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Leucobacter ruminantium sp. nov., isolated from the bovine rumen

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Abstract

A Gram-stain-positive, lemon yellow-pigmented, non-motile, rod-shaped bacterium, designated strain A2^T, was isolated from the rumen of cow. Cells were catalase-positive and weakly oxidase-positive. Growth of strain A2^T was observed at 25–45 °C (optimum, 37–40 °C), at pH 5.5–9.5 (optimum, pH 7.5) and in the presence of 0–3.5 % (w/v) NaCl (optimum, 1 %). Strain A2^T contained iso-$C_{16:0}$ and anteiso-$C_{15:0}$ as the major cellular fatty acids. Menaquinone-11 was detected as the sole respiratory quinone. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain A2^T formed a distinct phyletic lineage within the genus *Leucobacter*. Strain A2^T was most closely related to '*Leucobacter margaritiformis*' A23 (97.7 % 16S rRNA gene sequence similarity) and *Leucobacter tardus* K 70/01^T (97.2 %). The major polar lipids of strain A2^T were diphosphatidylglycerol, phosphatidylglycerol and an unknown glycolipid. Strain A2^T contained a B-type cross-linked peptidoglycan based on 2,4-diaminobutyric acid as the diagnostic diamino acid with threonine, glycine, alanine and glutamic acid but lacking 4-aminobutyric acid. The G+C content of the genomic DNA was 67.0 %. From the phenotypic, chemotaxonomic and molecular features, strain A2^T was considered to represent a novel species of the genus *Leucobacter*, for which the name *Leucobacter ruminantium* sp. nov. is proposed. The type strain is A2^T (=KACC 17571^T=JCM 19316^T).

The genus *Leucobacter*, a member of the family *Microbacteriaceae*, was first described by Takeuchi *et al.* [1]. At the time of writing, the genus *Leucobacter* includes 19 species with validly published names, including the recently described species *Leucobacter zeae* [2], *Leucobacter musarum* [3], *Leucobacter populi* [4] and *Leucobacter holotrichiae* [5]; three species, *L. musarum*, *Leucobacter celer* and *Leucobacter chromiireducens*, consist of two subspecies each. The genus *Leucobacter* accommodates aerobic, Gram-stain-positive, non-motile, non-sporulating, rod-shaped bacteria containing menaquinone-11 (MK-11) as the major isoprenoid quinone, 2,4-diaminobutyric acid (DAB) as the diagnostic cell-wall amino acid and iso-$C_{16:0}$ and anteiso-$C_{15:0}$ as the predominant fatty acids, which were retrieved from diverse environment habitats such as contaminated environment [6], soil [7, 8], fermented seafood [9], symptomatic bark [4] and the gut of larvae [5]. In this study, we isolated a putative novel *Leucobacter* strain, designated strain A2^T, from the bovine rumen and characterized its taxonomic properties.

Strain A2^T was isolated from a bovine rumen sample, which was collected from a cannulated 12-month-old HanWoo steer (Korean cow), as previously described [10]. The sample was serially diluted in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.2), spread on tryptic soy agar (TSA; BD) and aerobically incubated at 37 °C for 2 days. The 16S rRNA genes of colonies grown on TSA were PCR-amplified using primers F1 and R13 and the resulting PCR amplicons were double-digested with *Hae*III and *Hha*I, as previously described [11]. Representative PCR products showing unique fragment patterns were sequenced using the F1 primer and their resulting 16S rRNA gene sequences were compared with those of validated type strains using the nucleotide similarity search program in the EzTaxon-e server [12]. From the 16S rRNA gene sequence analysis, a putative novel strain belonging to the genus *Leucobacter*, designated strain A2^T, was selected for further phenotypic and phylogenetic analyses. Strain A2^T was stored at −80 °C in tryptic soy broth (TSB; BD) containing 15 % (v/v) glycerol for long-term preservation. '*Leucobacter margaritiformis*' A23, *Leucobacter tardus* KACC 21123^T and *Leucobacter komagatae* KACC 14453^T were used as reference strains for the comparison of phenotypic properties and fatty acid compositions.

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Abbreviations: DAB, 2,4-diaminobutyric acid; DDH, DNA–DNA hybridization; ML, maximum-likelihood; MP, maximum-parsimony; NJ, neighbour joining.
The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain A2^T is KC107829.
Three supplementary figures and one supplementary table are avialable with the online Supplementary Material.

The 16S rRNA gene of strain A2^T was cloned into the pCR2.1 vector using a TOPO cloning kit (Invitrogen) according to the manufacturer's instructions and sequenced using the M13 reverse and T7 primers of the TOPO cloning kit at Macrogen. The 16S rRNA gene sequence similarities between strain A2^T (1450 nt) and closely related *Leucobacter* species were analysed using the nucleotide similarity search program. The 16S rRNA gene sequences of strain A2^T and closely related type strains were aligned using the fast secondary-structure aware INFERAL aligner of the Ribosomal Database Project (RDP) [13]. Phylogenetic trees based on the neighbour-joining (NJ), maximum-parsimony (MP) and maximum-likelihood (ML) algorithms with bootstrap values (1000 replications) were reconstructed under the default options using the MEGA6 software [14].

Comparative analysis based on 16S rRNA gene sequences revealed that strain A2^T was most closely related to '*L. margaritiformis*' A23 and *L. tardus* K70/01^T with similarities of 97.7 and 97.2 %, respectively. Phylogenetic analysis based on the NJ algorithm showed that strain A2^T formed a distinct phylogenic lineage with a low bootstrap value within the genus *Leucobacter* (Fig. 1). Phylogenetic trees reconstructed using the ML and MP algorithms also showed that strain A2^T formed a distinct phylogenetic lineage within the genus *Leucobacter* (Fig. S1, available in the online Supplementary Material). Traditionally, 97 % 16S rRNA gene sequence similarity between two strains has been widely used as an alternative threshold to avoid laborious DNA–DNA hybridization (DDH) in bacterial classification [15]. However, recently 98.7 % 16S rRNA gene sequence similarity has been suggested as a new boundary to avoid DDH in species delineation because this value approximately equates to 70 % DNA–DNA relatedness between two strains [16]. The 16S rRNA gene sequence similarities between strain A2^T and validly recognized *Leucobacter* species were clearly lower (<97.2 %) than the new boundary value for species delineation, which suggests that strain A2^T may represent a novel species of the genus *Leucobacter* without the need to perform DDH.

Growth of strain A2^T was tested on R2A agar (BD), laboratory prepared Luria–Bertani (LB) agar, nutrient agar (NA; BD) and TSA (BD) at 37 °C for 3 days. Growth of strain A2^T at different temperatures (15, 20, 25, 30, 37, 40, 45 and 50 °C) and pH values (5.0–9.5 at 0.5 pH unit intervals) was evaluated in TSB for 3 days. TSB media of pH below 8.0 and pH 8.0–9.5 were prepared using Na_2HPO_4/NaH_2PO_4 and Tris/HCl buffers, respectively [17]. After sterilization (121 °C for 15 min), the pH values were adjusted again if necessary. Growth of strain A2^T at different NaCl concentrations [0, 0.5, 1, 2, 3, 3.5, 4 and 4.5 % (w/v)] was tested in TSB prepared in the laboratory according to the BD formula. Cell morphology and motility were investigated using phase-contrast microscopy and transmission electron microscopy (JEM-1010; JEOL) with cells from an exponentially grown culture on TSA at 37 °C. Anaerobic growth was assessed on TSA under anaerobic conditions (with 4–10 % CO_2) using the GasPak Plus system (BBL) at 37 °C for 21 days. The following physiological and biochemical properties of strain A2^T and the three reference strains were analysed in parallel under the same conditions. Oxidase activity was evaluated by the oxidation of 1 % (w/v) tetramethyl-*p*-phenylenediamine (Merck), and catalase activity was tested by the production of oxygen bubbles in 3 % (v/v) aqueous hydrogen peroxide solution [18]. Enzymatic activities, biochemical features and acid production from carbohydrates were evaluated using the API ZYM, API 20NE and API 50CH test strips (bioMérieux) at 37 °C, respectively, according to the manufacturer's instructions.

Strain A2^T grew well on TSA and NA (optimum, TSA), but did not grow on LB agar. Cells of strain A2^T were Gram-stain-positive and non-motile rods (0.2–0.5 µm in width and 1.0–1.6 µm in length) (Fig. S2). Anaerobic growth was not observed after 21 days of incubation at 37 °C. Many phenotypic properties of strain A2^T including catalase activity, and hydrolysis of Tween 20, Tween 80, urea, aesculin and casein were in common with those of the closely related reference *Leucobacter* species, but some other phenotypic properties such as activity of oxidase, α-glucosidase and β-galactosidase, hydrolysis of gelatin, and assimilation of D-mannose, D-mannitol and potassium gluconate differentiated strain A2^T from the closely related reference *Leucobacter* species. It has been reported that most *Leucobacter* species, including *L. komagatae* (the type species of the genus), but not *Leucobacter aerolatus* and *L. tardus,* are oxidase-negative [1, 3, 4, 7, 19, 20]. However, strain A2^T showed a weakly positive reaction for oxidase activity. Oxidase activities of *L. komagatae* and *L. tardus* were also positive in this study (Table 1) and the oxidase activity of *L. komagatae* was different from the previous result [1]. Physiological and biochemical characteristics of strain A2^T are additionally described in the species description and compared with those of closely related members of the genus *Leucobacter* in Tables 1 and S1.

The DNA G+C content of strain A2^T was determined by the fluorometric method [21] using SYBR Green I and a real-time PCR thermocycler (Bio-Rad). Isoprenoid quinones were extracted according to the method of Minnikin *et al.* [22] and were analysed using a model LC-20A HPLC system (Shimadzu) equipped with a diode array detector (SPD-M20A; Shimadzu) and a reversed-phase column (250×64.6 mm, Kromasil; Akzo Nobel), as described by Komagata and Suzuki [23]. The peptidoglycan of strain A2^T was isolated and purified by shaking cells with glass beads, trypsin digestion and subsequent SDS treatment, and its structure was analysed according to published protocols [24]. The total hydrolysate (100 °C, 4 M HCl, 16 h) of the peptidoglycans contained muramic acid (Mur) and the amino acids DAB, alanine (Ala), glutamic acid (Glu), glycine (Gly) and threonine (Thr). 4-Aminobutyric acid (occurring in the peptidoglycan of some *Leucobacter* type strains) was absent. Quantification of amino acids by GC/MS after derivatization to *N*-heptafluorobutyric amino acid

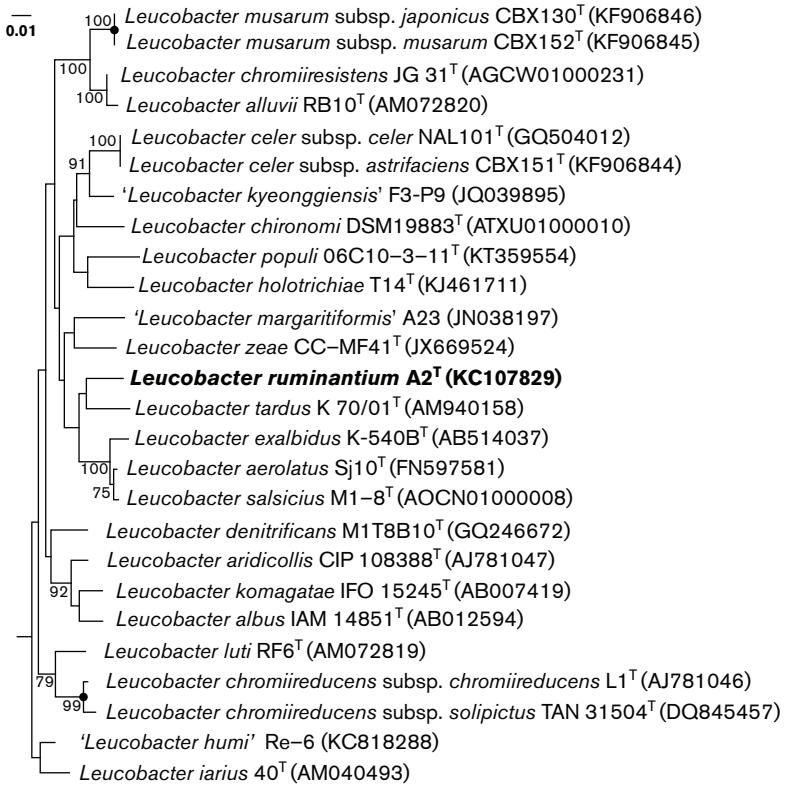

Fig. 1. NJ tree based on 16S rRNA gene sequences showing the phylogenetic relationships between strain A2^T and related taxa. Bootstrap values are shown at nodes as percentages of 1000 replicates; only values >70 % are shown. Filled circles indicate that the corresponding nodes were also recovered in the trees generated using the ML and MP algorithms. *Flavobacterium oceanosedimentum* ATCC 31317^T (EF592577) was used as an outgroup (not shown). Bar, 0.01 changes per nucleotide position.

isobutylesters (Protocol 10) resulted in the following molar ratio: 0.3 DAB : 1.9 Ala : 0.8 Gly : 1.0 Glu : 0.5 Thr : 1.1 Mur. The identity of all amino acids was confirmed by agreement in the GC retention time with those of authentic standards and by characteristic mass spectrometric fragment ions of the derivatives [24]. GC analysis of enantiomeric amino acids (Protocol 11) revealed the following amino acid ratio: 0.9 L-DAB : 1.4 D-Ala : 1.1 L-Ala : 0.4 L-Thr : 0.8 Gly : 1.0 D-Glu : 0.3 L-Glu. After hydrolysis under milder conditions (100 °C, 4 M HCl, 0.75 h), the hydrolysate contained (in addition to the amino acids) the peptide Gly–D-Glu, which typically occurs in peptidoglycans cross-linked according to the B-type. In addition, the peptides Ala–Ala and Thr–DAB were detected.

Strain A2^T, '*L. margaritiformis*' A23, *L. tardus* KACC 21123^T and *L. komagatae* KACC 14453^T were cultivated in TSB at respective optimal temperatures and their microbial cells were harvested at the same growth stage (exponential phase, optical density of 0.8 at 600 nm) for cellular fatty acid analysis. The cellular fatty acids of microbial cells were saponified, methylated and extracted using the standard MIDI protocol. The fatty acid methyl esters were analysed by GC (Hewlett Packard 6890) and identified by using the TSBA6 database of the Microbial Identification System (Sherlock ver. 6.0B; [25]). The polar lipids of strain A2^T were analysed by TLC using cells harvested during the exponential growth phase, according to the procedure described by Minnikin *et al.* [26].

The genomic DNA G+C content of strain A2^T was approximately 67.0 mol%. The only isoprenoid quinone detected in strain A2^T was MK-11. Strain A2^T contained a B-type cross-linked peptidoglycan containing DAB, glycine, alanine and glutamic acid similar to type e B2δ (B11 according to www.peptidoglycan-types.info) but with L-DAB instead of D-DAB as reported by Hensel [27]. Strain A2^T differed in containing threonine but lacking GABA in the peptidoglycan from *L. komagatae* [1], *Leucobacter albus* [28], *Leucobacter denitrificans* [29], *Leucobacter salsicius* [20], *L. margaritiformis* [30], *Leucobacter exalbidus* [8], *L. tardus* [7], *L. populi* [4], '*Leucobacter kyeonggiensis*' [31], *Leucobacter aridicollis* [6] and *Leucobacter chironomi* [32]. The major cellular fatty acids (>10 % of the total fatty acids) of strain A2^T were iso-$C_{16:0}$ and anteiso-$C_{15:0}$, which were in common with those of members of the genus *Leucobacter* (Table 2). The overall fatty acid profile of strain A2^T was similar to those of the reference *Leucobacter* strains, but there were some differences in their respective proportions of some fatty acid components. For example, $C_{10:0}$ was not

Table 1. Comparison of the phenotypic characteristics of strain A2^T and closely related members of the genus *Leucobacter*

Strains: 1, A2^T (this study); 2, '*L. margaritiformis*' A23 [30]; 3, *L. tardus* KACC 21123^T [6]; 4, *L. komagatae* KACC 14453^T [1]. All strains are positive for the following characteristics: activity* of catalase, alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, crystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase. All strains are negative for the following characteristics: indole production, urease activity, hydrolysis* of Tween 20, Tween 80, tyrosine, aesculin, starch and casein, activity* of α-galactosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-frucosidase, and assimilation* of *N*-acetyl-glucosamine, maltose, capric acid, adipic acid, trisodium citrate and phenylacetic acid. +, Positive; –, negative; ND, not described.

Characteristic	1	2	3	4
Colony colour	Lemon yellow	Creamy white	Lemon yellow	Pale brown
Range for growth:				
Temperature (°C)	25–45	4–42	10–30	ND
pH	5.5–9.0	5.0–10.0	ND	5.0–10.0
NaCl (%, w/v)	0–3.5	0–6.0	ND	2.0–5.0
Nitrate reduction	–	–	+	–
Oxidase*	+ (weak)	–	+ (weak)	+‡
Hydrolysis of gelatin (API 20NE)*	+	–	–	+
Enzyme activity (API ZYM and 20NE)* of:				
β-Glucuronidase, β-glucosidase	–	+	–	–
α-Glucosidase	+	+	–	–
D-Glucose, arginine dihydrolase	+	+	+	–
β-Galactosidase	+	–	–	–
Assimilation (API 20NE)* of:				
L-Arabinose	–	+	–	–
D-Mannose	+	+	–	–
D-Mannitol, malic acid	–	–	+	–
Potassium gluconate	+	–	+	–
DNA G+C content (mol%)	64.5	67.0	70.3†	66.2

*These analyses were conducted under the same conditions in parallel in this study.

†Analysis in this study.

‡Oxidase activity was negative in the previous study [1].

detected in strain A2^T, while all three reference strains did contain it. In addition, antesio-$C_{17:0}$ was detected in strain A2^T and '*L. margaritiformis*' A23^T as a minor component, while it was a major component in *L. tardus* KACC 21123^T and *L. komagatae* KACC 14453^T (Table 2). The major polar lipids of strain A2^T were diphosphatidylglycerol, phosphatidylglycerol and an unknown glycolipid (Fig. S3). Two phospholipids and three unidentified lipids were also detected as minor polar lipids. The physiological and chemotaxonomic features and the phylogenetic inference supported the proposition that strain A2^T represents a novel species of the genus *Leucobacter*, for which the name *Leucobacter ruminantium* sp. nov. is proposed.

DESCRIPTION OF *LEUCOBACTER RUMINANTIUM* SP. NOV

Leucobacter ruminantium (ru.mi.nan'ti.um. L. part. adj. *ruminans -antis* ruminating; N.L. gen. pl. n. *ruminantium* of ruminants).

Cells are Gram-stain-positive, strictly aerobic and non-motile rods (0.2–0.5 µm in width and 1.0–1.6 µm in length). Colonies on TSA are lemon yellow and circular. Growth occurs at 20–45 °C (optimum, 37 °C), at pH 5.5–9.0 (optimum, pH 7.5) and in the presence of 0–3.5 % (w/v) NaCl (optimum, 1 %). Catalase-positive and weakly oxidase-positive. Hydrolyses gelatin, but not aesculin, starch, casein, Tween 20, Tween 80, tyrosine or urea. Alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, crystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosidase, α-glucosidase and arginine dihydrolase activities are positive, but α-galactosidase, β-glucuronidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase activities are negative. Reduction of nitrate to nitrite is negative. Assimilation of D-glucose, D-mannose and potassium gluconate is positive, but assimilation of D-mannitol, maltose, L-arabinose, *N*-acetyl-glucosamine, capric acid, adipic acid, malic acid, trisodium citrate and phenylacetic acid is negative. Acids are produced from glycerol, L-arabinose, D-glucose, D-fructose and D-mannose. Major cellular fatty acids (>10 %) are iso-$C_{16:0}$ and anteiso-$C_{15:0}$. MK-11 is the sole isoprenoid quinone. Major polar lipids are diphosphatidylglycerol, phosphatidylglycerol and an unidentified glycolipid. Peptidoglycan is a B-type based on DAB as the diagnostic

Table 2. Cellular fatty acid compositions (%) of strain A2^T and closely related members of the genus *Leucobacter*

Strains: 1, A2^T; 2, '*L. margaritiformis*' A23; 3, *L. tardus* KACC 21123^T; 4, *L. komagatae* KACC 14453^T. All data were from this study. Data are expressed as percentages of the total fatty acids and fatty acids amounting to less than 0.5 % in all strains are not shown. Major components (>10.0 %) are highlighted in bold; TR, trace amount (<0.5 %); –, not detected.

Fatty acid	1	2	3	4
Saturated:				
$C_{10:0}$	–	2.2	1.8	2.0
$C_{14:0}$	–	–	0.7	TR
$C_{16:0}$	2.9	1.8	–	–
Unsaturated:				
$C_{15:1}\omega 8c$	2.5	TR	TR	TR
Branched:				
iso-$C_{10:0}$	–	0.6	–	0.6
iso-$C_{14:0}$	6.0	9.6	1.5	1.8
iso-$C_{15:0}$	2.5	0.5	0.8	2.1
anteiso-$C_{15:0}$	**23.9**	**31.0**	**49.9**	**51.2**
iso-$C_{16:0}$	**61.2**	**48.9**	**14.1**	**14.4**
anteiso-$C_{17:0}$	3.5	2.6	**21.3**	**18.1**
Hydroxy:				
$C_{11:0}$ 2-OH	–	0.7	0.5	–
$C_{13:0}$ 2-OH	–	0.7	0.6	–

diamino acid with threonine, glycine, alanine and glutamic acid but lacking 4-aminobutyric acid.

The type strain is A2^T (=KACC 17571^T=JCM 19316^T), isolated from the bovine rumen. The G+C content of the genomic DNA of the type strain is 67.0 mol%.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
The bovine rumen sampling in this study was undertaken in accordance with the *Guidelines for the Care and Use of Experimental Animals* of the Korean National Institute of Animal Science.

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