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NOTE

Leucobacter denitrificans sp. nov., Isolated from Cow Dung[§]

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The bacterial strain M1T8B10^T was isolated from cow dung in Suwon, Republic of Korea. The strain was a Gram stain-positive rod, nonmotile, and non-spore-forming. According to 16S rRNA gene sequence analysis, the strain fell within the clade of the genus *Leucobacter*, showing the highest sequence similarities with *Leucobacter aridicollis* L-9^T (98.7%), *Leucobacter iarius* 40^T (98.4%), and *Leucobacter komagatae* JCM 9414^T (98.2%). Cell-wall peptidoglycan contained the diagnostic diamino acid 2,4-diaminobutyric acid of the genus *Leucobacter*, showing B-type cross-linked peptidoglycans. The major fatty acids were anteiso-$C_{15:0}$, iso-$C_{16:0}$, and anteiso-$C_{17:0}$. The quinone system consisted of the menaquinones MK-11 (78%) and MK-10 (22%). The polar lipid profiles contained diphosphatidylglycerol, phosphatidylglycerol, and an unidentified glycolipid. Differences in several physiological features including nitrate reduction enabled the isolate to be differentiated from all recognized *Leucobacter* species. Based on these phylogenetic, chemotaxonomic, and phenotypic results, the isolate represents a novel species, for which the name *Leucobacter denitrificans* sp. nov. is proposed. The type strain is M1T8B10^T (=KACC 14055^T =NBRC 106309^T).

Keywords: *Leucobacter denitrificans* sp. nov., taxonomy, 16S rRNA gene, phylogeny, new species

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Introduction

The genus *Leucobacter* was proposed by Takeuchi *et al.* (1996) to describe a contaminant bacterium on an agar plate. The genus *Leucobacter* is a distinct phylogenetic lineage within the family *Microbacteriaceae*, and at the time of writing, the genus comprises 13 recognized species. *Leucobacter* has been isolated from various habitats, such as soil (Lin *et al.*, 2004), activated sludge (Morais *et al.*, 2004, 2006), river sediments (Morais *et al.*, 2006), a nematode (Muir and Tan, 2007; Somvanshi *et al.*, 2007), a chironomid egg mass (Halpern *et al.*, 2009), the phyllosphere of potato plants (Behrendt *et al.*, 2008), and the air of a duck barn (Martin *et al.*, 2010). All species of the genus are characterized by the presence of the diamino acid 2,4-diaminobutyric acid (DAB) in their peptidoglycans.

Bacterial strains were isolated from cow dung by serial dilution plating on tryptic soy agar (TSA), nutrient agar (NA), Luria-Bertani agar (LB), and R2A agar (all from Difco, USA). Plates were incubated for 4 days at 30°C. Fifty-four bacterial strains were isolated (data not shown), of which strain M1T8B10^T grew on R2A medium.

The colony morphology of M1T8B10^T on R2A was determined. Cell morphology was examined by light microscopy (AXIO; Zeiss). The Gram reaction was determined using the bioMérieux Gram staining kit (France) according to the manufacturer's instructions. Catalase, oxidase, and hydrolysis of casein, CM-cellulose, DNA, starch, tyrosine, and Tween 80 were conducted according to the methods of Smibert and Krieg (1994). Growth at 4, 10, 15, 20, 25, 28, 30, 37, 40, 45, and 50°C and at pH 5.0–12.0 (at intervals of 1.0 pH unit, at 30°C) was determined on R2A agar after 7 days of incubation. Salt tolerance was tested in R2A broth supplemented with 0–6% (w/v) NaCl (at 1.0% intervals) after 7 days of incubation at 30°C. Motility testing was performed on one-tenth strength R2A broth supplemented with 0.2% agar. Anaerobic growth was investigated using the GasPak anaerobic system (BBL) for 10 days at 30°C on R2A agar. Enzyme activities and other physiological and biochemical properties were determined using API 20NE, API ID 32GN, API 50 CH test strips (bioMérieux), and Biolog GP microplates (Biolog, USA) at 30°C according to the manufacturer's instructions. The results of API 20NE, API ID 32GN, API 50 CH test strips, and Biolog GP microplates were recorded for up to 5 days. Strain M1T8B10^T formed white-colored colonies with a round, convex shape after 2 days on R2A. Cells were rod-shaped, 0.4–0.6 μm in width, and 0.7–2.0 μm in length. The strain did not assimilate any

Table 1. Differential phenotypic characteristics of M1T8B10^T and closely related *Leucobacter* species

Strains: 1, strain M1T8B10^T; 2, *Leucobacter albus* DSM 17379^T (Lin *et al.*, 2004; Somvanshi *et al.*, 2007); 3, *Leucobacter alluvii* CIP 108819^T (Morais *et al.*, 2006; Somvanshi *et al.*, 2007); 4, *Leucobacter aridicollis* DSM 17380^T (Morais *et al.*, 2004; Somvanshi *et al.*, 2007); 5, *Leucobacter chromiiresistens* DSM 22788^T (Sturm *et al.*, 2010); 6, *Leucobacter iarius* DSM 17402^T (Somvanshi *et al.*, 2007); 7, *Leucobacter komagatae* DSM 8803^T (Takeuchi *et al.*, 1996; Morais *et al.*, 2004; Somvanshi *et al.*, 2007). All strains are non-motile, and utilize Tween 40, Tween 80, and putrescine on Biolog GP microplates. All strains were negative for all other substrates provided on the API 50CH and Biolog GP microplates. +, positive; -, negative; w, weakly positive; NA, data not available.

Characteristics	1	2	3	4	5	6	7
Source	Cow dung	Soil	River sediments	Activated sludge	Soil	Nematode	Contaminant
Colony colour	White	White	Cream	Cream	Yellow	White	Whitish brown
Catalase/oxidase	+/+	+/-	+/-	+/-	+/-	NA	+/-
Nitrate reduction	+	-	-	-	NA	-	-
Urease	-	-	+	+	-	-	-
Gelatin hydrolysis	-	-	+	-	-	-	-
Acid production from (API 50CH):							
Glycerol	-	w	-	-	-	+	+
D-Arabinose	-	w	-	-	-	-	-
L-Arabinose	-	-	-	-	-	-	w
D-Ribose	-	+	-	-	-	+	+
D-Adonitol	-	-	-	-	-	-	w
D-Fructose	-	-	-	-	-	+	-
Inositol	-	-	-	-	-	w	-
N-Acetylglucosamine	-	-	-	-	-	+	-
Esculin ferric citrate	-	-	-	-	+	-	-
Salicin	-	-	-	-	-	w	-
D-Trehalose	-	+	-	-	-	-	-
L-Fucose	-	+	-	-	-	-	-
Potassium 5-ketogluconate	+	-	-	-	-	w	-
Utilization of (Biolog GP2 microplates)							
Dextrin	-	-	+	-	-	-	-
N-Acetyl-D-glucosamine	-	-	-	-	-	+	-
D-Fructose	-	-	+	-	-	+	-
α-D-Glucose	-	-	+	-	-	-	-
D-Psicose	-	-	w	-	-	w	-
D-Ribose	w	+	-	+	-	+	+
D-Trehalose	-	+	-	-	-	-	-
Xylitol	-	-	+	-	+	-	-
β-Hydroxybutyric acid	+	-	-	+	-	-	-
ρ-Hydroxyphenylacetic acid	-	-	+	+	+	+	w
α-Ketovaleric acid	-	+	-	w	-	-	-
D-Lactic acid methyl ester	-	-	w	-	-	-	-
L-Lactic acid	-	-	+	-	-	-	-
Pyruvatic acid methyl ester	-	+	+	-	+	-	-
Pyruvic acid	+	+	w	+	+	-	-
N-Acetyl-L-glutamic acid	-	+	-	-	-	-	-
L-Alaninamide	-	+	+	+	+	+	+
L-Alanine	-	w	+	+	+	w	-
L-Alanyl-glycine	-	+	+	+	-	-	-
L-Asparagine	-	+	-	+	+	-	-
L-Glutamic acid	-	+	-	+	-	w	w
Glycyl-L-glutamic acid	-	+	-	+	-	+	w
L-Serine	-	+	-	+	-	w	-
Glycerol	-	+	+	+	+	+	+
Adenosine	-	w	+	+	+	+	-
2'-Deoxy-adenosine	-	+	-	+	+	-	+
Inosine	-	w	-	+	-	-	-
Thymidine	-	+	+	+	+	+	-
Uridine	-	+	-	+	-	+	-
Adenosine-5'-monophosphate	-	-	-	+	-	w	-

Table 1. Continued

Characteristics	1	2	3	4	5	6	7
Thymidine-5'-monophosphate	-	+	-	+	-	+	-
Uridine-5'-monophosphate	-	w	-	+	-	+	-
Menaquinone(s) (MK-)							
Major amounts	11	11	11	11	11	11	11
Minor amounts	10	9;12	10	10	10;9;8	10;9;12	10;12
Amino acids in the cell wall (molar ratios)							
DAB[a]	0.9	0.8	0.5	0.5	0.4	0.5	0.8
Alanine	2.8	1.8	2.4	2.0	1.8	1.5	1.9
Glycine	1.1	1.1	1.2	1.1	1.0	0.9	0.9
Glutamic acid	1.0	1.0	1.0	1.0	1.0	1.0	1.0
γ-aminobutyric acid	0.4	0.7	-	-	-	-	0.7
Threonine	-	-	0.7	-	0.6	0.7	-

[a] DAB, 2,4-diaminobutyric acid

substrate on API 20NE and API 32GN strips. The strain produced acid only from potassium 5-ketogluconate on the API 50 CH test strip using medium E, and utilized only Tween 40, Tween 80, D-ribose, β-hydroxybutyric acid, pyruvic acid, and putrescine on the Biolog microplate. These metabolic characteristics are shared with other *Leucobacter* species. The phenotypic characteristics of strain M1T8B10^T are given in Table 1 and the species description.

Cell biomass for the analysis of cell-wall and fatty acids was obtained from culture for 48 h in TSA at 30°C. Menaquinones and polar lipids were extracted and analyzed by the method of Minnikin *et al.* (1984). Peptidoglycan analysis was performed as described by Schleifer and Kandler (1972). The fatty acid profile was obtained according to the standard protocol of the Sherlock Microbial Identification System (Sasser, 1990). Strain M1T8B10^T contained the menaquinones MK-11 (78%) and MK-10 (22%). The polar lipid profile contained diphosphatidylglycerol, phosphatidylglycerol, and an unidentified glycolipid (see Supplementary data Fig. S1). The peptidoglycan of strain M1T8B10^T contained DAB, alanine, glycine, γ-aminobutyric acid, and glutamic acid at a molar ratio of 0.9:2.8:1.1:0.4:1.0. The major cellular fatty acids (>10% of the total fatty acids) of strain M1T8B10^T were anteiso-$C_{15:0}$ (40.0%), iso-$C_{16:0}$ (27.3%), and anteiso-$C_{17:0}$ (15.8%) (Table 2).

Isolation of chromosomal DNA, PCR amplification, and direct sequencing of the purified product were carried out as described previously (Weon *et al.*, 2006). The resultant 16S rRNA gene sequence (1,424 bp) was aligned with corresponding sequences of members of the genus *Leucobacter* retrieved from the GenBank database using the CLUSTAL W program (Thompson *et al.*, 1994). A phylogenetic tree was produced using the software package MEGA version 3.1 (Kumar *et al.*, 2004). Distances (using distance options according to Kimura's two-parameter model) and clustering using the neighbor-joining and maximum-parsimony methods were determined using bootstrap values based on 1,000 replicates. Sequence comparisons with 16S rRNA gene sequences from the EzTaxon database (Chun *et al.*, 2007) revealed that strain M1T8B10^T had 98.3–98.7% nucleotide sequence similarities with those of the type strains of all recognized species of the genus *Leucobacter*. The strain showed the highest sequence similarities with *Leucobacter aridicollis* L-9^T (98.7%), *Leucobacter iarius* 40^T (98.4%), and *Leucobacter komagatae* JCM 9414^T (98.2%), and less than 98% with other members of the genus *Leucobacter*. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain M1T8B10^T fell within the *Leucobacter* cluster (Fig. 1).

To determine genomic relatedness, *Leucobacter aridicollis* L-9^T, *Leucobacter iarius* 40^T, and *Leucobacter komagatae* JCM 9414^T, which showed the highest sequence similarities (>98%) with strain M1T8B10^T, were selected. The filter hybridization method was performed according to Seldin and Dubnau (1985). Probe labeling was conducted using the nonradioactive DIG-High prime system (Roche); hybridized DNA was visualized using the DIG luminescent detection kit (Roche). DNA–DNA relatedness was quantified using the Bio-1D Image analysis software (Vilber Lourmat,

Table 2. Fatty acid compositions (%) of *Leucobacter* species

Strains: 1, strain M1T8B10^T; 2, *Leucobacter albus* DSM 17379^T (Somvanshi *et al.*, 2007); 3, *Leucobacter alluvii* DSM 18279^T (Sturm *et al.*, 2010); 4, *Leucobacter aridicollis* DSM 17380^T (Somvanshi *et al.*, 2007); 5, *Leucobacter chromiiresistens* DSM 22788^T (Sturm *et al.*, 2010); 6, *Leucobacter iarius* DSM 17402^T (Sturm *et al.*, 2010); 7, *Leucobacter komagatae* DSM 8803^T (this study). -, <1.0% or not detected.

Fatty acids	1	2	3	4	5	6	7
iso-$C_{14:0}$	4.0	-	-	-	-	1.2	1.0
anteiso-$C_{15:0}$	40.0	54.2	55.6	50.9	52.5	47.8	47.4
iso-$C_{15:0}$	3.7	3.5	1.1	3.4	-	3.3	0.9
$C_{16:0}$	8.0	4.1	10.4	6.2	3.7	2.9	6.1
iso-$C_{16:0}$	27.3	17.0	11.1	11.4	15.0	17.6	13.8
anteiso-$C_{17:0}$	15.8	19.1	20.5	26.3	26.3	26.2	29.4

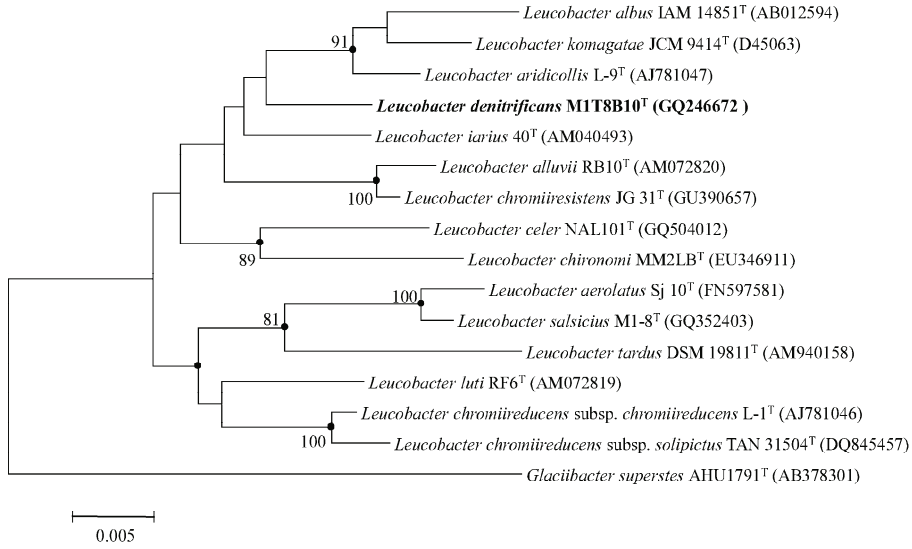

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships between strain M1T8B10^T and members of the genus *Leucobacter*. Filled circles indicate that the corresponding branches were also recovered in the maximum-parsimony tree. The bootstrap values below 70% were not indicated. Bar, 0.005 changes per nucleotide position.

France). Strain M1T8B10^T showed DNA–DNA relatedness of 40% (reciprocal 42%) to *Leucobacter aridicollis* L-9^T, 38% (reciprocal 45%) to *Leucobacter iarius* 40^T, and 39% (reciprocal 28%) to *Leucobacter komagatae* JCM 9414^T.

Strain M1T8B10^T shared the phenotypic, chemotaxonomic, and genetic properties of members of the genus *Leucobacter*. Phenotypically, the strain was unable to produce acid from most of the substrates and showed poor substrate utilization, and chemotaxonomically, possessed MK 11 as the major menaquinone, diphosphatidylglycerol, phosphatidylglycerol, and an unidentified glycolipid as the polar lipids, and anteiso-$C_{15:0}$, iso-$C_{16:0}$, and anteiso-$C_{17:0}$ as the predominant fatty acids. Peptidoglycan lysates also contained components indicating the B-type cross-linked peptidoglycans typical of the genus *Leucobacter*. However, the properties that distinguish this strain from closely related species include oxidase production and nitrate reduction, and its acid production and substrate utilization pattern differed from that of other species (Table 1).

On the basis of the phylogenetic evidence, together with the phenotypic characteristics presented in this study, strain M1T8B10^T is a representative of a novel species within the genus *Leucobacter*, for which the name *Leucobacter denitrificans* sp. nov. is proposed.

Description of *Leucobacter denitrificans* sp. nov.

Leucobacter denitrificans (de.ni.tri'fi.cans. L. prep. *de* away from; L. n. *nitrum* soda; N.L. n. *nitras* nitrate; N.L. v. *denitrifico* to denitrify; N.L. part. adj. *denitrificans* denitrifying).

Cells are rod-shaped and 0.4–0.6 μm in width and 0.7–2.0 μm in length. They are Gram-positive, and oxidase- and catalase-positive. Colonies are white-colored and round and convex in shape after 3 days of growth at 30°C on R2A agar. Growth occurs on TSA, NA, and R2A agar and at 15–37°C and pH 6.0–10.0; optimum growth occurs at 30°C and pH 7.0. It does not require NaCl for growth, but can tolerate up to 3% (w/v) NaCl. It does not hydrolyze casein, CM-cellulose, DNA, starch, tyrosine, or Tween 80. It is positive for nitrate reduction, but negative for idole production, glucose fermentation, arginine hydrolase, urease, aesculin hydrolysis, gelatin hydrolysis, and β-galactosidase (PNG) (API 20NE test strip). It does not assimilate any substrates on API 20NE and API ID 32GN test strips and produces acids only from potassium 5-ketogluconate among the substrates embedded in API 50CH. It utilizes Tween 40, Tween 80, β-hydroxybutyric acid, pyruvic acid, and putrescine, and weakly D-ribose, but no other substrates on Biolog GP microplates. The quinone system consists of the menaquinones MK-11 (78%) and MK-10 (22%). The polar lipid profile is composed of diphosphatidylglycerol, phosphatidylglycerol, and an unidentified glycolipid. Cell-wall peptidoglycan contains DAB, alanine, glycine, γ-aminobutyric acid, and glutamic acid at a molar ratio of 0.9:2.8:1.1:0.4:1.0. The major cellular fatty acids (>10% of the total fatty acids) of strain M1T8B10^T were anteiso-$C_{15:0}$, iso-$C_{16:0}$ (27.3%), and anteiso-$C_{17:0}$.

The type strain is M1T8B10^T (=KACC 14055^T =NBRC 106309^T), which was isolated from cow dung in Suwon, Republic of Korea.

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