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TAXONOMIC DESCRIPTION
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Flavobacterium fluminis sp. nov. to accommodate an aerobic, halotolerant and gliding flavobacterium isolated from freshwater

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Abstract

A Gram-stain-negative, aerobic, oxidase-positive, catalase-positive and rod-shaped bacterium designated strain 3R17^T was isolated from freshwater. Strain 3R17^T produced bright-yellow, circular, convex and smooth colonies on R2A agar, tryptic soy agar, potato dextrose agar, nutrient agar and brain–heart infusion agar media. The strain was motile by gliding. The strain grew at 4–30 °C (optimum, 25 °C), at pH 6–9 (optimum, pH 7) and in the presence of up to 3 % NaCl (optimum, 0 %) on R2A agar. The 16S rRNA gene sequence analysis indicated that 3R17^T represents a member of the genus *Flavobacterium* and is most closely related to *Flavobacterium resistens* BD-b365^T, with a sequence similarity of 97.78 %, but the strain formed a distinct phylogenetic lineage of its own. Fatty acid analysis indicated that a summed feature comprising $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$, iso-$C_{15:0}$, iso-$C_{15:1}$G, anteiso-$C_{15:0}$, $C_{16:0}$, iso-$C_{17:0}$ 3-OH and iso-$C_{15:0}$ 3-OH were the major components (>5 %). Strain 3R17^T contained phosphatidylethanolamine (PE) and several unidentified aminolipids as main polar lipids, and MK-6 as the predominant isoprenoid quinone. Flexirubin pigments were not produced. The DNA G+C content was 35.4 mol%. The combination of physiological and chemotaxonomic properties distinguished 3R17^T from related species of the genus *Flavobacterium*. On the basis of polyphasic taxonomy, 3R17^T evidently represents a novel species within the genus *Flavobacterium*, for which the name *Flavobacterium fluminis* sp. nov. is proposed. The type strain is 3R17^T (=KCTC 42062^T =JCM 30338^T).

The members of the genus *Flavobacterium* inhabit a broad range of ecosystems including freshwater, marine, soil, rhizosphere and fish [1]. Species of the genus *Flavobacterium* are characterized by rod-shaped cells, yellow colonies and aerobic, chemoorganotrophic and respiratory type metabolism [1]. Gliding motility, production of flexirubin type pigments, nitrate reduction and anaerobic growth are some of the main properties that differ among species. Members of the genus *Flavobacterium* may play important roles in freshwater habitats related to polymer degradation [2], proteorhodopsin-enhanced growth [3] or antibiotic resistance [4]. In a recent study, the genus was found to compose one of the main constituents of the freshwater microbiome [5].

In this study, an isolated strain of a member of the genus *Flavobacterium* showed distinct properties from other species of the genus with respect to phylogenetic relationships based on 16S rRNA gene sequences and also phenotypic characteristics. The polyphasic characterization of the novel strain following the standards proposed by Bernardet *et al.* [6] led to a proposal of a novel species of the genus *Flavobacterium*.

A freshwater sample was collected from the surface of the Keum River (36° 47′ 2″ N, 127° 40′ 33″ E) near Daejeon City, Republic of Korea where the freshwater microbiome had been previously examined and members of the genus *Flavobacterium* had been found to represent one of the main genera [5]. The sample was diluted and inoculated onto R2A agar (Difco) plates. The plates were incubated at 25 °C, and colonies with a yellowish colour were picked. Strain 3R17^T, one of these isolates, was routinely cultivated on R2A agar or in R2A broth at 25 °C, and also maintained in 20 % glycerol stock solution (in distilled water, v/v) at −70 °C.

DNA extraction and sequencing of the almost complete 16S rRNA gene were carried out as described by Kim *et al.* [7]. The 16S rRNA gene sequence similarities were estimated by EzBioCloud (www.ezbiocloud.net/). The 16S rRNA gene sequences of 3R17^T and closely related type strains of species of the genus *Flavobacterium* were aligned by EzEditor

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The GenBank accession number for the 16S rRNA gene sequence of *Flavobacterium fluminis* 3R17^T is KF891387.
One supplementary table and two supplementary figures are available with the online Supplementary Material.

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[8]. Phylogenetic trees were inferred using three different methods, neighbour-joining, maximum-likelihood and maximum-parsimony by MEGA 6.0 software [9]. Bootstrap analysis was carried out with 1000 resampled dataset to evaluate the neighbour-joining tree.

Strain 3R17^T was most closely related to *Flavobacterium resistens* BD-b365^T (97.78 %) and *Flavobacterium nitratireducens* N1^T (97.59 %), on the basis of 16S rRNA gene sequence similarity. This level of 16S rRNA gene sequence similarity is well below the levels of 98.5–98.7 % similarity for species distinction suggested by Stackebrandt and Ebers [10] and Kim *et al.* [11]. Moreover, 3R17^T formed a distinct phylogenetic lineage of its own, which was supported by the bootstrap analysis (Fig. 1). The topology between 3R17^T and neighbouring species was also conserved in the maximum-likelihood and maximum-parsimony trees.

The cell morphology of 3R17^T was observed by bright-field light microscopy (Nikon) and field-emission transmission electron microscopy (JEM-2100F, JEOL) with cultures grown on R2A broth at 25 °C for 3 days. Gram reaction was determined by the standard Gram staining method. Determination of gliding motility was based on comparative observation of a reference strain motile by gliding (*F. resistens* BD-b365^T) and a non-motile reference strain (*Limnohabitans curvus* MWH-C5^T) cultivated under reported optimal culture conditions. Oxidase activity by L,L,L′,L-tetramethyl-p-phenylenediamine (TMPD) reaction, catalase activity by 10 % (w/v) hydrogen peroxide, and production of flexirubin-type pigment with 1 M NaOH were examined with cells cultivated on R2A agar at 25 °C for 2 days.

Growth of 3R17^T was assessed on R2A, tryptic soy (TSA, Difco), nutrient (NA, Difco), brain–heart infusion (BHI, Difco), potato–dextrose (PDA, Difco), Luria–Bertani (LB, Difco) and marine agar plates. To examine range and optimal condition for growth, 3R17^T was incubated on R2A agar at 4, 10, 15, 20, 25, 30, 35, 37, 40 and 42 °C and on R2A broth at pH 4–11 (one unit intervals, adjusted with 1 M NaOH or HCl). After cultivation at different pHs for 7 days, OD_{600} was recorded. NaCl tolerance was examined on R2A agar containing 0–5 % NaCl (at 0.5 % intervals, w/v). The optimum conditions for temperature and NaCl concentration were determined by comparison of growth from serially diluted inocula after incubation for 7 days. To determine physiological properties of 3R17^T, API 20NE, API ZYM (bioMérieux) and Biolog microplate GN2 systems were used. Hydrolysis of DNA, casein, starch, tyrosine, Tween 80 and carboxymethylcellulose (CMC) by 3R17^T was assessed by culturing on DNase agar (BD Difco) and suitable media described by Rainey and Oren [12]. Anaerobic growth was examined on R2A agar plates placed in anaerobic box containing AnaeroPack (ThermoFisher Scientific). The box was incubated for up to 7 days at 25 °C.

Strain 3R17^T was Gram-stain-negative, rod-shaped, approximately 0.4–0.5 µm in width and 1.0–1.5 µm in length (Fig. S1, available with the online Supplementary Material), and motile by gliding. Oxidase and catalase activities were present. Colonies of 3R17^T were bright yellow pigmented, convex, circular, smooth and translucent on R2A agar at 25 °C, but did not produce flexirubin-type pigments. Strain 3R17^T could grow on R2A, TSA, NA, PDA and BHI agar media, but not on LB and marine agar media. Growth occurred at 4–30 °C (optimum, 25 °C) on R2A agar, and at pH 6–9 (optimum, pH 7–8) in R2A broth. The strain showed halotolerant growth in the presence of up to 3 % (w/v) NaCl. Anaerobic growth was not observed. The differential physiological properties of 3R17^T with other strains, as examined by API kits and Biolog GN2, are summarized in the species description, Tables 1 and S1.

The cellular fatty acid composition of 3R17^T and reference strains were analyzed using cells grown on R2A agar for 2 days at 25 °C. Fatty acids were extracted according to the protocols for the Sherlock Microbial Identification System (MIDI) and analyzed by gas chromatography (model 7890; Hewlett Packard) using the Microbial Identification software package with the Sherlock system MIDI 6.1 and RTSBA6 database. Chemotaxonomic properties were determined using cells grown on R2A broth for 3 days at 25 °C with agitation and freeze-dried. Polar lipids were extracted from freeze-dried cells and separated as described by Han *et al.* [13]. Respiratory quinones of 3R17^T were also extracted from freeze-dried cells with a chloroform/methanol mixture (2 : 1, v/v), purified by Sep-Pac Vac silica cartridge, and analyzed using a YL9100 HPLC system (Young Lin). HPLC analysis was carried out using isopropyl alcohol/methanol mixture (5 : 7, v/v) as mobile phase and Watchers 120 ODS-BP 4.6×250 mm×5 µm column. The DNA G+C content of 3R17^T was estimated using the methods of Gonzalez and Saiz-Jimenez [14].

The fatty acids of the novel strain and reference strains are summarized in Table 2. The major fatty acids of 3R17^T were a summed feature consisting of $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$ (35.8 %), iso-$C_{15:0}$ (15.9 %), anteiso-$C_{15:0}$ (7.8 %), iso-$C_{15:1}$ G (7.4 %), $C_{16:0}$ (7.0 %), iso-$C_{17:0}$ 3-OH (5.5 %), and iso-$C_{15:0}$ 3-OH (5.2 %). The major polar lipid of the novel strain was phosphatidylethanolamine (PE), and three unidentified aminolipids and two unidentified lipids were also present (Fig. S2). The major respiratory quinone of 3R17^T was MK-6. The DNA G+C content of 3R17^T was determined to be 35.4 mol%. The chemotaxonomic properties of 3R17^T were consistent with those of species of the genus *Flavobacterium* [1].

Notably, 3R17^T tolerated up to 3 % NaCl, which is not common among freshwater isolates [1]. Strain 3R17^T was distinguished from the most closely related species, *F. resistens* BD-b365^T, in habitat, NaCl tolerance, nitrate reduction, flexirubin type pigment production and hydrolysis of a number of polymeric substances as well as in carbohydrate assimilation and enzyme activities (Tables 1 and S1). The two strains also differed in the fatty acid composition, as the former contained a notably higher proportion of summed feature 3, whereas the latter contained higher

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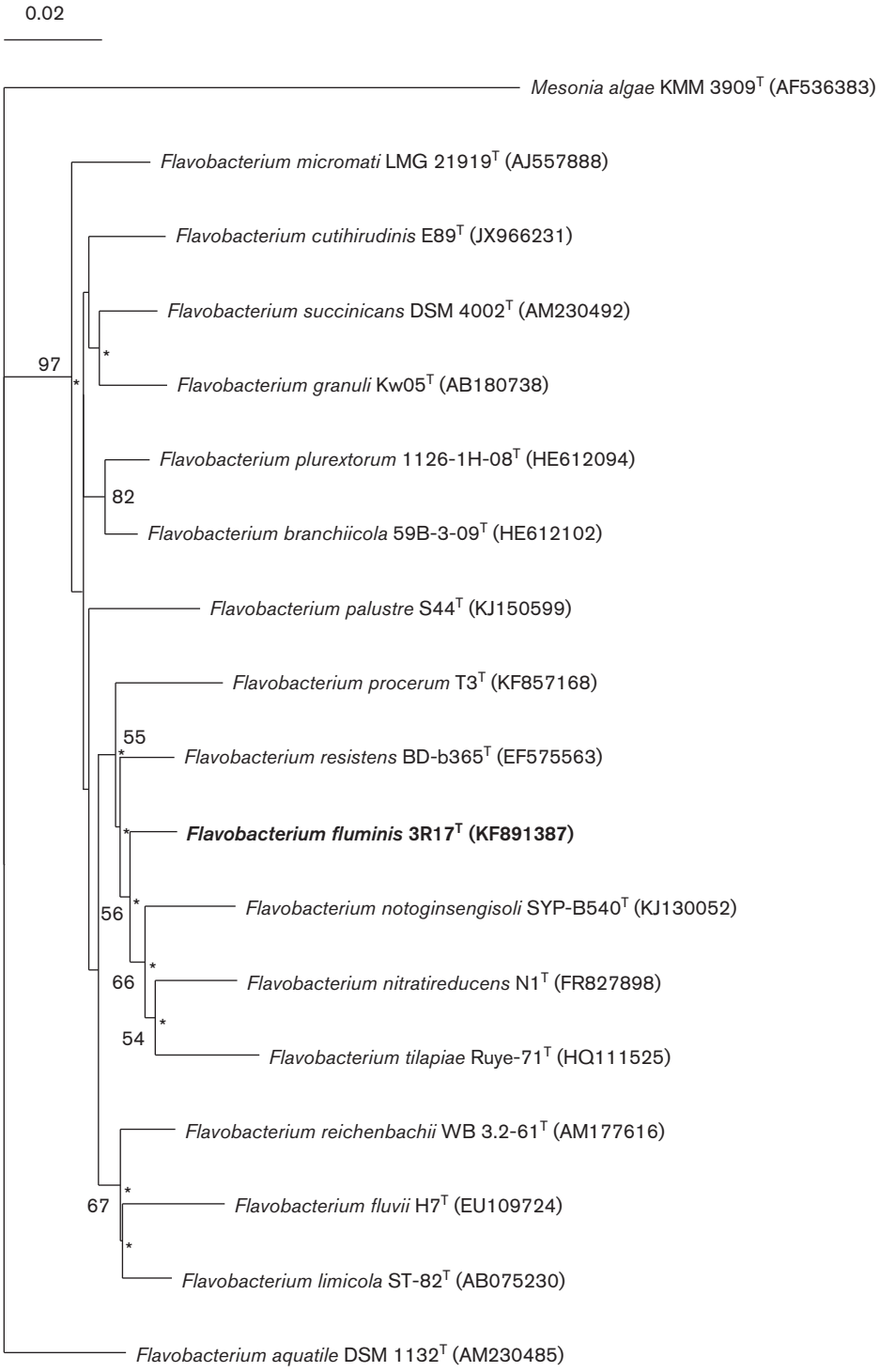

Fig. 1. Neighbour-joining tree based on 16S rDNA sequences (1351 nucleotides) showing relationships between 3R17^T and related species of the genus *Flavobacterium*. The consistency of each node is shown as a percentage value (>50 %) of bootstraps through 1000 resamplings above the nodes. Asterisks indicate the nodes also conserved in maximum-likelihood and maximum-parsimony trees. *Mesonia algae* KMM 3909^T was used as an outgroup. Bar, 0.02 changes per nucleotide position.

proportions of unsaturated fatty acids (Table 2). Based on the results from phylogenetic, phenotypic and chemotaxonomic studies, it is evident that 3R17^T merits recognition as representing a novel species of the genus *Flavobacterium*, for which the name *Flavobacterium fluminis* sp. nov. is proposed.

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Table 1. Differential phenotypic characteristics between 3R17^T and related species of the genus *Flavobacterium*

Strains: 1, *F. fluminis* 3R17^T; 2, *F. resistens* BD-b365^T; 3, *F. nitratireducens* N1^T. +, Positive; −, negative.

Property\Strain	1	2	3
Habitat*	River	Freshwater sediment	Seawater
Cell size:*			
Width (μm)	0.4–0.5	0.4–0.6	0.2–0.3
Length (μm)	1.0–1.5	1.6–3.2	1–1.5
Growth range (optimum):*			
Temperature (°C)	4–30 (25)	15–40 (20–30)	30–37 (30–37)
pH	6–9 (7–8)	6.0–9.5 (7–8)	7–9 (7.5–8.5)
NaCl concentration (%)	0–3 (0)	0–2 (0–0.5)	(0–1)
Oxygen requirement*	Aerobic	Facultatively anaerobic	Aerobic
Flexirubin-type pigment*	−	+	−
Gliding motility	+	+	−
Hydrolysis of:			
Aesculin	+	+	−
Arginine	−	−	+
Urea	−	−	+
Starch	−	+	+
Deoxyribonucleic acid	−	+	−
Casein	−	+	−
Tyrosine	−	+	−
Assimilation of (API 20NE):			
D-Glucose	+	+	−
D-Mannose	+	+	−
N-Acetyl-glucosamine	−	+	+
Maltose	−	+	−
Enzymatic activities (API ZYM):			
Cystine arylamidase	+	+	−
Trypsin	+	−	−
α-Mannosidase	+	−	−
β-Glucosidase	−	+	+
N-Acetyl-β-glucosaminidase	−	+	+
α-Galactosidase	−	−	+
α-Chymotrypsin	−	−	−
DNA G+C content (mol%)*	35.4	33.0	36.3

*Data for reference species were taken from previous studies [15, 16]. All other data were obtained from this study.

DESCRIPTION OF *FLAVOBACTERIUM FLUMINIS* SP. NOV.

Flavobacterium fluminis (flu′mi.nis. L. gen. n. *fluminis* of a river).

Cells are Gram-stain-negative, strictly aerobic, rod-shaped, measuring 0.4–0.5 μm in width and 1.0–1.5 μm in length, and motile by gliding. Colonies grown on R2A agar are bright yellow-pigmented, circular, convex, translucent and smooth with entire margins. Oxidase- and catalase-positive. Flexirubin-type pigments are not produced. Good growth occurs on R2A, NA, TSA, PDA and BHI media. Growth occurs at 4–30 °C (optimum, 25 °C), at pH 6–9 (optimum, 7–8), and in the presence of up to 3 % (w/v) NaCl (optimum, 0 %). DNA, casein, starch, tyrosine, Tween 80 and CMC are not hydrolyzed. Based on API 20NE, nitrate reduction, aesculin and gelatin hydrolysis, *p*-nitrophenyl-β-D-galactopyranosidase and assimilation of D-glucose, L-arabinose and D-mannose are positive. Based on API ZYM, alkaline and acid phosphatases, esterase (C4), esterase lipase (C8), leucine, valine and cystine arylamidases, trypsin, naphtol-AS-BI-phosphohydrolase, β-galactosidase, α-glucosidase and α-mannosidase activities are positive. Substrates including α-cyclodextrin, dextrin, glycogen, Tween 40, Tween 80, *N*-acetyl-D-glucosamine, cellobiose, D-fructose, D-galactose, gentiobiose, α-D-glucose, lactose, lactulose, maltose, D-mannose, turanose, methyl pyruvate, acetic acid, D-galacturonic acid, succinic acid, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-serine, L-threonine, uridine and glycerol are

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Table 2. Fatty acid profiles of 3R17^T and related species of the genus *Flavobacterium*

Strains: 1, *F. fluminis* 3R17^T; 2, *F. resistens* BD-b365^T; 3, *F. nitratireducens* N1^T. TR, Trace (<1 %); –, not detected. All data were obtained from this study.

Strain	1	2	3
Saturated:			
$C_{14:0}$	2.1	TR	1.6
$C_{16:0}$	7.0	5.1	3.4
Hydroxylated:			
$C_{15:0}$ 2-OH	1.6	1.6	TR
$C_{15:0}$ 3-OH	–	1.5	–
$C_{16:0}$ 3-OH	4.4	2.3	2.7
$C_{17:0}$ 3-OH	–	TR	–
Unsaturated:			
$C_{15:1}\omega6c$	2.0	5.5	4.6
$C_{17:1}\omega6c$	2.0	4.2	2.4
$C_{17:1}\omega8c$	–	1.5	TR
Branched:			
iso-$C_{13:0}$	–	1.3	1.3
iso-$C_{14:0}$	–	2.3	2.7
iso-$C_{15:0}$	15.9	15.1	19.2
anteiso-$C_{15:0}$	7.8	4.4	13.5
iso-$C_{15:0}$ 3-OH	5.2	7.6	5.7
iso-$C_{15:1}$G	7.4	8.4	8.6
iso-$C_{16:0}$	–	3.6	1.3
iso-$C_{16:0}$ 3-OH	–	1.8	1.7
iso-$C_{16:1}$H	–	1.3	TR
iso-$C_{17:0}$ 3-OH	5.5	6.3	4.6
Summed features*:			
2	1.4	TR	TR
3	35.8	17.9	21.1
9	1.9	4.5	1.3

*Summed features: 2, $C_{12:0}$ALDE and/or unknown ECL 10.928; 3, $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$; 9, 10-methyl $C_{16:0}$ and/or iso-$C_{17:1}\omega9c$.

utilized as sole carbon sources. The main fatty acids (>5 %) are a summed feature comprising $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$, iso-$C_{15:0}$, iso-$C_{15:1}$G, anteiso-$C_{15:0}$, $C_{16:0}$, iso-$C_{17:0}$ 3-OH and iso-$C_{15:0}$ 3-OH. The major isoprenoid quinone is MK-6. Phosphatidylethanolamine (PE) is the major polar lipid, and aminolipids and unidentified lipids are also present.

The type strain, 3R17^T (=KCTC 42062^T=JCM 30338^T), was isolated from the surface water of a river. The DNA G+C content of the type strain is 35.4 mol%.

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Conflicts of interest
The authors declare no conflicts of interest regarding the publication of this article.

References

1. Bernardet JF, Bowman JP. Genus I. *Flavobacterium* Bergey *et al.* 1923. In: Krieg NR, Staley JT, Brown DR, Hedlund BP, Paster BJ *et al.* (editors). *Bergey's Manual of Systematic Bacteriology*, 2nd ed. Baltimore, MD: Williams & Wilkins; 2011. pp. 112–154.
2. Tamaki H, Hanada S, Nakamura K, Nomura N, Matsumura M *et al.* The ecological role of a proteolytic psychrophile, *Flavobacterium limicola* belonging to the *Cytophaga–Flavobacteria* cluster, in the decomposition of particulate organic nitrogen in freshwater sediment. *Microbes Environ* 2004;19:147–153.
3. Kimura H, Young CR, Martinez A, Delong EF. Light-induced transcriptional responses associated with proteorhodopsin-enhanced growth in a marine *Flavobacterium*. *ISME J* 2011;5: 1641–1651.
4. Declercq AM, Boyen F, van den Broeck W, Bossier P, Karsi A *et al.* Antimicrobial susceptibility pattern of *Flavobacterium columnare* isolates collected worldwide from 17 fish species. *J Fish Dis* 2013;36:45–55.
5. Kim TW. Aquatic microbiome in the major water sources of Korea. MSc Thesis. Chungnam National University, Daejeon, Republic of Korea; 2014.
6. Bernardet JF, Nakagawa Y, Holmes B. Proposed minimal standards for describing new taxa of the family *Flavobacteriaceae* and emended description of the family. *Int J Syst Evol Microbiol* 2002; 52:1049–1070.
7. Kim TW, Joung Y, Han JH, Jung W, Kim SB. Antibiotic resistance among aquatic bacteria in natural freshwater environments of Korea. *J Water Health* 2015;13:1085–1097.
8. Jeon YS, Lee K, Park SC, Kim BS, Cho YJ *et al.* EzEditor: a versatile sequence alignment editor for both rRNA- and protein-coding genes. *Int J Syst Evol Microbiol* 2014;64:689–691.
9. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol* 2013;30:2725–2729.
10. Stackebrandt E, Ebers J. Taxonomic parameters revisited: tarnished gold standards. *Microbiol Today* 2006;33:152–155.
11. Kim M, Oh HS, Park SC, Chun J. Towards a taxonomic coherence between average nucleotide identity and 16S rRNA gene sequence similarity for species demarcation of prokaryotes. *Int J Syst Evol Microbiol* 2014;64:346–351.
12. Rainey F, Oren A. *Taxonomy of Prokaryotes*. Amsterdam: Elsevier.
13. Han JH, Kim TS, Joung Y, Kim MN, Shin KS *et al.* *Nocardioides endophyticus* sp. nov. and *Nocardioides conyzicola* sp. nov., isolated from herbaceous plant roots. *Int J Syst Evol Microbiol* 2013;63: 4730–4734.
14. Gonzalez JM, Saiz-Jimenez C. A fluorimetric method for the estimation of G+C mol% content in microorganisms by thermal denaturation temperature. *Environ Microbiol* 2002;4:770–773.
15. Nupur, Bhumika V, Srinivas TN, Kumar PA. *Flavobacterium nitratireducens* sp. nov., an amylolytic bacterium of the family *Flavobacteriaceae* isolated from coastal surface seawater. *Int J Syst Evol Microbiol* 2013;63:2490–2496.
16. Ryu SH, Park JH, Moon JC, Sung Y, Lee SS *et al.* *Flavobacterium resistens* sp. nov., isolated from stream sediment. *Int J Syst Evol Microbiol* 2008;58:2266–2270.

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