Luteimonas tolerans sp. nov., isolated from hexachlorocyclohexane-contaminated soil

Pooja Rani,†1 Udita Mukherjee,†1 Helianthous Verma,1 Komal Kamra2 and Rup Lal1

1Molecular Biology Laboratory, Department of Zoology, University of Delhi, Delhi-110007, India
2Ciliate Biology Laboratory, SGTB Khalsa College, University of Delhi, Delhi-110007, India

A Gram-stain-negative, aerobic, rod-shaped, non-spore-forming, yellow pigmented bacterial strain (UM1T) was isolated from the hexachlorocyclohexane (HCH)-contaminated dumpsite located at Ummari village in Lucknow, India. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain UM1T belongs to the genus Luteimonas with Luteimonas aestuarii B9 as the closest neighbour (97.2 % 16S rRNA gene sequence similarity). The DNA G+C content of strain UM1T was 64.3 mol%. The major polar lipids were diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE) and phosphatidylglycerol (PG). Main fatty acids were iso-C15:0, iso-C11:0, iso-C11:0 3-OH, iso-C17:0 and summed feature 9 (C16:0 10-methyl and/or iso-C17:0 10:0). Ubiquinone (Q-8) was the only respiratory quinone. Spermidine was detected as the major polyamine. The DNA–DNA relatedness value of strain UM1T with respect to its closest neighbour Luteimonas aestuarii B9 was well below 70 % (~49 %). Thus, data obtained from phylogenetic analysis, DNA–DNA hybridization, and chemotaxonomical and biochemical analyses supports classification of strain UM1T as representative of a novel species of the genus Luteimonas, for which the name Luteimonas tolerans sp. nov. is proposed. The type strain is UM1T (=DSM 28473T=MCC 2572T=KCTC 42936T).

The genus Luteimonas was first proposed by Finkmann et al. (2000), and belongs to the family Xanthomonadaceae of the class Gammaproteobacteria. At the time of writing, there are 14 species of the genus Luteimonas with validly published names: Luteimonas mephitis (Finkmann et al., 2000), L. composti (Young et al., 2007), L. aestuarii (Roh et al., 2008), L. aquatica (Chou et al., 2008), L. marina (Baik et al., 2008), L. terricola (Zhang et al., 2010), L. lutimaris (Park et al., 2011), L. cucumeris (Sun et al., 2012), L. vadosa (Romanenko et al., 2013), L. huabeiensis (Wu et al., 2013), L. abyssi (Fan et al., 2014), L. soli (Wang et al., 2015), L. pelagia (Lin et al., 2016) and L. notoginsengisoli (Cheng et al., 2016). These members of the genus Luteimonas have been isolated from various habitats, such as ammonia-supplied biofilter, food waste, tidal flat sediment, fresh water, seawater, soil, tidal flat, cucumber leaf, seashore sediment, stratum water, deep-sea sediment, farmland soil, marine sediment and rhizosphere soil, respectively.

We have been analysing bacterial diversity from the hexachlorocyclohexane (HCH)-dumpsite situated at Ummari village, Lucknow, India by using culture-dependent (Kumar et al., 2009; Singh & Lal, 2009; Kumari et al., 2009; Dadhwal et al., 2009; Bal et al., 2010; Sharma et al., 2010; Garg et al., 2012; Singh et al., 2013, 2014, 2015; Dwivedi et al., 2013; Kumar et al., 2015; Verma et al., 2015; Mahato et al., 2016) and culture independent (Sangwan et al., 2012, 2014) approaches. In order to continue our efforts to characterize bacteria, soil samples were collected from the HCH-contaminated field located at Ummari, Lucknow, India. The samples were serially diluted and plated on Luria–Bertani (LB) agar plates. Amplification of the 16S rRNA gene was carried out using colony PCR with universal primers 8F, 341F, 786F and 1542R. Sequencing was done using an ABI 3700/3700×1 sequencer (Applied Biosystems). The sequence obtained was assembled manually using Sequencing Analysis version 5.1.1 (Applied Biosystems) and Clone Manager software, version 5. A 1419 bp sequence of the 16S rRNA gene of strain UM1T was obtained.

Abbreviations: DPG, diphosphatidylglycerol; FAME, fatty acid methyl ester; HCH, hexachlorocyclohexane; PE, phosphatidylethanolamine; PG, phosphatidylglycerol.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain UM1T is KM888877.

A supplementary table and two supplementary figures are available with the online Supplementary Material.
Similarity searches of this strain were done using the BLASTN program (Altschul et al., 1990) and EzTaxon server (http://www.ezbiocloud.net/eztaxon; Kim et al., 2012). Strain UM1\textsuperscript{T} showed maximum 16S rRNA gene sequence similarity of 97.2% with \textit{Luteimonas aestuarii} B9\textsuperscript{T}.

For phylogenetic analysis, 16S rRNA gene sequences of strains that were closely related to strain UM1\textsuperscript{T} (sequence similarity 97.2–95.8%) were retrieved and aligned using the CLUSTAL X version 2.1 program (Larkin et al., 2007). Phylogenetic analysis was performed using the MEGA software package version 6 (Tamura et al., 2013). The distance model of Jukes & Cantor (1969) was used to calculate the evolutionary distance matrix and a phylogenetic tree was reconstructed using the neighbor-joining method of Saitou & Nei (1987) (Fig. 1). The evolutionary history was also inferred using the maximum-likelihood method (Felsenstein, 1981) and the maximum-parsimony method (Fig. 1). The robustness of the resultant tree topology was evaluated by bootstrap resampling analysis with 1000 replicates (Felsenstein, 1985). DNA–DNA hybridization was performed between strain UM1\textsuperscript{T} and its closest neighbour \textit{Luteimonas aestuarii} B9\textsuperscript{T}. The genomic DNA of both strains was isolated and DNA–DNA hybridization experiments were performed in triplicate using the protocol described by Kumar et al. (2008). Mean DNA–DNA hybridization values were well below 70% (~49%: Table S1, available in the online Supplementary Material) indicating that strain UM1\textsuperscript{T} represents a novel species of the genus \textit{Luteimonas}.

Cell morphological study of strain UM1\textsuperscript{T} was carried out by using transmission electron microscopy (Morgangi 269D; Fei), which revealed that strain UM1\textsuperscript{T} is

\begin{itemize}
  \item \textit{Xanthomonas fuscans} subsp. \textit{fuscans} LMG 826\textsuperscript{T} (JX986955)
  \item \textit{Xanthomonas perforans} DSM 18975\textsuperscript{T} (FR749910)
  \item \textit{Xanthomonas euvesicatoria} DSM 19128\textsuperscript{T} (FR733718)
  \item \textit{Xanthomonas alfalfa} subsp. \textit{citrumelonis} LMG 9325\textsuperscript{T} (JX986962)
  \item \textit{Xanthomonas alfalfa} subsp. \textit{alfalfa} LMG 495\textsuperscript{T} (JX986954)
  \item \textit{L. fuscans} subsp. \textit{aurantifolii} LMG 9179\textsuperscript{T} (JX986960)
  \item \textit{Xanthomonas cassavae} CFBP 4642\textsuperscript{T} (ATMC01000066)
  \item \textit{Xanthomonas phaseoli} ATCC 49119\textsuperscript{T} (GU993265)
  \item \textit{Xanthomonas citri} subsp. \textit{citri} LMG 941 (CAHO01000088)
  \item \textit{Xanthomonas bruni} LMG 947\textsuperscript{T} (Y10764)
  \item \textit{Xanthomonas pisi} DSM 18956\textsuperscript{T} (JPLE01000088)
  \item \textit{Xanthomonas codiae} LMG 8678\textsuperscript{T} (Y10765)
  \item \textit{Xanthomonas sacchari} LMG 471\textsuperscript{T} (Y10766)
  \item \textit{Xanthomonas halifax} YXH031\textsuperscript{T} (KC986351)
  \item \textit{Luteimonas aestuarii} B9\textsuperscript{T} (EF660758)
  \item \textit{L. cucumeris} Y4\textsuperscript{T} (HQ874629)
  \item \textit{Luteimonas vadosa} KMM 9005\textsuperscript{T} (AB704915)
  \item \textit{L. marina} FR1330\textsuperscript{T} (EU295459)
  \item \textit{Luteimonas composit} CC-Y255\textsuperscript{T} (D0846687)
  \item \textit{L. aquatica} RIB1-20\textsuperscript{T} (EF626688)
  \item \textit{Luteimonas huabeiensis} HB2\textsuperscript{T} (JX668136)
  \item \textit{Luteimonas terricola} BZ92\textsuperscript{T} (FJ948107)
  \item \textit{Luteimonas lucitamis} G3\textsuperscript{T} (GU199001)
  \item \textit{Luteimonas notoginsengisoli} SYP-B804\textsuperscript{T} (KP076295)
  \item \textit{Luteimonas mephitis} B1953/27.1\textsuperscript{T} (A1012228)
  \item \textit{Luteimonas soli} Y2\textsuperscript{T} (KP684142)
  \item \textit{Lysobacter ginsengisoli} Gsoil 357\textsuperscript{T} (AB245363)
  \item \textit{Lysobacter antibioticus} DSM 2044\textsuperscript{T} (AB019582)
  \item \textit{Lysobacter capsici} YC5194\textsuperscript{T} (EF488749)
  \item \textit{Escherichia coli} ATCC 11775\textsuperscript{T} (X80725)
\end{itemize}

\textbf{Fig. 1.} Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing relationship of strain UM1\textsuperscript{T} with the type strains of recognized species of the genus \textit{Luteimonas} and representative members of the family \textit{Xanthomonadaceae}. The sequence of \textit{Escherichia coli} ATCC 11775\textsuperscript{T} was taken as an outgroup. Numbers at nodes represent bootstrap values expressed as percentages of 1000 replicates. Asterisks indicate nodes that were also recovered using maximum-likelihood and maximum-parsimony method. GenBank accession numbers are shown in parentheses. Bar, 0.02 accumulated changes per nucleotide.
non-flagellated (Fig. S1). Prior to fixation of cells for transmission electron microscopy, strain UM1<sup>T</sup> was grown in LB broth at 28 °C for 48 h. The gliding motility test using the hanging-drop method on LB broth (Bowman et al., 2003) further confirmed that the strain was not motile. The Gram stain test was performed using a Gram staining kit (HiMedia) and strain UM1<sup>T</sup> was found to be Gram-stain-negative. Colonies were yellow, circular and smooth. Antibiotic sensitivity tests were performed on Mueller–Hinton agar (HiMedia) at 28 °C using antibiotic discs (HiMedia) with varying amounts of antibiotics. The antibiotics tested were polymixin B, tetracycline, oxytetracycline, ampicillin, penicillin, amikacin, gentamicin, rifampicin, kanamycin, ciprofloxacin, vancomycin, chloramphenicol and nalidixic acid. The oxidase test was performed using oxidase reagent (bioMérieux). Catalase activity was tested by adding 3 % (v/v) hydrogen peroxide solution to colonies that had been grown on LB agar (McCarthy & Cross, 1984). Other physiological and biochemical properties (Table 1) were examined with API 20NE and API 50CH systems (bioMérieux) according to the manufacturer’s instructions.

Growth at different temperatures was examined by inoculating strain UM1<sup>T</sup> in LB broth and keeping at 4, 15, 20, 28, 37, 42 or 45 °C for 48 h. Strain UM1<sup>T</sup> showed optimum growth at 28 °C. To determine the pH range for growth, strain UM1<sup>T</sup> was cultured in LB medium at 28 °C, after adjusting the pH of the medium to 2.0 (pH after autoclaving, 2.10), 3.0 (3.11), 4.1 (3.99), 5.0 (4.70), 5.5 (5.12), 6.0 (6.39), 6.5 (6.45), 7.0 (6.89), 7.5 (7.32), 8.0 (7.67), 8.5 (8.33), 9.0 (8.99), 10.0 (9.56), 11.0 (10.10), 11.5 (10.59) or 12.0 (11.15) using appropriate buffers. K<sub>2</sub>HPO<sub>4</sub>–KH<sub>2</sub>PO<sub>4</sub> buffer was used for pH ≤ 8, NaHCO<sub>3</sub>–NaOH buffer for pH 9–11 and Na<sub>2</sub>CO<sub>3</sub>–NaOH buffer for pH 12 (Arden Jones et al., 1979). The pH range for growth of strain UM1<sup>T</sup> was pH 3–10 (optimum pH 8). NaCl tolerance tests were performed by growing strain UM1<sup>T</sup> at different NaCl concentrations (0–10 %, at an interval of 0.5 %). The optimum NaCl concentration for growth of strain UM1<sup>T</sup> was 0–1 %. pH and NaCl tolerance tests were performed at 28 °C for 48 h. The DNA G + C content of strain UM1<sup>T</sup> was calculated using an Applied Biosystems 7500 Real-Time PCR by the method described by Gonzalez & Saiz-Jimenez (2002). The DNA G + C content of strain UM1<sup>T</sup> was 64.3 mol%.

Fatty acid methyl ester (FAME) analyses of strain UM1<sup>T</sup> and its closest phylogenetic neighbour, Luteimonas aestuarii B9<sup>T</sup>, were carried out at Royal Life Sciences, Secunderabad, India. For this purpose, both strains were grown on tryptic soy agar (HiMedia) at 28 °C for 48 h and two to four loopfuls of culture from the exponential phase of growth was scraped and subjected to saponification, methylation and extraction using the method of Miller (1982). FAME mixtures were separated using the Sherlock Microbial Identification System (MIDI) and fatty acids were identified using the Aerobe RTSBA database, version 6.0B. The FAME analysis revealed the presence of iso-C<sub>15 : 0</sub> (23.45 %), iso-C<sub>11 : 0</sub> (15.11 %), iso-C<sub>11 : 0</sub> 3-OH (22.43 %), iso-C<sub>17 : 0</sub> (10.77 %) and summed feature 9 (C<sub>16 : 0</sub> 10-methyl and/or iso-C<sub>17 : 1</sub> (97.2 %). DNA–DNA hybridization with L. aestuarii B9<sup>T</sup> was below 70 % (94 %) suggesting that strain UM1<sup>T</sup> represents a novel species of the genus Luteimonas. The FAME analysis of strain UM1<sup>T</sup> and L. aestuarii B9<sup>T</sup> was performed by two-dimensional thin-layer chromatography (TLC) as described by Bligh & Dyer (1959). The polar lipids were detected by spraying with 1 % (w/v) aqueous primulin dissolved in acetone followed by visualization under UV light. Diposphatidylglycerol (DPG), phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) were detected as major polar lipids present in both strains (Fig. S2). These polar lipids are concordant among all the known species of the genus Luteimonas. Strain UM1<sup>T</sup> showed a large amount of PE whereas the polar lipid profile of L. aestuarii B9<sup>T</sup> showed a large amount of PG. Polyamines were extracted as described by Busse & Auling (1988) and analysed by one-dimensional TLC. Ten microlitres of the extracted sample was applied to a TLC plate and the running solvent used was ethyl acetate/cyclohexane (2 : 3, v/v). Fluorescence was observed at a wavelength of 310 nm. Spermidine was found as the major polyamine in strain UM1<sup>T</sup>. Quinones were extracted from 200 mg dry cell mass with a 1 : 1 mixture of 10 % aqueous solution of 0.3 % (w/v) NaCl in methanol and petroleum ether. The upper phase was collected and dried using a Rotavapor (Buchi), and the dried extract was then dissolved in 100 ml aceton and separated on a TLC plate (silica gel 60 F<sub>254</sub>, 20 × 20 cm; Merck) using petroleum ether and diethyl ether (85 : 15, v/v) as the mobile phase. Purified ubiquinones were dissolved in 2-propanol and analysed by reversed-phase TLC according to Collins & Jones (1981). The predominant respiratory quinone observed in strain UM1<sup>T</sup> was ubiquinone (Q-8).

Based on 16S rRNA gene sequencing analysis, strain UM1<sup>T</sup> shared maximum similarity with Luteimonas aestuarii B9<sup>T</sup> (97.2 %). DNA–DNA hybridization with L. aestuarii B9<sup>T</sup> was below 70 % (~49 %) suggesting that strain UM1<sup>T</sup> represents a novel species of the genus Luteimonas. The fatty acid profile of both strains showed marked quantitative differences in fatty acids. The major polar lipids were DPG, PE and PG, while ubiquinone-8 (Q-8) and spermidine were the only respiratory quinone and polyamine, respectively. Thus based on genotypic, physiological, morphological and chemotaxonomic analyses, strain UM1<sup>T</sup> represents a novel species of genus Luteimonas for which the name Luteimonas tolerans sp. nov. is proposed.

**Description of Luteimonas tolerans sp. nov.**

*Luteimonas tolerans* (to’le.rans. L. part. adj. tolerans tolerating, referring to its ability to tolerate HCH).

Gram-stain-negative, aerobic, rod-shaped, non-sporeforming, non-motile bacterium. Colonies are circular, convex, opaque, smooth and yellow. Growth is observed at 25–40 °C (optimum 28 °C), at pH 3–10 (optimum...
**Table 1.** Differential morphological, physiological and biochemical characteristics of strains UM1<sup>T</sup> and related type strains of species of the genus *Luteimonas*

Strains: 1, UM1<sup>T</sup> (data from this study); 2, *L. aestuarii* B9<sup>T</sup> (this study); 3, *L. abyssi* XH031<sup>T</sup> (Fan **et al.,** 2014); 4, *L. aquatica* RIBI-20<sup>T</sup> (Chou **et al.,** 2008); 5, *L. huabeiensis* HB2<sup>T</sup> (Wu **et al.,** 2013); 6, *L. cucumeris* Y4<sup>T</sup> (Sun **et al.,** 2012); 7, *L. marina* FR1330<sup>T</sup> (Baik **et al.,** 2008); 8, *L. terricola* BZ92r<sup>T</sup> (Zhang **et al.,** 2010); 9, *L. vadosa* KMM 9005<sup>T</sup> (Romanenko **et al.,** 2013); 10, *L. lutimaris* G3<sup>T</sup> (Park **et al.,** 2011); 11, *L. composti* CC-YY255<sup>T</sup> (Young **et al.,** 2007); 12, *L. mephitis* B1953/27.1<sup>T</sup> (Finkmann **et al.,** 2000); 13, *L. soli* Y2<sup>T</sup> (Wang **et al.,** 2015); 14, *L. pelagia* CC-VAM-7<sup>T</sup> (Lin **et al.,** 2016); 15, *L. notoginsengisoli* SYP-B804<sup>T</sup> (Cheng **et al.,** 2016). All species are Gram-stain-negative, rod-shaped and catalase-positive. +, Positive; −, negative; W, weakly positive; NR, not reported.

<table>
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<th>Characteristics</th>
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Table 2. Cellular fatty acid CONTENTS (%) of strain UM1T and *Luteimonas aequarii* B9T

All data are from this study. –, Not detected.

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<th><em>L. aequarii</em> B9T</th>
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<td>iso-C_{11 : 0} 3-OH</td>
<td>22.43</td>
<td>12.44</td>
</tr>
<tr>
<td>C_{12 : 0}</td>
<td>1.78</td>
<td>0.81</td>
</tr>
<tr>
<td>iso-C_{15 : 0}</td>
<td>23.45</td>
<td>26.84</td>
</tr>
<tr>
<td>anteiso-C_{15 : 0}</td>
<td>–</td>
<td>7.25</td>
</tr>
<tr>
<td>iso-F-C_{15 : 0}</td>
<td>1.53</td>
<td>0.33</td>
</tr>
<tr>
<td>iso-C_{16 : 0}</td>
<td>1.43</td>
<td>4.34</td>
</tr>
<tr>
<td>C_{16 : 0}</td>
<td>1.45</td>
<td>0.76</td>
</tr>
<tr>
<td>iso-C_{17 : 0}</td>
<td>10.77</td>
<td>7.17</td>
</tr>
<tr>
<td>Summed feature*</td>
<td>–</td>
<td>1.86</td>
</tr>
<tr>
<td>3</td>
<td>22.04</td>
<td>21.49</td>
</tr>
<tr>
<td>9</td>
<td>21.49</td>
<td></td>
</tr>
</tbody>
</table>

*Summed features are groups of two or three fatty acids that could not be separated by GLC with the Microbial Identification System (MIDI). Summed feature 3 consists of C_{16 : 0}107c or C_{16 : 0}106c; summed feature 9 consists of C_{16 : 0}10-methyl and/or iso-C_{17 : 0}109c.*

pH 8) and in the presence of 0–1 % NaCl. Oxidase-and catalase-positive. Assimilates D-glucose and D-fructose. Does not hydrolyse aesculin or gelatin. Resistant to ampicillin, penicillin and rifampicin. The major polar lipids are DPG, PG and PE. Predominant fatty acids are iso-C_{15 : 0}, iso-C_{11 : 0}, iso-C_{11 : 0} 3-OH, iso-C_{17 : 0} and summed feature 9. Spermidine is detected as the major polyamine and ubiquinone (Q-8) is identified as the only respiratory quinone.

The type strain is UM1T (=DSM 28473T=MCC 2572T =KCTC 42936T), isolated from the hexachlorocyclohexane-contaminated dumpsite in Ummari village, Lucknow, India. The DNA G+C content of the type strain is 64.3 mol%.

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