of strain LP51<sup>T</sup> showed the presence of phosphatidylethanolamine, an unidentified aminophospholipid, unknown aminolipids, unknown polar lipids and unknown glycolipids. DNA–DNA relatedness of strain LP51<sup>T</sup> with respect to the most closely related type strain, *Pontibacter korlensis* X14-1<sup>T</sup>, 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<sup>T</sup> (=CCM 8436<sup>T</sup>=MCC 2070<sup>T</sup>).

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 saltrens (Nedashkovskaya et al., 2003; 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. korlensis* (Zhang et al., 2008), *P. niisensis* (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. populis* (Xu et al., 2012), *P. lucknowensis* (Dwivedi et al., 2013), *P. saemangeumensis* (Kang et al., 2013), *P. rambhanderi* (Singh et al., 2013), *P. toksonensis* (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. yuliensis* (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<sup>T</sup> 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<sup>T</sup>. A polyphasic approach (Vandamme et al., 1996; Prakash et al., 2007) was adopted for taxonomic classification of the novel bacterium LP51<sup>T</sup>.

16S rRNA gene sequence analysis of strain LP51<sup>T</sup> 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
of 1435 bp of the 16S rRNA gene of strain LP51\textsuperscript{T} was obtained, and this sequence was subjected to a similarity search using the Seqmatch tool of the RDP (http://rdp.cm.cmc.msu.edu/html/) and the BLAST program of the NCBI (http://www.ncbi.nlm.nih.gov). A non-redundant BLASTN 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 \textit{P. korlensis} X14-1\textsuperscript{T} were retrieved from the GenBank nucleotide database for the reconstruction of a phylogenetic tree. The 16S rRNA gene sequence similarity between strain LP51\textsuperscript{T} and the type strains of species of the genus \textit{Pontibacter} with validly published names ranged from 99.4 to 94.2 %. Strain LP51\textsuperscript{T} showed the highest sequence similarity to \textit{P. niitensis} NII-0905\textsuperscript{T} (99.4 %), followed by \textit{P. korlensis} X14-1\textsuperscript{T} (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 \textit{Erythrobacter litoralis} DSM 8509\textsuperscript{T} 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 evolutionary tree was reconstructed 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 MEGA software. Strain LP51\textsuperscript{T} falls in a clade exclusively containing members belonging to the genus \textit{Pontibacter} (Fig. 1).

DNA–DNA hybridization was carried out between LP51\textsuperscript{T} and \textit{P. korlensis} CCTCC AB 206081\textsuperscript{T} as suggested, as the latter strain showed 97 % 16S rRNA gene sequence similarity to strain LP51\textsuperscript{T}. Total genomic DNA of strain LP51\textsuperscript{T} and \textit{P. korlensis} X14-1\textsuperscript{T} was extracted and purified and hybridization was done by following the protocol described by Kumar 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 LP51\textsuperscript{T} represents a novel species of the genus \textit{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 LP51\textsuperscript{T} 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 LP51\textsuperscript{T} showed optimum growth on LB agar, nutrient agar, marine agar, tryptic soya 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 LP51\textsuperscript{T} were light red, circular and smooth. The Gram staining test was performed using a Gram staining kit (HiMedia), and strain LP51\textsuperscript{T} was found to stain Gram-negative. Antibiogram 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 oxidase discs from HiMedia and \textit{N},\textit{N},\textit{N},\textit{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 LP51\textsuperscript{T} was streaked on LB agar plates and incubated at 4, 28, 37, 45 and 55 °C; additionally, cell growth was monitored by measuring the OD\textsubscript{600} 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 LP51\textsuperscript{T} 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 LP51\textsuperscript{T} and its closest neighbour, \textit{P. korlensis} X14-1\textsuperscript{T}, are given in Table 1.
Pontibacter lucknowensis DM9T (JN561788)
Pontibacter ramchanderi LP43T (JQ806111)
Pontibacter indicus LP100T (KC469980)
Pontibacter ruber JC213T (HG008900)
Pontibacter populi HLY7-15T (HQ223078)
Pontibacter deserti JC215T (HG008011)
Pontibacter toksunensis ZLD-7T (KC894746)
Pontibacter niistensis NII-0905T (FJ897494)
Pontibacter chinhatensis LP51T (KF723288)
Pontibacter soli HYL7-26T (HQ223079)
Pontibacter korlensis X14-1T (DQ888330)
Pontibacter odishensis JC130T (HE681883)
Pontibacter roseus SRC-1T (AM049256)
Pontibacter jeungdoensis HMD3125 (GU339183)
Pontibacter actiniorum KMM6156T (AY989908)
Pontibacter akesuensis AKS 1T (DQ672723)
Pontibacter saemangeumensis GCM0142T (JN607163)
Pontibacter saxifragae H9X6T (FJ004994)
Pontibacter diazotrophicus HAXK (KF146887)
Adhaeribacter aerolatus 6515J-31T (GQ421846)
Adhaeribacter aerophilus 6424S-25T (GQ421850)
Adhaeribacter aquaticus MBRG1.5T (AJ626894)
Adhaeribacter terreus DNG6T (EU682684)
Hymenobacter deserti ZLB-3T (EU325941)
Hymenobacter ocellatus Myx2105T (Y18835)
Hymenobacter antarcticus VUG-A42aaT (EU155012)
Hymenobacter soli PB17T (AB251884)
Hymenobacter glaciei VUG-A130T (GQ454806)
Hymenobacter arizonensis OR362-8T (UJ294485)
Hymenobacter roseosalvarius AA718T (Y18833)
Hymenobacter elongatus VUG-A112T (GQ454797)
Hymenobacter chitinivorans Txc1T (Y18837)
Hymenobacter fastidiosus VUG-A124T (EU155015)
Hymenobacter algoricola VUG-A23aT (EU155009)
Hymenobacter daecheonensis Dae14T (EU370958)
Hymenobacter psychrophilus BZ393T (GQ131579)
Hymenobacter actinosclerus CCUG 39621T (Y17356)
Hymenobacter aerophilus DSM 13606T (EU155008)
Hymenobacter psychrotolerans Tibet-IIU11T (DQ177475)
Hymenobacter tibetensis XTM003T (EU382214)
Hymenobacter norwichensis NS/50T (AJ549285)
Hymenobacter yonginensis HMD1010T (GU808562)
Hymenobacter gelipurpurascens Tg9T (Y18836)
Hymenobacter rigui WPCB131T (DQ089669)
Hymenobacter xinjiangensis X2-1qT (DQ888329)
Erythrobacter litoralis DSM 8509T (AB013354)

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 LP51<sup>T</sup> 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 | R | P | P | P | P | P | P | R |
| Ranges for growth | | | | | | | | | | | | | | | | | | | | |
| NaCl concentration (% w/w) | 0–9 | 0–8 | 0–4 | 0–5 | 0–2.5 | 0–4 | 0–2.5 | 0–5 | 0–9 | 0–8 | 0–8 | 0–5 | 0–10 | 0–4 | 0–2 | 0–5 | 0–5 | 0–7.5 | 0–8 | 0–8 |
| Gliding motility | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| Hydrolysis of: | | | | | | | | | | | | | | | | | | | | |
| Gelatin | + | + | – | – | + | + | + | – | + | – | – | + | – | + | – | + | – | + | – |
| Casein | + | + | – | – | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Aesculin | + | + | – | – | NR | NR | + | NR | + | + | + | + | – | – | + | + | + | + | + |
| Starch | + | – | + | – | – | – | – | – | – | – | – | + | – | + | – | + | + | – | + |
| Tween 20 | + | NR | + | + | NR | NR | NR | NR | – | NR | + | – | NR | NR | NR | NR | NR | NR | NR | NR |
| Tween 80 | + | NR | + | – | + | – | – | – | – | NR | – | NR | NR | NR | NR | NR | NR | NR | NR | NR |
| Urea | – | – | – | + | + | NR | – | – | NR | + | – | NR | NR | NR | – | – | – | – | – |
| β-Galactosidase | + | + | – | – | NR | – | NR | – | NR | – | – | + | + | + | + | + | + | + | + | W |
| Carbon source utilization | | | | | | | | | | | | | | | | | | | | |
| L-Arabinose | – | + | NR | + | + | NR | – | NR | NR | – | – | – | – | + | + | + | + | – | W |
| Sorbitol | + | – | NR | + | – | + | + | NR | – | + | – | – | – | – | – | + | + | + | NR |
| Xyitol | + | – | NR | NR | + | + | NR | – | – | NR | – | – | – | – | – | – | – | NR | NR |
| myo-Inositol | + | – | W | + | – | – | NR | NR | – | + | – | – | – | – | + | + | + | – | NR |
| Rhamnose | – | – | W | + | + | – | NR | – | – | + | + | NR | + | – | + | – | + | – | – |
| D-Ribose | – | – | NR | NR | + | + | NR | – | – | NR | – | – | – | – | – | – | – | NR | NR |
| Inulin | + | + | NR | NR | – | – | NR | NR | – | – | NR | – | NR | NR | NR | NR | NR | NR | NR | NR |
| Citric acid | – | – | NR | NR | – | – | NR | NR | – | – | NR | – | NR | NR | NR | NR | NR | NR | NR | NR |
| 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 |

*B, Brown; LR, light red; O, orange; P, pink; R, red.
All tests were performed with the single reference strain P. korlensis X14-1T after taking the opinion of experts in the field. This is because efforts were made to obtain the type strain of 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 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 LP51T and P. niistensis NII-0905T as originally described include their differential ability to utilize different carbon sources and to grow at different ranges of pH, salinity and temperature. Growth of strain LP51T occurred at 5–45 °C, 0–9 % (w/v) NaCl and pH 5.0–10, while P. niistensis NII-0905T was reported to grow at 15–42 °C, 0–10 % NaCl and pH 5–12 (Dastager et al., 2010). P. niistensis NII-0905T assimilated starch, arabinose and mannitol, while strain LP51T assimilated inositol, trehalose, rhamnose and xyitol. β-Galactosidase activity was detected in strain LP51T, but this activity was absent from P. niistensis NII-0905T.

Hydrolysis of gelatin was observed in strain LP51T but not P. niistensis NII-0905T, while hydrolysis of Tween 80 and nitrate reduction were observed in P. niistensis NII-0905T but not in strain LP51T (Dastager et al., 2010).

For fatty acid analysis, cells of strain LP51T and P. korlensis X14-1T 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 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 method of Miller (1982) and Kuykendall (1988). Ten microlitres of extracted sample was loaded on a TLC plate (silica gel 60 F254, 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 Rf values of commercially prepared standards obtained from Sigma Life Science. syn-Homospermidine was detected as the major polyamine. The DNA G+C content of strain LP51T 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 LP51T 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 LP51T were phosphatidylethanolamine, an unidentified aminophospholipid, four unknown amino-lipids, six unknown polar lipids and three unknown glyco-lipids (Fig. S2).

On the basis of its morphological, physiological, genotypic and chemotaxonomic characteristics, strain LP51T is considered to represent a novel species of the genus Pontibacter, for which the name Pontibacter chinhatensis sp. nov is proposed.

Description of Pontibacter chinhatensis sp. nov

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 ascorbic acid but does not hydrolyse urea, Tween 80 and starch. Does not reduce nitrate. Production of indole and H2S is negative, but casein is decomposed. Flexirubin-type pigments are absent. Citrate, xanthine and hypoxanthine are not utilized. β-Galactosidase activity is detected. Assimilates maltose, D-fructose, melibiose, sorbitol, D-glucose, raffinose, myo-inositol, D-galactose, D-mannose, sucrose, lactose, xyitol, rhamnose, trehalose and inulin, but not D-mannitol, L-arabinose, D-ribose, sorbose, dulcitol or v/v). Purified menaquinone MK-7 was dissolved in diethyl ether and analysed by reversed-phase TLC according to Collins et al. (1977).

Bacterial polyamines were extracted as described by Busse & Auling (1988) and analysed by one-dimensional TLC. 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 LP51T were phosphatidylethanolamine, an unidentified aminophospholipid, four unknown amino-lipids, six unknown polar lipids and three unknown glyco-lipids (Fig. S2).

On the basis of its morphological, physiological, genotypic and chemotaxonomic characteristics, strain LP51T is considered to represent a novel species of the genus Pontibacter, for which the name Pontibacter chinhatensis sp. nov is proposed.
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 I and/or anteiso-C₁₇:₁ 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%.

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References


