Description of *Leucobacter holotrichiae* sp. nov., isolated from the gut of *Holotrichia oblita* larvae

Daochen Zhu,1 Peipei Zhang,1 Pingping Li,2 Jian Wu,1 Changxiao Xie,1 Jianzhong Sun1 and Lili Niu3

Correspondence
Daochen Zhu
dczhucn@hotmail.com
Lili Niu
lilyniu@126.com

1School of Environmental Engineering, Jiangsu University, 301 Xuefu Road, Zhenjiang 212013, PR China
2Institute of Agricultural Engineering, Jiangsu University, 301 Xuefu Road, Zhenjiang 212013, PR China
3Shanghai Key Laboratory of Bio-Energy Crops, School of Life Sciences, Shanghai University, Shanghai 200444, PR China

A Gram-stain-positive bacterium, designated T14T, was isolated from the gut of *Holotrichia oblita* larvae and was subjected to a taxonomic study. The isolate was rod-shaped, aerobic, non-motile, non-spore-forming and yellow-pigmented. Phylogenetic analysis based on 16S rRNA gene sequence comparison indicated that the isolate is related to the genus *Leucobacter*. Its closest neighbours were the type strains ‘*Leucobacter kyeonggiensis*’ F3-P9 (96.8 % 16S rRNA gene sequence similarity), *Leucobacter celer* NAL101T (96.2 %) and *Leucobacter chironomi* DSM 19883T (95.5 %). The DNA G+C content of strain T14T was 69.3 mol%, and DNA–DNA hybridization values with closely related strains were <32 %. The predominant cellular fatty acids were anteiso-C15:0 (49.3 %), iso-C16:0 (16.4 %) and anteiso-C17:0 (16.8 %). The major polar lipids were aminolipid, diphosphatidylglycerol, phosphatidylglycerol, phospholipid, phosphoglycolipid and unidentified glycolipids. The predominant respiratory quinone was MK-11. Based on these phylogenetic and phenotypic results, strain T14T can be clearly distinguished from all of the recognized species of the genus *Leucobacter* and is considered to represent a novel species of the genus *Leucobacter*. The name *Leucobacter holotrichiae* sp. nov. is proposed, with the type strain T14T (=DSM 28968T=JCM 30245T).

The genus *Leucobacter* was first described by Takeuchi et al. (1996). At the time of writing, the genus *Leucobacter* comprises 15 species and two subspecies (http://www.bacterio.net/leucobacter.html). The genus *Leucobacter* was proposed to accommodate Gram-positive rods with 2,4-diaminobutyric acid (DAB) as the diagnostic diamino acid in the peptidoglycan and a high percentage of menaquinone-11 (MK-11). Species of the genus *Leucobacter* have been isolated from a wide variety environments such as wastewater, compost, soil, fermented foods, animal faeces and sediments etc. (Halpern et al., 2009; Kim & Lee, 2011; Morais et al., 2006; Shin et al., 2011; Sturm et al., 2011; Ue, 2011; Weon et al., 2012). In the course of screening micro-organisms from the gut of *Holotrichia oblita* larvae, a *Leucobacter*-like isolate, T14T, was isolated.

Samples of *H. oblita* (a species of scarab beetle) larvae were collected from the soil of the Zhuwan farm of Yuncheng county, Shandong province, China. For isolation, larvae were surface-sterilized with 75 % ethanol for 10 s, with 0.1 % mercuric chloride for 10 min, and rinsed several times in sterile distilled water to remove contaminants from the body surface. Gut contents were dissected and crushed. The samples were streaked on LB agar medium at 30 °C. The LB medium contained (g l−1): 10.0 Bactopeptone (DFcO); 5.0 yeast extract (DFcO); 5.0 NaCl. Based on the phenotypic including chemotaxonomic characteristics studied and phylogenetic analysis of 16S rRNA gene sequences, the new isolate, T14T, should be classified in the genus *Leucobacter*. The reference strains used in this study were type strains belonging to the species of the genus *Leucobacter* including ‘*Leucobacter kyeonggiensis*’ JCM 17539 (=F3-P9), *Leucobacter celer* JCM 16465T (=NAL101T) obtained from the Japan Collection of Microorganisms and *Leucobacter chironomi* DSM 19883T.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain T14T is KJ461711.

Three supplementary figures and one supplementary table are available with the online Supplementary Material.

Abbreviation: DAB, 2,4-diaminobutyric acid.
Cells of strain T14T were Gram-stain-positive, non-motile, rod-shaped, 0.4–0.6 μm wide and 0.7–2.5 μm long without flagella (Fig. S1 available in the online Supplementary Material). The isolate was non-spore-forming and chemoheterotrophic, and yellow-coloured colonies appeared on LB agar medium (incubated for 1 day at 30 °C) which were 0.7–1.2 mm in diameter with entire margins. It grew aerobically in LB medium with a temperature range of 10–45 °C containing 0–10 % (w/v) NaCl at pH 5.5–10.0, with optimum growth at 30 °C, pH 7.0 and 0–1 % NaCl. Differential phenotypic characteristics of strain T14T and other species of the genus Leucobacter are summarized in Table 1.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony pigmentation</td>
<td>Yellow</td>
<td>Cream</td>
<td>Cream</td>
<td>Yellow</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Hydrolysis of gelatin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>NaCl tolerance (%)</td>
<td>8</td>
<td>10</td>
<td>12</td>
<td>7</td>
</tr>
<tr>
<td>pH range for growth</td>
<td>5.5–10.0</td>
<td>6.0–10.0</td>
<td>5.0–10.0</td>
<td>4.5–9.5</td>
</tr>
<tr>
<td>Optimal pH for growth</td>
<td>7</td>
<td>8</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>10.0–45.0</td>
<td>4.0–42.0</td>
<td>4.0–55.0</td>
<td>17.0–37.0</td>
</tr>
<tr>
<td>Utilization of (Biolog GP2): d-Ribose</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>N-Acetyl-d-glucosamine</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>l-Alaninamide</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>l-Alanine</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>l-Alanyl glycine</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Maltose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Melibiose</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Melizitose</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Pyruvic acid</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Raffinose</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>l-Rhamnose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Turanose</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Thymidine</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>69.3</td>
<td>67.5a</td>
<td>68.8b</td>
<td>70.7c</td>
</tr>
</tbody>
</table>

Table 1. Phenotypic properties of strain T14T and reference strains

Strains: 1, T14T; 2, ‘L. kyeonggiensis’ JCM 17539; 3, L. celer JCM 16465; 4, L. chironomi DSM 19883. +, Positive; –, negative. All data were obtained in this study unless otherwise stated. All strains were positive for catalase activity and negative for oxidase. All strains were negative for hydrolysis of urea and β-galactosidase (ONPG). All of the micro-organisms utilized glucose, sucrose, glycerol, Tween 40 and Tween 80 as sole carbon sources.

Leucobacter by the multiple sequence alignment program CLUSTAL W2 (Thompson et al., 1994). Sequences of type strains closely related to isolate were retrieved from the EzTaxon-e database. Phylogenetic trees were reconstructed by the neighbour-joining method and maximum-parsimony method based on sequence analyses with the software MEGA6 (Kumar et al., 2004).

An almost complete gene sequence of strain T14T was obtained. Phylogenetic trees reconstructed using the neighbour-joining and maximum-parsimony methods showed the position of strain T14T was within the genus Leucobacter. The resulting 16S rRNA gene sequence was used for a phylogenetic analysis comparing with those of species belonging to the genus Leucobacter.

Leucobacter was retrieved from the German Culture Collection (DSMZ, Braunschweig, Germany).

Morphological characteristics were observed in cells grown on LB agar at 30 °C for 2 days by light microscopy and transmission electron microscopy (H-800; Hitachi). Gram staining was performed by using cell pellets from 18 h of cultivation in LB medium and examined as described by Reddy (2007). Spore formation was determined by malachite green staining of cells grown on LB agar medium for 6 days. A motility test was performed using semisolid agar LB medium (0.5 % agar, w/v). Growth under anaerobic conditions was determined in LB medium for 3 days by the Hungate roll-tube technique under a gas phase of O2-free N2 (Hungate, 1969). Growth was determined at 4, 10, 30, 37, 45 and 50 °C on LB medium and then at pH 5–7 (buffered by 50 mM citrate/Na2HPO4), pH 7–8 (buffered by 100 mM NaH2PO4/Na2HPO4) and pH 9–10.5 (buffered by 100 mM NaHCO3/Na2CO3). Evaluation of the tolerance to NaCl was performed by inoculating 60 μl of inocula to 6 ml of LB medium with NaCl concentration of 0–10 % (w/v) at pH 7.0 in test tubes. Growth was considered positive if the OD600 was greater than that of the negative control. Tests to examine a broad range of phenotypic properties were performed on strain T14T, and additional biochemical tests were performed using the API 20E and API 20NE (bioMérieux) microtest systems and Biolog GP2 system following the manufacturers’ protocols.

Extraction of genomic DNA from the isolate was performed using a Wizard Genomic DNA Purification kit as described by the protocol of the manufacturer (Promega). Amplification of the 16S rRNA gene was carried out as described by Zhu et al. (2014) using the two universal primers 27F (5′-AGAGTTTGATCCTGCGCAAG-3′) and 1541R (5′-AAGAGGTTGATCCTGCGCC-3′). The PCR product was purified with a PCR purification kit (Qiagen) following the manufacturer’s protocol and sequenced using an ABI PRISM 377 sequencer. The resulting 16S rRNA gene sequence was compared with those of recognized type strains using the EzTaxon-e server (Kim et al., 2012). EzTaxon-e analysis indicated that strain T14T belonged to the genus Leucobacter. The resulting 16S rRNA gene sequence was used for a phylogenetic analysis comparing with those of species belonging to the genus Leucobacter. The result...
**Leucobacter** (Figs 1 and S2). The 16S rRNA gene sequence similarity calculations showed strain T14T was most closely related to ‘L. kyeonggiensis’ F3-P9 (96.8 % similarity), *L. celer* NAL101T (96.2 %) and *L. chironomi* DSM 19883T (95.5 %) and shared less than 95.5 % similarity with other members of the genus *Leucobacter*. Sequence analysis of strain T14T showed none of the species with validly published names shared greater than 97 % similarity, which provided evidence that strain T14T represented a novel genomic species.

To determine the taxonomic relatedness of the new isolate with reference strains, whole genome DNA–DNA hybridization experiments were performed using the renaturation rate method (De Ley et al., 1970). The hybridization values of T14T with related strains were 31.2 % (‘L. kyeonggiensis’ F3-P9), 25.6 % (L. celer NAL101T) and 22.3 % (L. chironomi DSM 19883T), which were well below the 70 % cut-off point recommended for the assignment of the strain to the same genus species (Wayne et al., 1987). The DNA G+C content (mol%) of the entire genome was determined from the midpoint of the thermal denaturation profile *Tm* (Marmur & Doty, 1962) obtained with a UV-VIS DU800 spectrophotometer (Beckman) at 260 nm. The *Tm* of reference DNA from *Escherichia coli* NCTC 9001T was 74.6 °C in 0.1 × SSC and the reference DNA G+C content was 50.9 mol% (Owen & Pitcher, 1985).

For analysis of cell-wall peptidoglycan, menaquinones, polar lipids and major fatty acids, cells were cultivated on LB medium at 30 °C and harvested at the end of exponential growth then freeze-dried. Peptidoglycan was extracted and purified by shaking with glass beads to disrupt cells and subsequently digested by trypsin according to a previously described method and analysed by TLC (Schleifer, 1985; Halpern et al., 2009). The molar ratios of amino acids were determined by GC (HP-6890, Agilent) and GC-MS (HP-6890/HP-5793, Agilent) of N-heptafluorobutyryl amino acid isobutyl esters (Groth et al., 1996). Cellular respiratory quinones were extracted, purified and analysed by reversed-phase HPLC as described by Collins & Jones (1981). The cellular fatty acid methyl esters were prepared, purified and identified with the Microbial Identification System (MIS; Microbial ID) according to the manufacturer’s protocol. Polar lipids of strain T14T were extracted, purified and analysed by two-dimensional silica-gel TLC according to the procedure described by Tindall (1990). Lipid material was detected using dodecamolybdophosphoric acid (total lipids), Zinzadze reagent (phosphate), ninhydrin (free amino groups),

![Phylogenetic tree reconstructed by the neighbour-joining method based on complete 16S rRNA gene sequences showing the relationship between strain T14T and other species of the genus Leucobacter. Microbacterium esteraromaticum DSM 8609T and Mycetocola saprophilus NBRC 16274T were used as the outgroup. Bar, 0.5 % sequence divergence. Bootstrap percentages greater than 50 %, based on 1000 resamplings, are shown.](http://ijs.microbiologyresearch.org)
periodate–Schiff (glycols), Dragendorff (quaternary nitrogen) and anisaldehyde sulfuric acid (glycolipids).

The DNA G+C content of strain T14T was 69.3 mol%, which was within the range of DNA G+C contents of species of the genus Leucobacter (62.8–70.7 mol%) described previously (Lee & Lee, 2012; Shin et al., 2011). The hydrolysate (4 M HCl, 100 °C, 16 h) of the purified peptidoglycan of strain T14T contained the amino acids alanine, glycine, threonine, DAB and glutamic acid in a molar ratio of approximately 2:1.1:0.8:0.6:0.9, respectively. Strain T14T possessed a B-type cross-linked peptidoglycan (Schleifer & Kandler, 1972). The predominant menaquinone of strain T14T was MK-11 (61.7 %) with small amounts of MK-9 (16.2 %) and MK-10 (29.4 %). The major cellular fatty acids of T14T were anteiso-C15:0 (49.3 %), iso-C16:0 (16.4 %), anteiso-C17:0 (16.8 %) and iso-C15:0 (9.4 %) and there were two kinds of minor fatty acids.

The fatty acid content of strain T14T is shown in Table S1 in comparison with those of related species of the genus Leucobacter. The main fatty acid profiles of strain T14T were similar to those of other members of the genus Leucobacter. Strain T14T contained aminolipid, diphosphatidylglycerol, phosphatidylglycerol, phospholipid, phosphoglycolipid and unidentified glycolipids as the typical polar lipids (Fig. S3).

The main differential phenotypic characteristics of strain T14T with the closely related species of the genus Leucobacter were compared and are shown in Table 1. Several phenotypic characteristics clearly distinguished strain T14T from other species of the genus Leucobacter. For example, strain T14T was positive for utilization of pyruvic acid but ‘L’kyeonggiensis’ JCM 17539 and Leucobacter chironomi DSM 19883T were negative. Taking together the results of 16S rRNA gene sequence analysis, DNA–DNA hybridization, chemotaxonomic analyses and current taxonomic norms, we propose that strain T14T be classified a representative of a novel species, Leucobacter holotrichiae sp. nov.

**Description of Leucobacter holotrichiae sp. nov.**

Leucobacter holotrichiae sp. nov. (ho.lo.tri’chi.ae. N.L. neut. Holotrichia the scientific name of a genus of beetle; N.L. gen. n. holotrichiae referring to the isolation of the type strain from the gut of larvae of Holotrichia).

Cells are Gram-stain-positive, non-spor-forming, non-motile, short rod-shaped with mean size 0.4–0.6 μm in width and 0.7–2.5 μm in length. It can grow in the presence of 0–8 % (w/v) NaCl at 30 °C. Growth occurs at 10–45 °C with an optimum of 30 °C, and from pH 5.5 to 10.0 (optimum pH 7.0). In API 20E and 20NE kit tests, catalase is detected but oxidase is absent, and hydrolysis of gelatin, enzyme activities for β-glucosidase and arginine dihydrolase are positive. Hydrolysis of casein and starch, nitrate reduction, Voges–Proskauer reaction, enzyme activities for urease, β-galactosidase (ONPG), β-galactosidase (PNPG), lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase are negative. Assimilation occurs for gluconate, glucose and mannose, but no assimilation of arabinose, caprate, mannotol, maltose, adipate, malate, citrate, N-acetylgalactosamine and phenylacetate.

In the Biolog GN2 microtitre test system, the following compounds are utilized: N-acetyl-D-glucosamine, N-acetyl-L-glutamic acid, N-acetyl-β-D-mannosamine, L-alanine, D-arabitol, L-α-lactyl glycerine, L-asparagine, 2,3-butanediol, D-fructose, D-fructose 6-phosphate, D-galactose, gentiobiose, α-D-glucose, D-glucose 1-phosphate, D-glucose 6-phosphate, 3-methyl-D-glucose, D-gluconic acid, D-gluconic acid, glycerol, D-L-α-glycerol phosphate, glycogen, myo-inositol, D-lactic acid methyl ester, D,L-malic acid, malteose, mannans, D-mannose, putrescine, L-rhamnose, L-serine, D-sorbitol, succinic acid, sucrose, thymidine and Tweens 40 and 80. Cannot utilize adenosine, adenine-5’-monophosphate, amygdalin, L-arabinose, acetic acid, L-alanine, D-alanine, arbutin, cellobiose, β-cyclodextrin, 2-deoxyadenosine, dextrose, d-fructose, methyl β-D-galactoside, methyl β-D-galactoside, D-galacturonic acid, methyl β-D-glucoside, L-glutamic acid, glycol-L-glutamic acid, α-, β- and γ-hydroxybutyric acids, p-hydroxyphenylactic acid, inosine, inulin, α-ketogluconic acid, α-ketovaleric acid, α-lactose, lactamid, L-lactic acid, lactulose, maltooltriose, α-D-mannitol, methyl β-D-mannoside, melezitose, melibiose, palatinose, propionic acid, D-psicose, L-proglu- taminic acid, pyruvic acid methyl ester, raffinose, D-ribose, salicin, sedoheptulose, stachyose, succinic acid, succinic acid monomethyl ester, D-tagatose, thymidine-5’-monophosphate, trehalose, turanose, uridine, uridine-5’-monophosphate, xylitol or D-xyllose. Cell-wall amino acids are alanine, glycine, threonine, DAB and glutamic acid. Has a B-type cross-linked peptidoglycan. The major cellular fatty acids are anteiso-C15:0, iso-C16:0, anteiso-C17:0 and iso-C15:0 with small amounts of iso-C14:0 and C16:0. The predominant respiratory quinone is MK-11 and the major polar lipids are aminolipid, diphosphatidylglycerol, phosphatidylglycerol, phospholipid, phosphoglycolipid and unidentified glycolipids.

The type strain is T14T, deposited at the Japan Collection of Micro-organisms as JCM 30245T and the German Collection of Microorganisms and Cell Cultures as DSM 28968T, which was isolated from the gut of Holotrichia oblitae larvae, and the larvae was collected from soil, Yuncheng County, Shanxi province, China. The DNA G+C content of the type strain is 69.3 mol%.

**Acknowledgements**

This work was supported by a grant from the Technology Innovation Fund for Small and Medium-sized Enterprises of Jiangsu Province, China (code: BC2013209), a grant of the Research and development fund project of Panshizhua, China (code: 2015CY-N-10), the Research Innovation Program for College Graduates of Jiangsu Province, the Scientific Research Foundation for the Returned Overseas Chinese Scholars, State Education Ministry, China, as well as a project funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions.


References


