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Confluentibacter flavum sp. nov., Isolated from the Saline Lake

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Abstract

A Gram-stain-negative, rod-shaped, non-motile, bacterial isolate designated 3B^T, was isolated from a saline lake, and subjected to a polyphasic taxonomic investigation. The phylogenetic analysis based on 16S rRNA gene sequence clearly showed an allocation to the genus *Confluentibacter* with similarity ranging from 95.1 to 98%. OrthoANI values between strain 3B^T and related strains of *Confluentibacter* (< 90%) were lower than the threshold value of 95% ANI relatedness recommended for species demarcation. Strain 3B^T grew at 4–35 °C and pH 6.0–8.0 (optimum, 28 °C and pH 6.5) and with 0–3% (w/v) NaCl (optimum, 0.5%). The predominant respiratory quinone was menaquinone-6 (MK-6) and the major fatty acids were iso-$C_{15:0}$, iso-$C_{15:1}$ G, iso-$C_{15:0}$ 3-OH, and iso-$C_{17:0}$ 3-OH. The polar lipid profile of strain 3B^T comprised phosphatidylethanolamine, one unidentified aminolipid, one aminophospholipid, and three unidentified lipids (L1–3). The DNA G+C content was 33.1 mol%. On the basis of morphological, physiological, and chemotaxonomic characteristics, together with the results of phylogenetic analysis, strain 3B^T is described as a novel species in genus *Confluentibacter*, for which the name *Confluentibacter flavum* sp. nov. (type strain 3B^T = CGMCC115960^T = KCTC52969^T) is proposed.

Introduction

The genus *Confluentibacter*, a member of the family *Flavobacteriaceae* in the phylum *Bacteroidetes*, was established by Vandamme et al. [1]. At the time of writing, the genus *Confluentibacter* contained two species: *Confluentibacter citreus* and *Confluentibacter lentus*. *C. lentus* was isolated from the junction between the ocean and a freshwater lake at Hwajinpo on the South Sea of South Korea [2]. *C. citreus* was isolated from lake sediment [3]. Here we present a novel strain named 3B^T, which was isolated from saline lake in the northwest China. Based on phylogenetic, genomic, chemotaxonomic, and phenotypic characteristics, we proposed that this strain should be assigned as a novel species.

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Materials and Methods

Strains and Culture Conditions

A bottle of saline lake water was collected in October 2015 from a saline lake (37°38′N, 89°54′E) located in Xinjiang province, China. The isolate was obtained by the standard dilution plating technique [4] at 28 °C on modified marine agar 2216 and cultivated routinely on modified marine agar 2216 (MA) or in modified marine broth 2216 (MB) at 28 °C [5]. The modified MA contained the same ingredients of marine agar 2216 (MA; Difco), except that peptone and yeast extract were reduced to 0.1 and 0.5 g, respectively. Based on colony morphology, an opaque, regular edge, and yellowish colony was picked up and named strain 3B^T. After being purified, the strain was preserved as suspensions with 30% (v/v) glycerol at − 80 °C and was freeze-dried for preservation.

According to phylogenetic analysis based on 16S rRNA gene sequences, strain 3B^T was assigned to the genus *Confluentibacter* with highest 16S rRNA gene sequence similarity of 98% to *C. citreus* KCTC52638^T, followed by *C. lentus* KCTC42777^T (97.5%). *C. citreus* KCTC52638^T and *C. lentus* KCTC42777^T (obtained from the Korean Collection for Type Cultures) were chosen as reference strains.

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They were routinely cultured and preserved in the same way as strain $3B^T$.

For cellular fatty acids analysis, cell mass of the isolate was harvested from MA plates after incubation for 4 days at 28 °C. Cell biomass of the strain for DNA extraction and for the analyses of isoprenoid quinones and polar lipids was obtained from cultures grown for 5 days in MB at 28 °C. Strain $3B^T$ has been deposited at the CGMCC (China General Microbiological Culture Collection Center) and the KCTC (Korean Collection for Type Cultures).

Determination of 16S rRNA Gene Sequence and Phylogenetic Analysis

A quick bacteria genomic DNA extraction kit (Dongsheng Biotech) was used to obtain genomic DNA. The 16S rRNA gene was amplified by PCR using bacterial universal primers 27F (5′-GACGATTGAGAGTTTGATC-3′) and 1492R (5′-CGTAAATCTCTAGAGG-3′). The almost complete sequence was compared with sequences of closely related reference organisms obtained from the EzTaxon-e database [6]. Multiple sequence alignment was performed with ClustalW 1.8 [7]. Phylogenetic trees were constructed using the neighbor-joining [8], maximum-likelihood [9], and the maximum-parsimony [10] methods with the MEGA 5 program package. Evolutionary distances were calculated according to the algorithm of Kimura's two-parameter model [11] for the neighbor-joining method. The DNA G+C content was determined by the method of Tamaoka and Komagata [12].

The draft genome of $3B^T$ was sequenced using an Illumina HiSeq 4000 system (Illumina, San Diego, CA, USA) at the Beijing Genomics Institute (Shenzhen, China). The paired-end fragment libraries were sequenced according to the Illumina HiSeq 4000 system's protocol. Raw reads of low quality from paired-end sequencing (those with consecutive bases covered by fewer than five reads) were discarded. The sequenced reads were assembled using ABySS software [13]. The whole genome accession number of strain $3B^T$ was PJEO00000000. The whole genome accession number of *C. lentus* KCTC42777^T was NIHE00000000. The whole genome accession number of *C. citreus* KCTC52638^T was NIXN00000000.

Phenotypic Characterization

The cell morphology, Gram reaction, and anaerobic growth were determined as described by Park et al. [14]. Gliding motility was investigated as described by Bowman [15]. Motility was determined by microscopic observation and inoculation in semi-solid agar medium. Cells of the exponential growth phase, grown on MA plates, were used to determine the temperature range for growth. The growth temperature was tested by incubation at 4, 10, 15, 20, 25, 28, 30, 35, 40, 45, and 50 °C. The pH range for growth in MB was measured from 4.5 to 10.0 with an interval of 0.5 pH and 40 mM MES (pH 4.5–6.0), PIPES (pH 6.5–7.5), Tricine (pH 8.0–8.5), and CAPSO (pH 9.0–10.0) added to maintain every pH system stable, respectively. Tolerance of NaCl was tested with the concentrations of NaCl at 0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, 18, and 20% (w/v) in MB at 28 °C. Growth in the MB with 2% (w/v) NaCl as the sole salt was also determined. The requirement for Mg^{2+} ions was investigated by using MB prepared according to the formula of the BD Difco medium that comprised all the constituents except $MgCl_2$ and $MgSO_4$.

Catalase and oxidase activities were determined by using 3% (v/v) hydrogen peroxide solution and oxidase reagent (bioMérieux), respectively. Hydrolysis of hypoxanthine, Tween 80, aesculin, casein, starch, L-tyrosine, and xanthine were tested based on methods of Han et al. [16]. Nitrate reduction was investigated as described by Lányí with the modification that artificial seawater was used for the preparation of media [17]. The presence of flexirubin-type pigments was investigated as described by Reichenbach [18] and Bernardet et al. [19]. All experiments were performed in triplicate. For acid production test, Leifson-modified MOF medium was used to suspend the cells for the inoculation of API 50CH (bioMérieux) strips [20]. Enzyme activities was tested using API ZYM following the manufacturer's instructions. and these tests were performed at 28 °C for 96 h.

Exponential phase cells spread and were incubated on MA at 28 °C for 6 h, then the MA plate were used for antimicrobial susceptibility test. The pretreated plate attached with antibiotics discs was incubated at 28 °C for 7 days. The following antibiotic discs were used (μg per disc, unless indicated): ampicillin (10), carbenicillin (100), cefalotin (30), chloramphenicol (100), gentamicin (30), kanamycin (30), lincomycin (15), neomycin (30), novobiocin (5), oleandomycin (15), erythromycin (15), penicillin G (20 IU), polymyxin B (100 IU), streptomycin (50), and tetracycline (30).

Determination of Isoprenoid Quinones, Fatty Acids, and Polar Lipids

After incubation in MB at 28 °C for 5 days, cells in exponential phase were freeze-dried and used for fatty acid methyl esters (FAMEs), polar lipid, and isoprenoid quinone analyses. Isoprenoid quinones were extracted from freeze-dried cells with chloroform/methanol (2:1), purified by TLC, and analyzed by reversed-phase HPLC [7]. FAMEs were analyzed according to the instructions of the Microbial Identification System (MIDI; Microbial ID). The fatty acids were analyzed by GC (Hewlett Packard 6890N) and identified by using the TSBA40 database. Polar lipids were extracted by 80 ml of chloroform/methanol/water (1:2:1, by

vol), separated by two-dimensional TLC on silica gel 60 F254 plates (Merck), and then analyzed as described by Xu et al. [21].

Results and Discussion

Genotypic Characterization and Taxonomic Conclusion

On the basis of 16S rRNA gene sequence alignment, strain 3B^T was most closely related to *C. citreus* KCTC52638^T with 98.0% sequence similarity, and of 97.5% sequence similarity to *C. lentus* KCTC42777^T, 95.69% to *Mariniflexile ostreae* KCTC 42113^T, and 95.55% to *Yeosuana aromativorans* JCM 12862^T. Phylogenetic analysis, based on nearly full-length 16S rRNA gene sequences (1500 bp), showed that strain 3B^T belonged to the genus *Confluentibacter* in the neighbor-joining, maximum-likelihood, and maximum-parsimony trees (Fig. 1).

Average nucleotide identity (ANI) was calculated with OrthoANI. The ANI values of strain 3B^T compared with the type strains of *C. lentus* KCTC42777^T (86.6%) and *C. citreus* KCTC52638^T (88.1%) were lower than the threshold value of 95% ANI relatedness for species demarcation [22, 23]. The DNA G+C content of strain 3B^T was 33.1 mol%, the value similar to that of the type strain *C. lentus* KCTC42777^T and strain *C. citreus* KCTC52638^T (Table 1). This result clearly indicated that strain 3B^T should represent a novel species of genus *Confluentibacter*.

Phenotypic Characterization

Cells of the strain 3B^T were Gram-negative and non-motile without flagellum. Cells are approximately 0.13–0.17 μm in width and 1.5–1.7 μm in length (Supplementary Fig. S2). Strain 3B^T grew at 4–35 °C (optimum 28 °C), pH 6.0–8.0 (optimum 6.5), and with 0–3% NaCl (optimum 0.5%, w/v). Flexirubin-type pigments are not produced. Under the optimal growth conditions, cells reached the logarithmic phase in MB at the fifth day and on MA at the fourth day. A comparison of the physiological and biochemical characteristics between 3B^T and the related type strains in our study showed that they shared some similarities, such as they were all positive for catalase activity and hydrolysis of starch. Despite some similarities, strain 3B^T displayed certain distinct characteristics from other two reference strains. In the API ZYM tests, activity of Cystine arylamidase was present in strain 3B^T. But activity of Cystine arylamidase were absent in related type strains. Besides, susceptible to erythromycin and producing acid from D-Maltose could also be distinguished from the related reference strains (Table 1). Detailed results of physiological and biochemical tests are given in species description. The different characteristics between strain 3B^T and its reference strains are summarized in Table 1.

Isoprenoid Quinones, Fatty Acids, and Polar Lipids Characterization

The only respiratory quinone detected in strain 3B^T was MK-6, which was in accordance with the genus

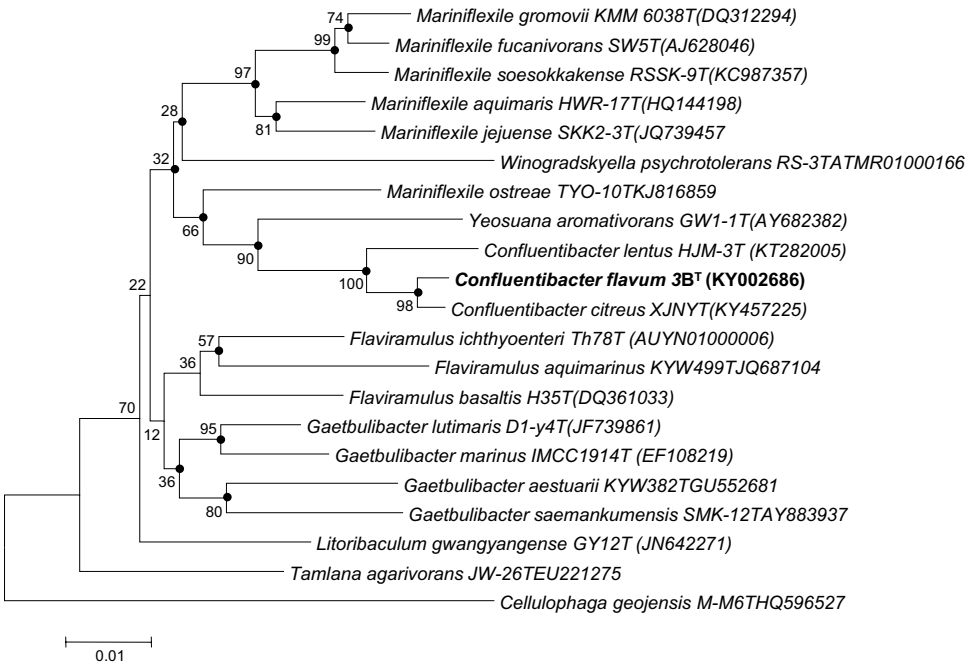

Fig. 1 Neighbor-joining phylogenetic tree of strain 3B^T and some closely related taxa, based on 16S rRNA gene sequences. Numbers at branching points represent bootstrap values (%) from 1000 replicates; only values ≥ 50% are shown. Solid circles indicate that the corresponding nodes were also recovered in maximum-likelihood and maximum-parsimony trees. Bar, 0.01 substitutions per nucleotide position

Table 1 Differential phenotypic characteristics of strain 3B^T, *C. citreus* KCTC52638^T, and *C. lentus* KCTC42777^T

Characteristics	1	2	3
Cell size (width × length; μm)	0.13–0.17 × 1.5–1.7	0.35–0.42 × 1.20–1.33[a]	0.3–0.4 × 0.9–1.9[b]
Color	Yellow	Strong yellow	Yellow
Temperature range (optimum) (°C)	4–35 (28)	15–37 (30)[a]	4–37 (33)[b]
pH range (optimum)	6.0–8.0 (6.5)	4.5–8.0 (7.5)[a]	6.5–8.5 (7.5)[b]
NaCl tolerance (%, w/v)	< 3.0	< 4.0[a]	< 4.0[b]
Nitrate reduction	+	+	−
Acid production from			
D-Maltose	+	−	−
Xylose	+	+	+
L-Rhamnose	−	+	−
Raffinose	−	+	+
Mannitol	−	−	−
Sucrose	−	+	−
L-Arabinose	−	+	+
D-Fructose	−	+	−
Enzyme activity (API ZYM)			
Cystine arylamidase	+	−	−
β-Galactosidase	W	−	+
α-Glucosidase	+	−	+
β-Glucosidase	−	W	−
N-Acetyl-β-glucosaminidase	−	−	+
α-Fucosidase	−	−	−
Susceptibility to			
Chloramphenicol	−	+	−
Tetracycline	−	−	+
Neomycin	+	−	+
Erythromycin	+	−	−
Penicillin G	+	−	+
Streptomycin	−	−	+
Oleandomycin	−	+	−
DNA G+C content (mol%)	33.1	34.7[a]	34.5[b]

Strains: 1, strain 3B^T; 2, *C. lentus* KCTC42777^T; 3, *C. citreus* KCTC52638^T. All data are obtained from this study unless indicated. +, Positive; −, negative. W, weakly positive; ND, no data available

[a]Data from Park et al. [2]

[b]Data from Han et al. [3]

Confluentibacter. The cellular fatty acid profiles of strain 3B^T and the reference strains are compared in Table 2. The major fatty acids (≥ 10%) of strain 3B^T were iso-$C_{15:0}$, iso-$C_{15:1}$ G, iso-$C_{15:0}$ 3-OH, and iso-$C_{17:0}$ 3-OH, similar with *C. citreus* KCTC52638^T and *C. lentus* KCTC52638^T. A comparison of the fatty acid profiles showed a few difference between strain 3B^T and the reference strains, less $C_{16:0}$ 3-OH, less $C_{16:0}$, and more anteiso-C15:0 in strain 3B^T. The polar lipids of strain 3B^T and the reference strains are shown in Supplementary Fig. S1. The polar lipid profile of 3B^T comprised phosphatidylethanolamine (PE), one unidentified aminolipid (AL), one aminophospholipid (APL), and three unidentified lipids (L1–3). Similarly, strain 3B^T as well as *C. citreus* KCTC52638^T and *C. lentus* KCTC42777^T had the same polar lipids, liked PE. However, strain 3B^T had more polar lipid type than that found in *C. lentus* KCTC42777^T. For example, the APL, L2, and L3 were absent in strain *C. lentus* KCTC42777^T. In addition, one unidentified phospholipid was detected in strain *C. citreus* KCTC52638^T but not in strain 3B^T.

On the basis of the phenotypic, phylogenetic, and chemotaxonomic characteristics described above, strain 3B^T is considered to represent a novel species within the genus *Confluentibacter* for which the name *Confluentibacter flavum* sp. nov. is proposed.

Table 2 Fatty acid compositions of strain $3B^T$ and its related reference strains

Fatty acid	1	2	3
Straight-chain			
$C_{18:0}$	tr	1.4	–
$C_{16:0}$	4.3	10.0	6.3
Branched			
Iso-$C_{15:1}$ G	10.1	9.6	8.0
Anteiso-$C_{15:1}$ A	2.7	1.4	tr
Iso-$C_{15:0}$	17.0	16.5	11
Anteiso-$C_{15:0}$	9.5	6.5	14.3
Unsaturated			
$C_{17:1}$ ω6c	1.0	–	tr
$C_{15:1}$ ω6c	5.9	–	tr
Hydroxy			
$C_{15:0}$ 2-OH	2.9	3.5	2.4
$C_{15:0}$ 3-OH	–	6.7	–
$C_{16:0}$ 3-OH	1.4	4.3	tr
$C_{17:0}$ 2-OH	–	5.4	6.3
$C_{17:0}$ 3-OH	tr	2.5	1.1
Iso-$C_{17:0}$ 3-OH	15.8	13.6	11.9
Iso-$C_{16:0}$ 3-OH	2.0	1.6	tr
Iso-$C_{15:0}$ 3-OH	13.2	10.8	6.0
Summed feature 3[a]	4.2	6.1	4.3

Summed feature 3 comprises $C_{16:1}$ *ω7c* and/or $C_{16:1}$ *ω6c*

Strains: 1, $3B^T$; 2, *C. lentus* KCTC42777^T; 3, *C. citreus* KCTC52638^T; All data are from this study. Fatty acids that represented less than 1.0% in all strains were omitted. Fatty acids that represented > 1.0% are indicated. tr, Traces (< 1.0%); –, not detected

[a]Summed features represent one or more fatty acids that cannot be separated by GLC with the MIDI system

Description of *Confluentibacter flavum* sp. nov.

Confluentibacter flavum (fla¢vum. L. neut. adj. flavum, yellow, referring to the colony color).

Cells are Gram-negative, non-flagellated, non-gliding, and rod-shaped, approximately 0.13–0.17 μm in width and 1.5–1.7 μm in length. Colonies are circular, convex with a shiny, smooth surface, entire edges, and yellow after incubation for 6 days at 28 °C. Flexirubin-type pigments are not produced. Cells grow at 4 °C, but not at 35 °C. The optimum temperature for growth is 28 °C. The pH range for growth is 6.0–8.0, and optimum is pH 6.5. Growth occurs with 0–3.0% (w/v) NaCl concentration with an optimum of 0.5%. Mg^{2+} ions are not required for growth. No growth is observed under anaerobic condition in modified MB. Nitrate can be reduced to nitrite. Positive results for catalase and oxidase activities. Gelatin, aesculin, Tween 80, L-tyrosine, urea, and starch are hydrolysed, but casein, hypoxanthine, and xanthine are not. Acid is produced from maltose and xylose, but not from rhamnose, raffinose, mannitol, sucrose, L-arabinose, and fructose. In API ZYM tests, production of alkaline phosphatase, esterase (C4), leucine arylamidase, α-glucosidase, cysteine arylamidase, and acid phosphatase are positive; production of esterase lipase (C8), trypsin, and β-galactosidase are weakly positive; production of α-galactosidase, valine arylamidase, lipase (C14), naphthol–AS-BI–phosphohydrolase, β-glucuronidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, chymotrypsin, α-mannosidase, and α-fucosidase activities are negative. Additionally, Strain $3B^T$ was susceptible to neomycin and penicillin G, and sensitive to chloramphenicol, tetracycline, streptomycin, ampicillin, carbenicillin, cefalotin, gentamicin, kanamycin, lincomycin, novobiocin, oleandomycin, polymyxin B, and streptomycin. The predominant menaquinone is MK-6. The major fatty acids (> 10.0%) are iso-$C_{15:0}$, iso-$C_{15:1}$ G, iso-$C_{15:0}$ 3-OH, and iso-$C_{17:0}$ 3-OH. The major polar lipids are PE and one unidentified AL. The genomic DNA G+C content is 33.1 mol% (from HPLC).

The type strain, $3B^T$ (= CGMCC115960^T = KCTC52969^T), was isolated from a saline lake located in Xinjiang Province, China. The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence and the whole genome project of strain $3B^T$ are KY002686 and PJEO00000000. The DPD TaxonNumber for strain $3B^T$ is TA00400.

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