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Leucobacter salsicius sp. nov., from a salt-fermented food

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Strain M1-8^T was isolated from jeotgal, a Korean salt-fermented food. Cells were aerobic, non-motile, Gram-reaction-positive and rod-shaped. Colonies were cream-coloured and circular with entire margins. Strain M1-8^T exhibited optimal growth at 25–30 °C and pH 7.0–8.0 and in 0–4 % (w/v) NaCl. The strain tolerated up to 10.0 mM Cr(VI). Phylogenetic analyses of 16S rRNA gene sequences indicated that strain M1-8^T represents a novel species in the genus *Leucobacter*. The 16S rRNA gene sequence of M1-8^T exhibited 98.1 % similarity to that of *Leucobacter chromiireducens* subsp. *chromiireducens* L-1^T. The new isolate was clustered with *Leucobacter* species on a 16S rRNA gene sequence-based phylogenetic tree. The chromosomal DNA G+C content of strain M1-8^T was 62.8 mol%. Its cell-wall peptidoglycan contained 2,4-diaminobutyric acid, glutamic acid, alanine, glycine and γ-aminobutyric acid. The major menaquinone was MK-11 and the predominant fatty acids were anteiso-$C_{15:0}$ (63.6 %), anteiso-$C_{17:0}$ (16.7 %) and iso-$C_{16:0}$ (14.2 %). The polar lipid profile of strain M1-8^T contained diphosphatidylglycerol and one unknown glycolipid. Significant genotypic and phenotypic differences were found between strain M1-8^T and other *Leucobacter* species. These differentiating characteristics indicate that strain M1-8^T represents a novel species of the genus *Leucobacter*, for which the name *Leucobacter salsicius* sp. nov. is proposed. The type strain is M1-8^T (=KACC 21127^T =JCM 16362^T).

The genus *Leucobacter* was first proposed by Takeuchi *et al.* (1996) to allow taxonomic dissection of distinct phylogenetic lineages within the family *Microbacteriaceae*. All *Leucobacter* species contain 2,4-diaminobutyric acid (DAB) in their peptidoglycan. At present, the following 10 members of *Leucobacter* have been defined: *Leucobacter komagatae* (the type species; Takeuchi *et al.*, 1996), *L. albus* (Lin *et al.*, 2004), *L. aridicollis*, *L. chromiireducens* subsp. *chromiireducens* (Morais *et al.*, 2004), *L. alluvii*, *L. luti* (Morais *et al.*, 2006), *L. chromiireducens* subsp. *solipictus* (Muir & Tan, 2007), *L. iarius* (Somvanshi *et al.*, 2007), *L. tardus* (Behrendt *et al.*, 2008) and *L. chironomi* (Halpern *et al.*, 2009). Strains of *Leucobacter* have been recovered from a variety of ecological niches, including activated sludge from soil (Lin *et al.*, 2004) and chromium-contaminated wastewater (Morais *et al.*, 2004, 2006), river sediments containing chromium (Morais *et al.*, 2006), nematodes (Muir & Tan, 2007; Somvanshi *et al.*, 2007), the potato plant phyllosphere (Behrendt *et al.*, 2008) and chironomid egg masses (Halpern *et al.*, 2009).

We isolated strain M1-8^T from jeotgal, a traditional Korean salt-fermented seafood. Shrimp jeotgal is made by mixing fresh tiny shrimps with rock salt, followed by fermenting the mixture for several months (Suh & Yoon, 1987). A sample of shrimp jeotgal was diluted 10^{-6}-fold with PBS and cultured on marine agar (MA; BBL) containing 50 mg mupirocin l^{-1} in an aerobic incubator at 30 °C. Mupirocin was used against the dominant Gram-positive lactic acid bacteria. A pure culture was obtained by repeated restreaking. The isolate was characterized as representing a novel species in the genus *Leucobacter*.

For morphological and physiological characterization, strain M1-8^T and reference strains were grown on MA (pH 7.3) at 30 °C for 2 days. Cell morphology was examined by light microscopy and the Gram reaction was determined by using a non-staining method described previously (Buck, 1982). Motility was examined on semi-solid MA plates (Tittsler & Sandholzer, 1936). Catalase activity was determined by the formation of bubbles in a 3 % H_2O_2 solution and oxidase activity was determined

Abbreviations: DAB, 2,4-diaminobutyric acid; GABA, γ-aminobutyric acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain M1-8^T is GQ352403.

Two supplementary figures are available with the online version of this paper.

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using an oxidase reagent (bioMérieux). Hydrolysis of Tweens 20, 40, 60 and 80 (each 1 % on MA plates) was tested and observed after 1 week. In order to determine the temperature range for growth, the isolate was grown in marine broth (MB) and then incubated for 48 h at 4, 15, 25, 30, 37 or 45 °C. Tolerance of NaCl was determined by growing cells in the presence of 0–10 (at 1 % intervals), 15 or 20 % NaCl (w/v) using medium that contained all of the constituents of MB except NaCl, supplemented with appropriate concentrations of NaCl for 48 h at 30 °C. The pH range for growth was examined by growing cells for 48 h at 30 °C in MB adjusted to pH 2.0–13.0 (at 1.0 pH unit intervals) with HCl or KOH. Members of the genus *Leucobacter* are known to exhibit chromate resistance (Morais *et al.*, 2004). To determine chromium tolerance, strain M1-8^T was cultured at 30 °C for 48 h in MB supplemented with K_2CrO_4 at 0–8 (at 1 mM intervals), 10–20 (at 2 mM intervals), 25 or 30 mM. Anaerobic growth on tryptic soy agar (BBL) plates was analysed at 30 °C for 7 days in an anaerobic chamber with an atmosphere of $N_2/CO_2/H_2$ (8 : 1 : 1). The strain was characterized biochemically using API 20NE and API ZYM strips (bioMérieux), according to the manufacturer's instructions. Substrate utilization was analysed by using the API 50CH strip with API AUX medium (bioMérieux), according to the manufacturer's directions. Table 1 summarizes the data obtained from these comparative studies.

Chromosomal DNA was isolated using the G-spin DNA extraction kit (iNtRON Biotechnology). Phenotypic characterization and phylogenetic analyses were used to determine the taxonomic position of strain M1-8^T. The 16S rRNA gene sequence was amplified by PCR using the universal bacterial primers 8f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-AAGGAGGTGATCCAGCCGC-3′). A PCR purification kit (Qiagen) was used to prepare the template for sequencing. 16S rRNA gene sequences for other members of *Leucobacter* were obtained from the EzTaxon server (http://www.eztaxon.org/; Chun *et al.*, 2007). The multiple sequence alignment program CLUSTAL_X version 1.8 (Thompson *et al.*, 1997) was used to align 16S rRNA gene sequences from the novel isolate and related taxa. Strain M1-8^T was related closely to *L. chromiireducens* subsp. *chromiireducens* L-1^T, *L. chromiireducens* subsp. *solipictus* TAN 31504^T, *L. luti* RF6^T, *L. tardus* K 70/01^T and *L. komagatae* IFO 15245^T (98.1, 97.8, 97.5, 97.4 and 97.3 % similarity, respectively). MEGA4 (Tamura *et al.*, 2007) was used to construct phylogenetic trees with neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Kluge & Farris, 1969) algorithms. Bootstrap analysis was performed using 1000 replications. Phylogenetic trees constructed with both the neighbour-joining (Fig. 1) and maximum-parsimony (not shown) algorithms indicated that strain M1-8^T was associated with the *Leucobacter* cluster, sharing a branching point with *L. tardus* K 70/01^T.

L. komagatae JCM 9414^T, *L. chromiireducens* subsp. *chromiireducens* JCM 13322^T, *L. luti* JCM 14920^T and *L. chromiireducens* subsp. *solipictus* JCM 15573^T, obtained

Table 1. Characteristics that differentiate strain M1-8^T from type strains of closely related taxa

Strains: 1, strain M1-8^T; 2, *L. komagatae* JCM 9414^T; 3, *L. chromiireducens* subsp. *chromiireducens* JCM 13322^T; 4, *L. luti* JCM 14920^T; 5, *L. chromiireducens* subsp. *solipictus* JCM 15573^T; 6, *L. tardus* DSM 19811^T. For reference strains, API kit results were determined in this study; the remaining data were obtained from Takeuchi *et al.* (1996), Morais *et al.* (2004, 2006), Muir & Tan (2007) and Behrendt *et al.* (2008). All strains were positive for activities of catalase, esterase C4, leucine arylamidase and naphthol-AS-BI-phosphohydrolase and utilization of glycerol, D-ribose and starch. All strains were negative for indole production, D-glucose fermentation, activities of L-arginine dihydrolase, urease, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase and utilization of erythritol, D-adonitol, methyl β-D-xylopyranoside, D-galactose, methyl α-D-glucopyranoside, melezitose, raffinose and xylitol. +, Positive; −, negative; W, weakly positive; PNPG, *p*-nitrophenyl β-D-galactopyranoside; NR, not reported.

Characteristic	1	2	3	4	5	6
DNA G+C content (mol%)	62.8	66	66.7	68.8	69.5	NR
Major menaquinone(s)	11	11	11	11	11	10, 11
Hydrolysis of:						
Tween 80	−	+	+	−	+	NR
Starch	−	−	+	+	+	−
Oxidase	−	−	−	−	−	W
Reduction of nitrate to nitrite	−	−	−	−	−	+
β-Glucosidase (aesculin hydrolysis)	W	W	+	W	W	−
Protease (gelatin hydrolysis)	−	+	−	−	+	+
β-Galactosidase (PNPG hydrolysis)	W	W	W	+	W	−
Enzyme activity (API ZYM)						
Alkaline phosphatase	−	+	−	+	−	+
Esterase lipase (C8)	−	W	W	W	W	W
Lipase (C14)	−	+	−	−	−	−
Valine arylamidase	+	W	W	−	−	W
Cystine arylamidase	+	W	W	−	W	−
Acid phosphatase	−	+	−	+	+	+
β-Glucosidase	+	−	−	−	−	−
Utilization of (API 50CH):						
D-Arabinose	−	+	−	−	−	−
L-Arabinose	−	−	W	−	−	−
D-Xylose	−	−	W	−	−	W
L-Xylose	−	−	−	−	+	+
D-Glucose	+	−	−	−	+	+
D-Fructose	−	−	−	−	−	+
D-Mannose	−	−	−	−	W	W
L-Sorbose	−	−	−	−	−	W
L-Rhamnose	−	+	+	+	+	+
Dulcitol	−	−	+	−	−	−

Table 1. cont.

Characteristic	1	2	3	4	5	6
Inositol	+	−	+	+	+	+
D-Mannitol	−	−	+	−	+	+
D-Sorbitol	+	−	−	−	−	+
Methyl α-D-mannopyranoside	−	−	+	−	w	−
N-Acetylglucosamine	−	−	−	+	−	−
Amygdalin	−	−	−	−	+	−
Arbutin	−	−	−	+	−	−
Aesculin	+	−	+	+	+	+
Salicin	w	−	−	−	+	−
Cellobiose	−	−	w	−	+	−
Maltose	−	−	−	−	+	−
Lactose	+	−	−	−	−	−
Melibiose	−	−	+	−	−	−
Sucrose	w	−	+	−	−	−
Trehalose	+	−	−	−	+	+
Inulin	+	−	w	w	+	+
Glycogen	−	w	+	+	+	+
Gentiobiose	−	−	+	−	−	−
Turanose	+	w	+	−	−	w
D-Lyxose	+	−	+	w	w	−
D-Tagatose	−	−	+	−	+	−
D-Fucose	−	−	+	−	w	−
L-Fucose	−	+	w	−	w	−
D-Arabitol	−	−	w	w	w	+
L-Arabitol	−	−	+	−	−	−
Potassium gluconate	−	−	w	−	−	+
Potassium 2-ketogluconate	+	−	−	−	−	+
Potassium 5-ketogluconate	w	w	−	−	+	+

from the Japan Collection of Microorganisms, and *L. tardus* DSM 19811^T, obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, were used as reference strains.

A fluorometric method using SYBR green I and real-time PCR (Gonzalez & Saiz-Jimenez, 2002) was used to determine the G+C content of the genome of strain M1-8^T. Genomic DNA from *Escherichia coli* K-12 and *Bacteroides finegoldii* DSM 17565^T was used for calibration. Each strain was tested in triplicate. Members of the genus *Leucobacter* exhibit genomic DNA G+C contents of 66–69.5 mol% (Muir & Tan, 2007; Takeuchi *et al.*, 1996). However, the DNA G+C content of strain M1-8^T was 62.8 mol%, which is lower than the accepted values for existing species of the genus *Leucobacter*. DNA–DNA hybridization was performed fluorometrically, according to the method described by Ezaki *et al.* (1989) with modifications (Hirayama *et al.*, 1996). DNA–DNA hybridization was performed with five repetitions to determine the relatedness between strain M1-8^T and the reference strains *L. chromiireducens* subsp. *chromiireducens* JCM 22506^T (16.0±7.0 %), *L. chromiireducens* subsp. *solipictus* JCM 15573^T (16.0±6.7 %), *L. luti* JCM 14920^T (10.6±6.9 %), *L. tardus* DSM 19811^T (5.2±2.6 %) and *L. komagatae* JCM 9414^T (13.2±5.8 %). Based on DNA–DNA relatedness values falling well below the threshold of 70 % (Wayne *et al.*, 1987), we propose that strain M1-8^T represents a distinct genospecies.

The amino acid composition of cell-wall hydrolysates was analysed by one-dimensional TLC on cellulose sheets (Bousfield *et al.*, 1985). Hydrolysates from strain M1-8^T and the reference strain *L. komagatae* JCM 9414^T were compared against standard amino acids [DAB, γ-aminobutyric acid (GABA), alanine, glutamic acid, glycine and threonine]. Peptidoglycan composition represents a diagnostic tool for characterizing species in the family *Microbacteriaceae* (Takeuchi *et al.*, 1996). Similarly to the *Leucobacter* reference strain, the peptidoglycan hydrolysate from strain M1-8^T contained DAB, alanine, glycine, glutamic acid and GABA (Supplementary Fig. S1, available in IJSEM Online). Cellular fatty acids were prepared using standard protocols of the Sherlock Microbial Identification system (MIDI). To determine fatty acid compositions, strain M1-8^T and reference strains were grown on MA at 30 °C for 2 days. The predominant fatty acids (>10 % of the total) of M1-8^T were anteiso-$C_{15:0}$ (63.6 %), anteiso-$C_{17:0}$ (16.7 %) and iso-$C_{16:0}$ (14.2 %). Cellular fatty acid profiles of each strain are shown in Table 2. Quinone extraction and identification were performed as described

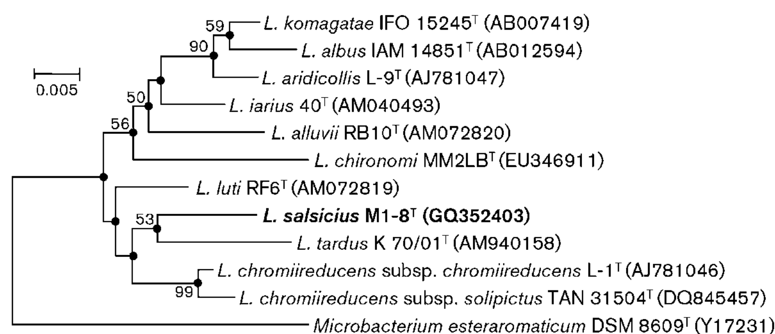

Fig. 1. Phylogenetic tree derived from 16S rRNA gene sequences from strain M1-8^T and closely related *Leucobacter* type strains. Filled circles indicate generic branches present in phylogenetic trees generated by both the neighbour-joining and maximum-parsimony algorithms. Numbers at nodes indicate bootstrap percentages, calculated by neighbour-joining. Bootstrap analyses were performed using 1000 replicates and values greater than 50 % are shown. Bar, 0.005 changes per nucleotide position.

Table 2. Cellular fatty acid contents of strain M1-8^T and reference type strains

Strains: 1, strain M1-8^T; 2, *L. komagatae* JCM 9414^T; 3, *L. chromiireducens* subsp. *chromiireducens* JCM 13322^T; 4, *L. luti* JCM 14920^T; 5, *L. chromiireducens* subsp. *solipictus* JCM 15573^T; 6, *L. tardus* DSM 19811^T. Data are from this study; values are percentages of total fatty acids. tr, Trace (<1.0 %); −, not detected.

Fatty acid	1	2	3	4	5	6
iso-$C_{14:0}$	tr	4.1	1.3	4.7	1.8	1.6
$C_{14:0}$	−	−	tr	1.2	tr	−
anteiso-$C_{15:1}$ A	−	−	−	−	tr	1.1
iso-$C_{15:0}$	tr	8.5	2.7	4.9	2.7	2.9
anteiso-$C_{15:0}$	63.6	53.4	61.8	49.6	60.1	55.2
$C_{15:0}$	−	−	−	tr	1.1	−
iso-$C_{16:0}$	14.2	14.2	13.5	27.2	18.3	14.3
$C_{16:0}$	3.7	5.7	6.4	5.9	5.2	2.6
iso-$C_{17:0}$	−	1.2	−	tr	−	−
anteiso-$C_{17:0}$	16.7	12.8	13.7	5.2	9.5	22.4

previously (Komagata & Suzuki, 1987). The major menaquinone of M1-8^T was MK-11 and minor menaquinones were MK-10 and MK-7. Polar lipids were extracted by a modification of the method of Xin *et al.* (2000), separated by one-dimensional TLC on a Merck silica gel 60 F_{254} glass-backed plate with the solvent system chloroform/methanol/acetic acid/water (85 : 22.5 : 10 : 4, by vol.) and detected by spraying the plate with specific reagents, as described by Tindall (1990). The designation of all spots was compared with reference strains *L. komagatae* JCM 9414^T and *L. chromiireducens* subsp. *solipictus* JCM 15573^T. The polar lipid profile contained diphosphatidylglycerol and an unknown glycolipid (Supplementary Fig. S2).

Strain M1-8^T and related species showed the same general characteristics: non-motile, catalase-positive, rod-shaped cells, the presence of DAB, alanine, glycine and glutamic acid in the peptidoglycan, anteiso-$C_{15:0}$, anteiso-$C_{17:0}$ and iso-$C_{16:0}$ as dominant fatty acids and MK-11 as the major menaquinone. However, strain M1-8^T differed from recognized *Leucobacter* species based on several physiological characteristics (Tables 1 and 2). Our phenotypic, chemotaxonomic, genotypic and phylogenetic analyses suggest that strain M1-8^T represents a novel species of the genus *Leucobacter*, for which the name *Leucobacter salsicius* sp. nov. is proposed.

Description of *Leucobacter salsicius* sp. nov.

Leucobacter salsicius (sal.si′ci.us. L. masc. adj. *salsicius* salted, salty).

Forms circular, cream-coloured colonies with entire margins. Cells are aerobic, Gram-reaction-positive, non-motile (on semi-solid agar medium) rods, 1.0–1.5 µm long. Catalase-positive and oxidase-negative. Optimal growth occurs at 25–30 °C and at pH 7.0–8.0 and in the presence of 0–4 % (w/v) NaCl. Can tolerate up to 10.0 mM Cr(VI). Produces esterase C4, leucine arylamidase, valine arylamidase, cystine arylamidase, naphthol-AS-BI-phosphohydrolase and β-glucosidase. Does not produce alkaline phosphatase, esterase lipase C8, lipase C14, trypsin, α-chymotrypsin, acid phosphatase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase or α-fucosidase. Negative for reduction of nitrate to nitrite and does not produce indole. Hydrolyses casein, aesculin, Tweens 20, 40 and 60 and *p*-nitrophenyl β-D-galactopyranoside. Does not hydrolyse starch, urea, gelatin or Tween 80. Negative for D-glucose fermentation, L-arginine dihydrolase and urease. Utilizes glycerol, D-ribose, D-glucose, inositol, D-sorbitol, aesculin, salicin, lactose, sucrose, trehalose, inulin, starch, turanose, D-lyxose, potassium 2-ketogluconate and potassium 5-ketogluconate but not erythritol, D- or L-arabinose, D- or L-xylose, D-adonitol, methyl β-D-xylopyranoside, D-galactose, D-fructose, D-mannose, L-sorbose, L-rhamnose, dulcitol, D-mannitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, *N*-acetylglucosamine, amygdalin, arbutin, cellobiose, maltose, melibiose, melezitose, raffinose, glycogen, xylitol, gentiobiose, D-tagatose, D- or L-fucose, D- or L-arabitol or gluconate. The dominant cellular fatty acids are anteiso-$C_{15:0}$, anteiso-$C_{17:0}$ and iso-$C_{16:0}$. The cell-wall peptidoglycan contains DAB, alanine, glycine, glutamic acid and GABA. The chromosomal DNA G+C content of the type strain is 62.8 mol%. Polar lipids include diphosphatidylglycerol and an unknown glycolipid.

The type strain, M1-8^T (=KACC 21127^T =JCM 16362^T), was isolated from jeotgal, a Korean salt-fermented food containing tiny shrimps.

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