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

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A Gram-stain-positive bacterium, designated T14 T, 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* NAL101 T (96.2 %) and *Leucobacter chironomi* DSM 19883 T (95.5 %). The DNA G+C content of strain T14 T was 69.3 mol%, and DNA–DNA hybridization values with closely related strains were <32 %. The predominant cellular fatty acids were anteiso-C 15 : 0 (49.3 %), iso-C 16 : 0 (16.4 %) and anteiso-C 17 : 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 T14 T 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 T14 T ( = DSM 28968 T = JCM 30245 T ).

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 of 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, T14 T, 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 Bacto peptone (Difco); 5.0 yeast extract (Difco); 5.0 NaCl. Based on the phenotypic including chemotaxonomic characteristics studied and phylogenetic analysis of 16S rRNA gene sequences, the new isolate, T14 T, 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 16465 T (= NAL101 T ) obtained from the Japan Collection of Microorganisms and *Leucobacter chironomi* DSM 19883 T .

Abbreviation: DAB, 2,4-diaminobutyric acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain T14 T is KJ461711

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

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Cells of strain T14T were Gram-stain-positive, non-motile, obtained 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.

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 chemo-organotrophic, 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 0–8 % (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.

DNA 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’-AGAGTTTGATCCTTGGCTCAG-3’) and 1541R (‘5’-AAGGAAGGTGATCCAGCC-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 RNA gene sequence was used for a phylogenetic analysis comparing with those of species belonging to the genus 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).

Table 1. Phenotypic properties of strain T14T and reference strains

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<td>Yellow</td>
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<td>Nitrate reduction</td>
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<td>67.5a</td>
<td>68.8b</td>
<td>70.7c</td>
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</table>

*Data from: a, Kim & Lee (2011); b, Shin et al. (2011); c, Halpern et al. (2009).
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 genome species (Wayne et al., 1987). The DNA G + C content (mol%) of the entire genome 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 and identified by separation using 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),

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Fig. 1. 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 Mycelocola saprophilus NBRC 16274T were used as the outgroup. Bootstrap percentages greater than 50 %, based on 1000 resamplings, are shown.

Leucobacter holotrichiae T14T (KJ461711)

‘Leucobacter kyeonggiensis’ F3-P9 (JQ039895)

Leucobacter celer NAL101T (GQ504012)

Leucobacter chironomi DSM 19883T (EU346911)

Leucobacter chromiresistens JG31T (GU390657)

Leucobacter alluvii RB10T (AM072820)

Leucobacter iarius 40T (AM040493)

Leucobacter denitrificans M1TB10T (GQ246672)

Leucobacter aridicollis CIP 108388T (AJ781047)

Leucobacter komagaiae NBRC 15245T (AB007419)

Leucobacter albus IAM 14851T (AB012594)

Leucobacter luti RF6T (AM072819)

Leucobacter chromireducens subsp. chromireducens L-1T (AJ781046)

Leucobacter chromireducens subsp. solipictus TAN31504T (DQ845457)

Leucobacter tardus K70/01T (AM940158)

Leucobacter aerolatus SJ10T (FN597581)

Leucobacter saliscius M1-8T (GQ352403)

Leucobacter exalbidus K-540B T (AB514037)

Microbacterium esteraromaticum DSM 8609T (Y17231)

Mycelocola saprophilus NBRC 16274T (AB012647)
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 as 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, mannitol, 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-β-glutamic acid, N-acetyl-β-D-mannosamine, L-alanine, D-arabitol, L-α-aminolevulinic acid, 2,3-butanediol, D-fructose, D-fructose 6-phosphate, D-galactose, gentiobiose, D-galactose, D-glucose 1-phosphate, D-glucose 6-phosphate, 3-methyl-D-glucose, D-gluconic acid, D-glucuronic acid, glycerol, D,L-α-galactosyl phosphate, glucose, myo-inositol, D-lactic acid methyl ester, D,L-malic acid, malate, mannitol, putrescine, L-rhamnose, L-serine, L-sorbitol, succinic acid, sucrose, thymidine and Tween 40 and 80. Cannot utilize adenosine, adenosine-5’-monophosphate, amydalin, L-arabinose, acetic acid, α-D-mannanide, D-mannose, arbutin, cellobiose, β-cyclodextrin, 2-deoxyadenosine, dextrin, D-fucose, methyl β-D-galactoside, methyl β-D-galactoside, D-galacturonic acid, methyl α-D-glucoside, D-glutamic acid, glycoly-β-glutamic acid, α-L- and β-γ-hydroxybutyric acids, p-hydroxyphenylactic acid, inosine, inulin, α-ketoglutaric acid, α-ketovaleric acid, α-lactate, lactamide, L-lactic acid, lactulose, maltotriose, D-mannitol, methyl β-D-mannoside, melezitose, melibiose, palatinose, propionic acid, D-psicose, D-xylose, D-xylose, pyruvic acid, α-D-mannose, ribose, D-rhamnose, salicin, sedoheptulosan, stachyose, sucinic acid, succinic acid monomethyl ester, D-tagatose, thymidine-5’-monophosphate, trehalose, turanose, uridine, uridine-5’-monophosphate, xylitol or D-xylose. 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-C 14 : 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 obilta* larvae, and the larvae was collected from soil, Yuncheng County, Shandong province, China. The DNA G+C content of the type strain is 69.3 mol%.

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References


