Leucobacter celer sp. nov., isolated from Korean fermented seafood

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A novel, Gram-reaction-positive, aerobic, rod-shaped, non-motile bacterial strain, designated NAL101^T, was isolated from gajami-sikhae, a traditional Korean fermented seafood made of flatfish. Growth occurred at 4–45 °C, at pH 5–10 and in 0–12 % (w/v) NaCl. Optimum growth occurred at 30–37 °C, at pH 8 and in 0–1 % (w/v) NaCl. The cell-wall amino acids were 2,4-diaminobutyric acid, alanine, glycine, threonine and glutamic acid and the major fatty acids were anteiso-$C_{15:0}$, iso-$C_{16:0}$ and anteiso-$C_{17:0}$. The predominant menaquinone was MK-11. The major polar lipids were diphosphatidylglycerol, phosphatidylglycerol and an unknown glycolipid. The 16S rRNA gene sequence of strain NAL101^T showed 97.7 % similarity to that of *Leucobacter chironomi* MM2LB^T, its closest relative. The DNA G+C content was 68.8 mol% and DNA–DNA hybridization values with closely related strains were <22 %. Phylogenetic analyses based on 16S rRNA gene sequences as well as differences in its physiological and biochemical characteristics indicated that strain NAL101^T represents a novel species of the genus *Leucobacter* in the family *Microbacteriaceae*, for which the name *Leucobacter celer* sp. nov. is proposed. The type strain is NAL101^T (=KACC 14220^T =JCM 16465^T).

Sikhae, a kind of traditional Korean food, also known as jeotgal, consists of fermented fish without intestines and grain-derived lactic acid. In order to understand the fermentation caused by a diverse range of micro-organisms, this fermented food was investigated. A recent study reported the isolation of a novel bacterium, *Alishewanella jeotgali* (Kim *et al.*, 2009), from gajami-sikhae. During a study of the microbial diversity of fermented seafood, a *Leucobacter*-like strain was isolated. The taxonomic position of the isolate was then determined using a polyphasic approach.

The liquid portion of sikhae was diluted with filtered PBS and spread on marine agar (MA, BBL). To produce a pure culture, the isolate was transferred to fresh MA several times. The 16S rRNA gene sequence of the isolate was amplified by colony PCR using a PCR pre-mix (iNtRon Biotechnology) with four universal bacteria-specific primers, 8F, 518R, 968F and 1492R (Baker *et al.*, 2003). After purification (QIAquick PCR Purification kit, Qiagen), the PCR product was sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems). The reaction mixture was analysed using an automated system (PRISM 3730XL DNA analyser, Applied Biosystems). Fragments of the 16S rRNA gene sequence were assembled using SeqMan (DNASTAR) and comparisons of the 16S rRNA gene sequence were performed using the EzTaxon server (Chun *et al.*, 2007). EzTaxon analysis indicated that strain NAL101^T belonged to the genus *Leucobacter*. Strain NAL101^T showed 16S rRNA gene sequence similarities of 97.7, 97.3, 97.2, 97.2, 97.1 and 97.0 % with *Leucobacter chironomi* MM2LB^T, *L. chromiireducens* subsp. *chromiireducens* L-1^T, *L. chromiireducens* subsp. *solipictus* TAN 31054^T, *L. chromiiresistens* JG 31^T, *L. iarius* 40^T and *L. alluvii* RB10^T, respectively. Some of these strains were obtained from the DSMZ and used as reference strains. The 16S rRNA gene sequence of the isolate was aligned with those of the reference strains using the multiple sequence alignment program CLUSTAL_X v. 1.83 (Thompson *et al.*, 1997). Phylogenetic relationships between the novel isolate and its closely related strains were determined using MEGA4 (Tamura *et al.*, 2007). Phylogenetic distances were determined based on the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) algorithms with 1000, 1000 and 300 randomly chosen bootstrap replications (Felsenstein, 1985), respectively. In the resulting tree, the novel isolate formed a cluster with *L. chironomi* MM2LB^T, *L. chromiireducens* subsp. *chromiireducens* L-1^T, *L. chromiireducens* subsp. *solipictus* TAN 31054^T, *Leucobacter chromiiresistens* JG 31^T, *L. iarius* 40^T and *L. alluvii* RB10^T (Fig. 1).

Abbreviations: DAB, 2,4-diaminobutyric acid; DPG, diphosphatidylglycerol; GL, glycolipid; PG, phosphatidylglycerol.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain NAL101^T is GQ504012.

A supplementary figure is available with the online version of this paper.

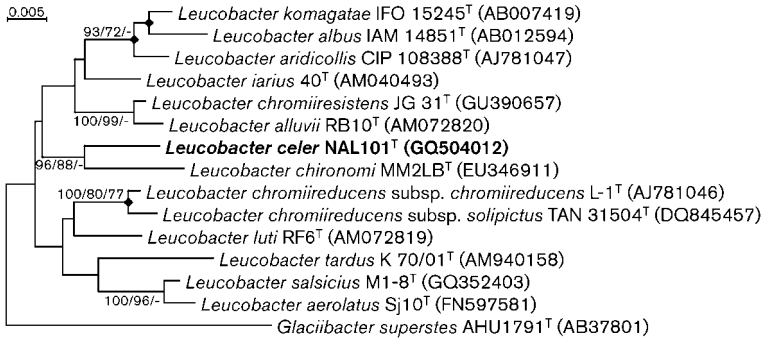

Fig. 1. Phylogenetic consensus tree based on 16S rRNA gene sequence comparisons, reconstructed using the neighbour-joining, maximum-parsimony and maximum-likelihood methods. Filled diamonds indicate generic branches that were present in phylogenetic consensus trees generated by all three methods. Numbers at the nodes represent bootstrap values based on neighbour-joining, maximum-parsimony and maximum-likelihood algorithms as percentages of 1000, 1000 and 300 replicates, respectively. Bar, 0.005 substitutions per nucleotide position.

A motility test was performed according to the method of Tittsler & Sandholzer (1936) on semi-solid agar medium containing 0.3 % beef extract, 1 % pancreatic digest of casein, 0.5 % sodium chloride and 0.4 % agar. Gram staining was performed using a Gram Staining kit (bioMérieux). Morphology and Gram staining were observed using a light microscope (ECLIPSE 50*i*, Nikon). Motility was not observed and cells were Gram-reaction-positive. All physiological and biochemical tests were performed on isolates that had been incubated on Trypticase soy agar (TSA) or Trypticase soy broth (TSB) at 37 °C for 24 h. Growth was determined at 4, 25, 30, 37, 45 and 60 °C on TSB and then at pH 5–7 (buffered by 10 mM MES), pH 8 and 9 (buffered by 10 mM TAPS) (Morais *et al.*, 2006) and pH 10 (buffered by 10 mM Na_2HPO_4) on TSB at 37 °C. Salt tolerance was determined in TSB containing 0, 1, 2, 3, 4, 5, 8, 10, 12, 15 and 20 % (w/v) NaCl. The chromium resistance of some species of the same genus was reported by Morais *et al.* (2006), Muir & Tan (2007) and Takeuchi *et al.* (1996). Thus, chromium resistance was determined by incubating in TSB supplemented with different concentrations of K_2CrO_4 [0, 1, 2, 3, 4, 5, 10, 15, 20, 25 and 30 mM Cr (VI)] at 37 °C for 7 days. OD_{600} was measured using a spectrophotometer (SYNERGY MX, BioTek) after 24 h, 48 h and 7 days of culture. Growth of the isolate was observed at 4–45 °C, at pH 5–10 and in 0–12 % (w/v) NaCl. Optimal growth conditions were 30–37 °C, pH 8 and 0–1 % (w/v) NaCl. Although the isolate was obtained from a chromium-free environment, strain NAL101^T exhibited resistance to chromium up to 20 mM Cr (VI). The phenotypic differences between the isolate and reference strains are presented in Table 1. Growth under anaerobic conditions was determined by incubating the isolate in a chamber filled with mixed gas (N_2 : H_2 : CO_2; 90 : 5 : 5) for 7 days. Growth did not occur under anaerobic conditions.

The assimilation of various substrates by the isolate was examined using a GP2 MicroPlate (Biolog) and API 50 CH strips (bioMérieux), according to the manufacturers' instructions, using minimal medium containing 0.02 % D-glucose, 0.01 % yeast extract, 0.01 % trypticase, 0.1 % K_2HPO_4 and 0.5 % NaCl (Takeuchi *et al.*, 1996). The novel isolate and closely related reference strains differed in their ability to use D-ribose, D-xylose, aesculin and gluconate as carbon and energy sources (Table 2). Enzyme activities were characterized using API 20 NE strips (bioMérieux), according to the manufacturer's instructions. In API 20 NE tests, the isolate was positive for hydrolysis of aesculin ferric citrate and assimilation of gelatin, D-glucose, D-mannose, D-mannitol, potassium gluconate and trisodium citrate and assimilation of Catalase and oxidase activities were identified by using solutions of 3 % (v/v) hydrogen peroxide and 1 % (w/v) p-tetramethylphenylenediamine (bioMérieux), respectively. The novel isolate was oxidase-negative and catalase-positive.

For analysis of cellular fatty acid contents, strains NAL101^T, *L. chironomi* MM2LB^T, *L. chromiireducens* subsp. *chromiireducens* L-1^T, *L. chromiireducens* subsp. *solipictus* TAN 31054^T, *L. iarius* 40^T and *L. alluvii* RB10^T were cultivated on TSA at 30 °C for 48 h. The fatty acids were then extracted using the protocol of MIDI (1999). The fatty acids were analysed using GC (Hewlett Packard 6890) and identified with the Sherlock Microbial Identification Systems (MIDI, 1999). The predominant fatty acids of the novel isolate were anteiso-$C_{15:0}$ (46.53 %), anteiso-$C_{17:0}$ (21.10 %), and iso-$C_{16:0}$ (16.05 %), as for the other species of the genus *Leucobacter*. The novel isolate also contained iso-$C_{15:0}$ (9.79 %), $C_{16:0}$ (3.49 %) and iso-$C_{17:0}$ (3.04 %) as minor fatty acids.

The composition of amino acids in the cell wall was analysed using one-dimensional TLC on cellulose sheets (Bousfield *et al.*, 1985). 2,4-Diaminobutyric acid (DAB), γ-aminobutyric acid (GABA), alanine, glutamic acid, glycine and threonine were used as standard amino acids to analyse the amino acid composition of the novel isolate and to compare it with those of the reference strains. The hydrolysate of the peptidoglycan of strain NAL101^T was composed of DAB, alanine, threonine, glycine and glutamic acid. The menaquinone composition was analysed by TLC by using the methods of Hiraishi *et al.* (1996). The isolate contained respiratory quinone MK-11 as a major component and MK-9 and MK-10 in minor amounts. Polar lipids were extracted according to the procedures described by Xin *et al.* (2000), separated by two-dimensional TLC and detected by spraying the plate with appropriate detection reagents, as described

Table 1. Characteristics differentiating strain NAL101^T from closely related members of the genus *Leucobacter*

Strains: 1, NAL101^T (data from this study); 2, *L. chironomi* MM2LB^T (Halpern *et al.*, 2009); 3, *L. chromiireducens* subsp. *chromiireducens* L-1^T (Morais *et al.*, 2004); 4, *L. chromiireducens* subsp. *solipictus* TAN 31504^T (Muir & Tan, 2007); 5, *L. iarius* 40^T (Somvanshi *et al.*, 2007); 6, *L. alluvii* RB10^T (Morais *et al.*, 2006). +, Positive; w, weakly positive; −, negative; ND, no data.

Characteristics	1	2	3	4	5	6
Temperature range (°C)	4–55	17–37	4–37	10–37	4–37	4–40
Temperature optimum (°C)	37	30	28	25	30	30
pH range	5–10	4.0–9.5	5–9	5.5–9.0	5–9	ND
pH optimum	8	6–8	7	7.5–8.5	ND	ND
NaCl range (%)	0–12	0–7	0–9	0–8	0–7	0–8
NaCl optimum (%)	0	0–1	ND	0	ND	ND
Chromium range (mM)	0–20	0–18	0–5	0–5	ND	0–4
Cellular fatty acids* (%):						
anteiso-$C_{15:0}$	46.53	43.52	55.95	62.80	46.56	57.97
anteiso-$C_{17:0}$	21.10	12.50	23.55	17.45	32.18	21.00
iso-$C_{16:0}$	16.05	36.05	12.28	12.64	15.50	13.26
iso-$C_{15:0}$	9.79	−	0.62	−	2.12	1.67
DNA G+C content (mol%)	68.8	70.7	66.7	69.5	ND	68.9
Isolation source	Korean fermented food	Chironomid egg mass	Activated sludge	*C. elegans*	Nematode juveniles	Activated sludge

*Data are from the current study.

by Tindall (1990). The polar lipids in the isolate were diphosphatidylglycerol (DPG), phosphatidylglycerol (PG) and an unknown glycolipid (GL) (Supplementary Fig. S1, available in IJSEM Online). Based on the menaquinone types and polar lipid composition, the novel isolate belonged to the genus *Leucobacter*.

Table 2. Substrate utilization pattern of strain NAL101^T and closely related members of the genus *Leucobacter*

Strains: 1, strain NAL101^T; 2, *L. chironomi* MM2LB^T; 3, *L. chromiireducens* subsp. *chromiireducens* L-1^T; 4, *L. chromiireducens* subsp. *solipictus* TAN 31504^T; 5, *L. iarius* 40^T; 6, *L. alluvii* RB10^T. Data for carbon source utilization obtained from GP2 MicroPlate (Biolog). All data are from this study. All strains were positive for utilization of Tween 40, Tween 80 and glycerol. +, Positive; w, weakly positive; −, negative.

Carbon source	1	2	3	4	5	6
D-Fructose	−	−	−	−	+	+
α-D-Glucose, adenosine	−	−	−	−	−	+
β-Methyl-D-glucoside, D-psicose, trehalose, lactamide	−	−	−	−	+	−
L-Rhamnose	−	−	−	w	+	−
D-Ribose, D-xylose	w	−	−	−	−	−
Xylitol	−	−	−	−	+	w
p-Hydroxy-phenylacetic acid	−	−	+	+	−	+
L-Lactic acid	−	−	−	−	−	w
Pyruvatic acid methyl ester	−	−	+	−	w	w
Pyruvic acid	w	−	+	−	−	w
L-Alaninamide	+	−	+	+	+	+
D-Alanine	−	−	−	−	w	−
L-Alanine	−	−	+	+	−	−
L-Alanyl-glycine	−	−	w	−	−	+
L-Glutamic acid	−	−	w	+	−	−
Glycyl-L-glutamic acid	−	−	−	w	−	−
Putrescine	+	+	+	+	−	+
2′-Deoxyadenosine	−	−	+	−	−	+
Thymidine	−	−	w	−	−	w
Uridine, inosine	−	−	w	−	−	−

Extraction of chromosomal DNA from the isolate was performed according to the method described by Rochelle *et al.* (1992). The DNA G+C content of the entire genome was defined by a fluorimetric method using SYBR Green I and a real-time PCR thermocycler (Gonzalez & Saiz-Jimenez, 2002). *Escherichia coli* K-12 DNA was used as reference DNA. The DNA G+C content of strain NAL101^T was found to be 68.8 mol%. To determine the genetic relatedness between the novel isolate and the reference species, DNA–DNA hybridizations were performed using a microarray spotted with genomic DNA (Bae *et al.*, 2005; Chang *et al.*, 2008). DNA–DNA hybridization values obtained between the novel isolate and the reference strains were as follows: *L. chironomi* MM2LB^T, 22 %; *L. chromiireducens* subsp. *chromiireducens* L-1^T, 13 %; *L. chromiireducens* subsp. *solipictus* TAN 31054^T, 6 %; *L. iarius* 40^T, 10 %; and *L. alluvii* RB10^T, 13 %. Based on the recommended criterion of distinct species having DNA–DNA hybridization values <70 %, strain NAL101^T was determined to be a novel species.

On the basis of its distinctive phylogenetic, physiological and biochemical characteristics, strain NAL101^T represents a novel species of the genus *Leucobacter* (family *Microbacteriaceae*), for which the name *Leucobacter celer* sp. nov. is proposed.

Description of *Leucobacter celer* sp. nov.

Leucobacter celer (ce′ler. L. masc. adj. *celer* rapid, indicating rapid growth)

Cells are Gram-reaction-positive, aerobic, irregular-rod-shaped and non-motile. After 48 h of incubation on TSA at 37 °C, colonies are circular, cream coloured, smooth, opaque, convex and 0.8–1.2 mm in diameter with entire margins. Growth occurs at 4–45 °C, at pH 5–10 and in the presence of 0–12 % (w/v) NaCl. Optimal growth occurs at 37 °C, pH 8 and 0 % NaCl. Grows on TSB containing up to 20 mM Cr (VI). Oxidase-negative and catalase-positive. Utilizes Tweens 40 and 80, D-ribose, D-xylose, pyruvic acid, L-alaninamide, putrescine and glycerol (Biolog GP2). Aesculin ferric citrate and gelatin are hydrolysed (API 20 NE) and D-glucose, D-fructose, D-mannose, inositol, aesculin, gluconate and 5-ketogluconate (API 50 CH) are assimilated. The major cellular fatty acids are anteiso-$C_{15:0}$, anteiso-$C_{17:0}$ and iso-$C_{16:0}$. The cell-wall amino acids are DAB, alanine, threonine, glycine, and glutamic acid. The predominant menaquinone is MK-11; minor components are MK-9 and MK-10. The polar lipids are DPG, PG, and GL.

The type strain, NAL101^T (=KACC 14220^T =JCM 16465^T), was isolated from gajami-sikhae, a traditional Korean fermented food. The DNA G+C content of the type strain is 68.8 mol%.

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