Luteolibacter yonseiensis sp. nov., isolated from activated sludge using algal metabolites

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A Gram-negative, rod-shaped, aerobic bacterial strain, designated EBTL01^T, was isolated from activated sludge by using metabolites of microalgae *Ankistrodesmus gracilis* SAG278-2. Phylogenetic analyses based on 16S rRNA gene sequence showed that strain EBTL01^T belongs to the family *Verrucomicrobiaceae*, class *Verrucomicrobiae*, and is related most closely to *Luteolibacter pohnpeiensis* A4T-83^T (95.5 % sequence similarity) and *Luteolibacter algae* A5J-41-2^T (95.2 %). The G+C content of the genomic DNA of strain EBTL01^T was 56.3 mol% and the menaquinone MK-9 was detected as the predominant quinone. Major fatty acid components were iso-$C_{14:0}$, $C_{16:1}\omega7c$ and $C_{16:0}$. The amino acids of the cell-wall peptidoglycan contained muramic acid and *meso*-diaminopimelic acid. These profile results supported the affiliation of strain EBTL01^T to the genus *Luteolibacter*. On the other hand, based on chemotaxonomic properties and phenotypic characteristics, strain EBTL01^T could be clearly differentiated from its phylogenetic neighbours. Therefore, strain EBTL01^T represents a novel species of the genus *Luteolibacter*, for which the name *Luteolibacter yonseiensis* sp. nov. is proposed. The type strain is EBTL01^T (=KCTC 23678^T=JCM 18052^T).

The genus *Luteolibacter*, a member of the family *Verrucomicrobiaceae*, class *Verrucomicrobiae*, was proposed by Yoon *et al.* with the description of *Luteolibacter pohnpeiensis* A4T-83^T in 2008. At the time of writing, the genus *Luteolibacter* comprises two species with validly published names, *Luteolibacter pohnpeiensis* A4T-83^T and *Luteolibacter algae* A5J-41-2^T (Yoon *et al.*, 2008). Members of the genus *Luteolibacter* have the following characteristics: they are Gram-negative, non-endospore-forming, non-motile rods and positive for catalase and oxidase. They contain iso-$C_{14:0}$ as the predominant cellular fatty acid and the genomic DNA G+C contents of known strains range from 55.8 to 55.9 mol%. During the screening of bacterial strains from activated sludge of industrial wastewater treatment by using algal metabolites, a Gram-negative, rod-shaped bacterial strain, designated EBTL01^T, was isolated. On the basis of 16S rRNA gene sequence analysis, this isolate was considered to be a *Luteolibacter*-like strain. To determine its exact taxonomic position, EBTL01^T was subjected to a detailed investigation using a polyphasic taxonomic approach, including genotypic, chemotaxonomic and classical phenotypic analyses. These results indicate that EBTL01^T should be placed in the genus *Luteolibacter* as a representative of a novel species.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain EBTL01^T is JQ319003.

Algal metabolites were obtained by culturing microalgae *Ankistrodesmus gracilis* SAG278-2 in artificial wastewater for 2 weeks. The artificial wastewater contained following components (in mg l^{-1}): NaCl (7), $CaCl_2$ (4), $MgSO_4 . 7H_2O$ (2), K_2HPO_4 (21.7), KH_2PO_4 (8.5), Na_2HPO_4 (33.4) and NH_4Cl (3). Algal metabolites agar media were prepared for isolating micro-organisms that can utilize algal metabolites as their carbon and nutrient sources. An activated sludge sample from wastewater treatment (Seo-nam, Seoul, Republic of Korea) was initially serial diluted and aliquots of each serial dilution were spread on algal metabolites agar media. After being incubated at 30 °C for 5 days, single colonies on the plates were purified by transferring them onto fresh plates and incubating them again under the same conditions. The isolate was routinely cultured on R2A agar at 30 °C and maintained as a glycerol suspension (20 %, w/v) at −80 °C.

For the phylogenetic analysis of strain EBTL01^T, genomic DNA was extracted using a genomic DNA-extraction kit (Solgent). PCR amplification of 16S rRNA and sequencing after purifying PCR product were conducted as described by Kim *et al.* (2005). After preliminary sequence identification using the EzTaxon-e database (Kim *et al.*, 2012), the almost-complete 16S rRNA sequence of strain EBTL01^T and sequences of relatives obtained from GenBank database were compiled using SeqMan Software (DNASTAR). Multiple alignments were carried out by CLUSTAL_X

(Thompson *et al.*, 1997) and gaps were eliminated by using the BioEdit program (Hall, 1999). By employing the Kimura two-parameter model (Kimura, 1983), evolutionary distances were calculated and phylogenetic trees were constructed using the neighbour-joining (Saitou & Nei, 1987) and the maximum-parsimony (Fitch, 1971) algorithms in the MEGA4 program (Tamura *et al.*, 2007) and using the maximum-likelihood (Felsenstein, 1981) algorithm in PHYLIP, version 3.69 (Felsenstein, 2009), based on bootstrap values of 1000 replications (Felsenstein, 1985).

For the Gram test, a Gram-stain kit (Difco) was employed according to the manufacturer's instructions. Using the hanging-drop technique, cell morphology and motility were observed in cells grown on R2A (Difco) agar media at 30 °C for 5 days with an Olympus light microscope (1000×; model CX31). Endospores were examined after negative staining according to the method suggested by Cappuccino & Sherman (2002). Catalase and oxidase tests were assessed as described by Cappuccino & Sherman (2002). Assimilation tests, enzyme activities and physiological characteristics were determined by API 20NE (48 h, 30 °C) and API ZYM (48 h, 30 °C) kits according to the manufacturer's instructions (bioMérieux) using cells grown on R2A media at 30 °C for 5 days. Growth at different temperatures (4, 10, 15, 20, 25, 30, 37 and 42 °C) was assessed on R2A, NB, LB, TSA and MacConkey media by incubating for 5 days. Growth at various pH values (pH 5.0–11.0 at 0.5 pH unit intervals) was determined by incubating in R2A broth at 30 °C for 5 days. Salt tolerance was examined in R2A with 0, 1, 2, 3, 4, 5, 6, 7, 8 and 10 % (w/v) NaCl by incubating at 30 °C for 5 days. Tests for hydrolysis of DNA [DNase agar (Scharlau)] (Collins & Lyne, 1984), skimmed milk, chitin, starch, xylan and hydroxyethyl (HE)-cellulose (Ten *et al.*, 2004) were performed and examined after 7 days incubation.

Chromosomal DNA G+C content was determined as described previously by Moore & Dowhan (1995). After the

Table 1. Differential phenotypic characteristics of strain EBTL01^T and its closest phylogenetic neighbours in the genus *Luteolibacter*

Strains: 1, EBTL01^T; 2, *L. pohnpeiensis* A4T-83^T; 3, *L. algae* A5J-41-2^T. All data are from the present study. All strains were positive for alkaline phosphatase but negative for indole production, glucose acidification, lipase (C14), cystine arylamidase, α-chymotrypsin, β-glucuronidase, α-glucosidase, β-glucosidase and α-fucosidase. In substrate-assimilation tests, all strains showed identical biochemical characteristics except those indicated here (see Description of *Luteolibacter yonseiensis* sp. nov.). +, Positive; −, negative.

Characteristic	1	2	3
Isolation source	Algal metabolites media	Driftwood	Red algae
Cell morphology	Rod	Rod	Rod
Colony colour	Pale yellow	Pale yellow	Pale yellow
Temperature range (°C)	10–30	20–37	4–37
Growth at 4 °C	−	−	+
Growth at 37 °C	−	+	+
pH range	5.5–8	6.5–8.5	6.5–8
NaCl tolerance (%)	0	7	5
Reduction of nitrate	−	−	−
Enzyme activities:			
Arginine dihydrolase	−	+	−
Urease	−	+	−
Protease	+	−	−
Esterase (C4)	+	−	−
Esterase lipase (C8)	−	+	+
Leucine arylamidase	+	+	−
Valine arylamidase	+	−	−
Trypsin	+	−	−
Acid phosphatase	+	+	−
Naphthol-AS-BI-phosphohydrolase	−	+	+
α-Galactosidase	−	+	−
β-Galactosidase	−	+	−
N-Acetyl-β-glucosaminidase	−	+	−
α-Mannosidase	−	+	−
Assimilation:			
Arabinose	−	+	+
N-Acetylglucosamine	+	−	−
Maltose	+	+	−
Malate	+	−	−
Citrate	+	−	−

genomic DNA of the strain had been extracted and purified, it was enzymically degraded into nucleosides and then the G+C content was measured using reverse-phase HPLC according to the method described by Mesbah *et al.* (1989). Isoprenoid quinones were extracted using chloroform/methanol (2 : 1, v/v) with cells grown on R2A at 30 °C for 5 days. After evaporating solvent under vacuum conditions, isoprenoid quinones were re-extracted using n-hexane. Crude quinones dissolved in n-hexane were purified using Sep-Pak Vac silica cartridges (Waters) and identified by HPLC (Hiraishi *et al.*, 1996). Cellular fatty acids were analysed using cell biomass grown on R2A at 30 °C for 5 days. Saponification, methylation and extraction for analysing cellular fatty acids were performed using the methods of Kuykendall *et al.* (1988). Extracted fatty acid methyl esters were separated by GC (Agilent 6890; Hewlett Packard) using the Microbial Identification software package (Sasser, 1990) and identified with Sherlock version 2.95 and the TSBA 3.9 database. Purified cell-walls were obtained by disruption of the cells with glass beads and subsequent digestion according to the method of Schleifer (1985). Amino acids and peptides in the cell-wall hydrolysates were analysed by using 2-D TLC on cellulose plates with the solvent systems described by Schleifer & Kandler (1972).

Cells of strain EBTL01^T were Gram-negative, aerobic, non-spore-forming, non-motile rods and positive for catalase and oxidase. Colonies grown on R2A agar plates for 5 days at 30 °C were 0.5–2 mm in diameter, circular, shiny and pale yellow. On R2A agar, strain EBTL01^T was able to grow at 10–30 °C, but not at 4 °C or 37 °C. On TSA, LB agar, MacConkey agar and nutrient agar (Difco), no growth was observed at any temperature. As a result of salt tolerance and pH range tests, strain EBTL01^T was not grown on R2A agar media with 1 % (w/v) NaCl but was grown in R2A broth with a pH range of 5.5–8. Strain EBTL01^T and two closely related *Luteolibacter* species (*L. pohnpeiensis* A4T-83^T and *L. algae* A5J-41-2^T) were positive for alkaline phosphatase but negative for lipase (C14), cystine arylamidase, α-chymotrypsin, β-glucuronidase, α-glucosidase, β-glucosidase and α-fucosidase. These results are in good

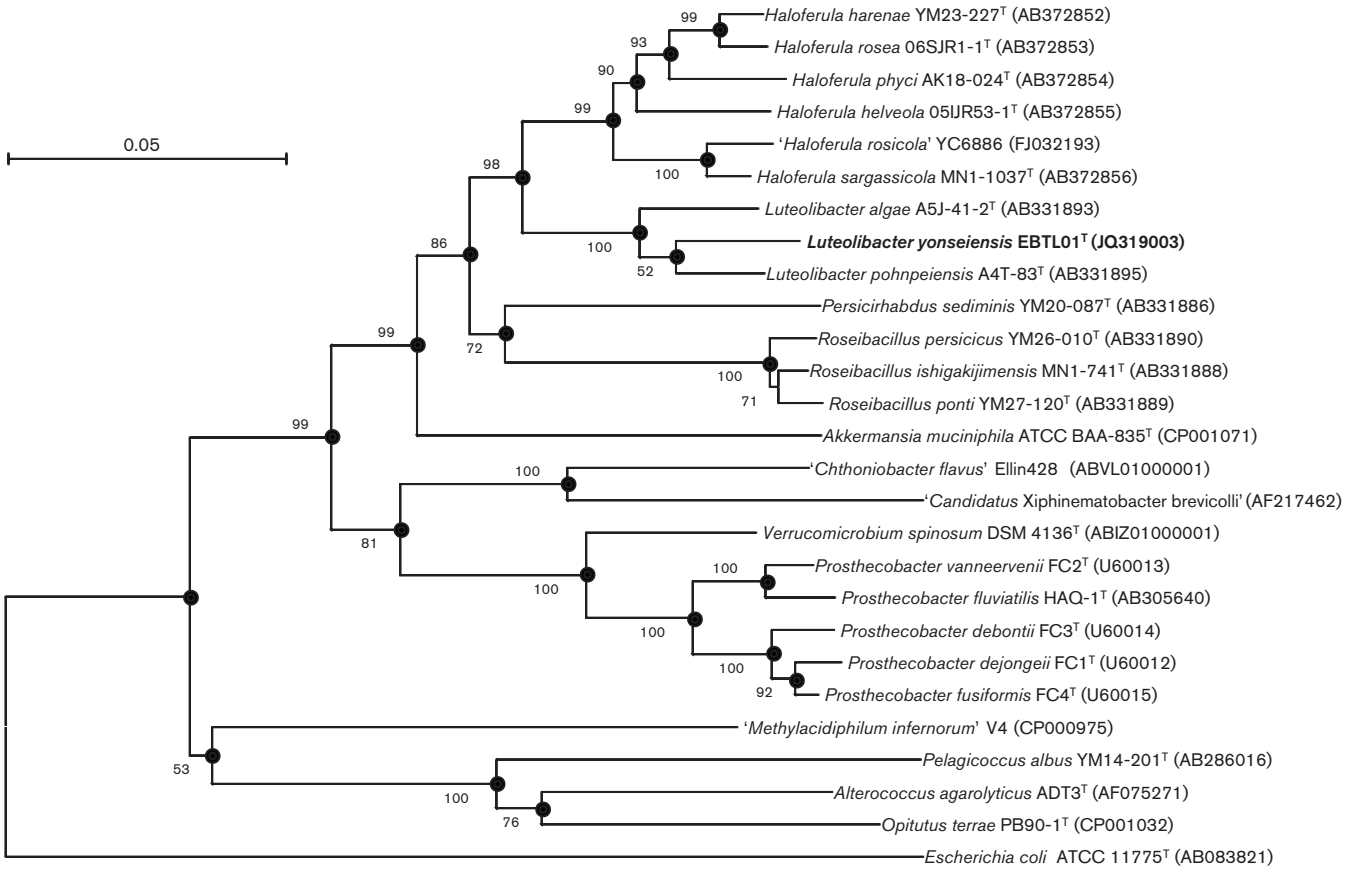

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences, showing the phylogenetic position of the strain EBTL01^T among recognized *Luteolibacter* species and some other related taxa. Bootstrap values higher than 50 % (percentages of 1000 replications) are shown at branching points. The corresponding nodes recovered in the tree generated with the maximum-parsimony and maximum-likelihood algorithms were marked with filled circles. Bar, 0.05 substitutions per nucleotide position. *Escherichia coli* ATCC 11775^T was used as an outgroup.

agreement with previously reported data and supported the affiliation of the isolate to the genus *Luteolibacter* (Yoon *et al.*, 2008). Phenotypic and chemotaxonomic characteristics that differentiate strain EBTL01^T from its closest neighbours in the genus *Luteolibacter* are listed in Table 1. In particular, strain EBTL01^T could be differentiated readily from both above-mentioned *Luteolibacter* species based on its enzyme activities of protease, esterase (C4), esterase lipase (C8), valine arylamidase, trypsin and naphthol-AS-BI-phosphohydrolase, and assimilation ability or inability of arabinose, *N*-acetylglucosamine, malate and citrate.

The length of the almost-complete 16S rRNA gene sequence obtained from strain EBTL01^T was 1407 bp. Based on phylogenetic analysis, it was revealed that highest sequence similarity between strain EBTL01^T and the closest relatives were *L. pohnpeiensis* A4T-83^T (95.5 % sequence similarity) and *L. algae* A5J-41-2^T (95.2 %). By several algorithms for phylogenetic tree analysis (neighbour-joining, maximum-parsimony and maximum-likelihood), it was revealed that the phylogenetic position of strain EBTL01^T was within the genus *Luteolibacter* with a high bootstrap value of 98 % (Fig. 1). It is generally accepted that novel bacterial species can be delineated by one of the following criteria: either a DNA–DNA relatedness value below 70 % determined by DNA–DNA hybridization or a similarity of 16S rRNA gene sequence below 3 % with the novel strain's nearest relatives (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994; Stackebrandt *et al.*, 2002). By this definition, the above-mentioned phylogenetic analyses suggest that strain EBTL01^T should be considered to represent a novel species in the genus *Luteolibacter*.

The amino acids muramic acid and *meso*-diaminopimelic acid were detected in total hydrolysate of the cell-wall preparation of strain EBTL01^T. Strain EBTL01^T contained MK-9 as the predominant menaquinone. The DNA G+C content of strain EBTL01^T was 56.3 mol%. These data are in good agreement with those of other members of the genus *Luteolibacter* (Yoon *et al.*, 2008). Cellular fatty acid profiles of strain EBTL01^T and the type strains of phylogenetically related *Luteolibacter* species are shown in Table 2. All strains contained iso-$C_{14:0}$ as the dominant component and $C_{16:1}\omega7c$, $C_{16:0}$, anteiso-$C_{15:0}$ and iso-$C_{16:0}$ as the major components. However, some minor qualitative and quantitative differences in fatty acid content could be observed between strain EBTL01^T and the phylogenetically closest relatives. In particular, strain EBTL01^T can be distinguished from other *Luteolibacter* species by the presence of $C_{16:0}$ N alcohol and by the absence of iso-$C_{16:1}$ I.

The phenotypic and phylogenetic data presented here indicate that strain EBTL01^T belongs to the genus *Luteolibacter*. The phylogenetic distinctiveness of EBTL01^T confirmed that this strain represents a species that is distinct from the recognized *Luteolibacter* species. Strain EBTL01^T can be differentiated from phylogenetically related *Luteolibacter* species based on several phenotypic characteristics (Table 1). Therefore, on the basis of the data presented, strain EBTL01^T should be classified within the genus *Luteolibacter* as the type strain of a novel species, for which the name *Luteolibacter yonseiensis* sp. nov. is proposed.

Table 2. Cellular fatty acid profiles of strain EBTL01^T and its closest phylogenetic neighbours in the genus *Luteolibacter*

Strains: 1, EBTL01^T; 2, *L. pohnpeiensis* A4T-83^T; 3, *L. algae* A5J-41-2^T. All data are from the present study. All strains were grown on R2A at 30 °C for 5 days prior to fatty acids analysis. Values are percentages of total fatty acids; −, not detected. Data below 1 % are not shown in this table.

Fatty acid	1	2	3
Saturated:			
$C_{14:0}$	3.2	11.5	7.1
$C_{15:0}$	−	1.0	−
$C_{16:0}$	10.9	26.6	21.4
$C_{16:0}$ N alcohol	4.3	−	−
iso-$C_{14:0}$	47.7	34.8	42.0
iso-$C_{16:0}$	7.2	3.3	3.7
anteiso-$C_{15:0}$	7.6	7.1	6.1
Unsaturated:			
iso-$C_{16:1}$ I	−	3.2	2.9
iso-$C_{17:1}$ I	7.2	1.4	4.9
$C_{16:1}\omega7c$	15.1	10.3	11.9

Description of *Luteolibacter yonseiensis* sp. nov.

Luteolibacter yonseiensis (yon.sei.en′sis. N.L. masc. adj. *yonseiensis* pertaining to Yonsei University, Seoul, Korea, in recognition of its efforts for the research of microalgae and bacteria).

Cells are Gram-negative, aerobic, non-spore-forming rods, positive for catalase and oxidase, 0.5–0.8 μm in width and 1.2–1.5 μm in length. After 5 days incubation at 30 °C on R2A agar, colonies are 0.5–2 mm in diameter, circular, shiny and pale yellow. Grows at 10–30 °C and at pH 5.5–8, with optimum growth occurring at 30 °C and at pH 6.5–7.5. Xylan is hydrolysed, but skimmed milk, chitin, DNA, HE-cellulose and starch are not. Nitrate is not reduced. In substrate-assimilation tests, glucose, mannose, *N*-acetylglucosamine, maltose, malate and citrate are utilized for growth but arabinose, mannitol, gluconate, caprate, adipate, phenylacetate are not. MK-9 is the predominant menaquinone. The major cellular fatty acids are iso-$C_{14:0}$, $C_{16:1}\omega7c$, $C_{16:0}$, anteiso-$C_{15:0}$ and iso-$C_{16:0}$.

The type strain, EBTL01^T (=KCTC 23678^T=JCM 18052^T), was isolated from an activated sludge sample by using media containing algal metabolites. The DNA G+C content of the type strain is 56.3 mol%

Acknowledgements

This work was supported by Mid-career Researcher Program through an NRF (National Research Foundation) grant funded by the MEST (Ministry of Education, Science and Technology) (no. 2009-0081153).

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