Exiguobacterium aestuarii sp. nov. and *Exiguobacterium marinum* sp. nov., isolated from a tidal flat of the Yellow Sea in Korea

In-Gi Kim, Mi-Hwa Lee, Seo-Youn Jung, Jae Jun Song, Tae-Kwang Oh and Jung-Hoon Yoon

Correspondence
Jung-Hoon Yoon
jhyoon@kribb.re.kr

Korea Research Institute of Bioscience and Biotechnology (KRIBB), PO Box 115, Yusong, Taejeon, Korea

Three Gram-variable, rod-shaped bacterial strains, TF-16^T, TF-19 and TF-80^T, were isolated from a tidal flat of Daepo Beach (Yellow Sea) near Mokpo City, Korea, and their taxonomic positions were investigated by a polyphasic approach. These isolates grew optimally in the presence of 2% NaCl and at 30 °C. Their peptidoglycan types were based on L-Lys–Gly. The predominant menaquinone detected in the three strains was MK-7. The three strains contained large amounts of the branched fatty acids iso-C_{17:0}, anteiso-C_{13:0}, iso-C_{13:0} and iso-C_{15:0}. The DNA G + C contents of strains TF-16^T, TF-19 and TF-80^T were 48.6, 48.4 and 48.0 mol%, respectively. The three strains formed a coherent cluster with *Exiguobacterium* species in a phylogenetic tree based on 16S rRNA gene sequences. They showed closest phylogenetic affiliation to *Exiguobacterium aurantiacum*, with 16S rRNA gene sequence similarity values of 98.1–98.3%. The three strains exhibited 16S rRNA gene sequence similarity values of 94.0–94.6% to the type strains of other *Exiguobacterium* species. Levels of DNA–DNA relatedness indicated that strains TF-16^T and TF-19 and strain TF-80^T are members of two species that are separate from *E. aurantiacum*. On the basis of phenotypic, phylogenetic and genetic data, strains TF-16^T and TF-19 and strain TF-80^T represent two novel species in the genus *Exiguobacterium*; the names *Exiguobacterium aestuarii* sp. nov. (type strain TF-16^T = KCTC 19035^T = DSM 16306^T; reference strain TF-19) and *Exiguobacterium marinum* sp. nov. (type strain TF-80^T = KCTC 19036^T = DSM 16307^T) are proposed.

The genus *Exiguobacterium* was proposed by Collins *et al.* (1983) for alkaliphilic strains isolated from potato-processing effluent (Gee *et al.*, 1980). The first species of this genus to be described was *Exiguobacterium aurantiacum*, the type species. The second species of the genus, *Exiguobacterium acetylicum*, was created by reclassification of *Brevibacterium acetylicum* (Farrow *et al.*, 1994). Recently, three more *Exiguobacterium* species, *Exiguobacterium antarcticum* and *Exiguobacterium undae* (Frühling *et al.*, 2002) and *Exiguobacterium oxidotolerans* (Yumoto *et al.*, 2004), have been described. 16S rRNA gene sequence analyses have shown that the genus *Exiguobacterium* is related phylogenetically to the genus *Bacillus* and related taxa (Farrow *et al.*, 1992, 1994; Yumoto *et al.*, 2004). In this study, three slightly halophilic bacterial strains, TF-16^T, TF-19 and TF-80^T, which were isolated from a tidal flat of Daepo Beach (Yellow Sea), near Mokpo City in Korea, are described. 16S rRNA gene sequence comparisons indicated that the three

isolates are *Exiguobacterium*-like organisms. The aim of the present work was to determine the exact taxonomic positions of the three isolates by detailed characterization combining phenotypic, chemotaxonomic and genetic analyses.

Tidal flat sediment was collected from a tidal flat and used to isolate bacterial strains. Strains TF-16^T, TF-19 and TF-80^T were isolated by the usual dilution plating method on marine agar 2216 (MA; Difco) at 30 °C. To investigate their morphological and physiological characteristics, strains TF-16^T, TF-19 and TF-80^T were routinely cultivated at 30 °C on MA under aerobic conditions. Reference strains *E. aurantiacum* KCTC 9244^T and *E. acetylicum* KCTC 3255^T were obtained from the Korean Collection for Type Cultures (KCTC), Taejeon, Korea. For chemical and genetic analyses, cell mass of strains TF-16^T, TF-19 and TF-80^T and *E. aurantiacum* KCTC 9244^T was obtained from marine broth 2216 (MB; Difco) cultures at 30 °C. For fatty acid methyl ester analysis, cell mass of strains TF-16^T, TF-19 and TF-80^T and *E. aurantiacum* KCTC 9244^T was harvested from MA plates after 3 days cultivation at 30 °C and cell

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains TF-16^T, TF-19 and TF-80^T are AY594264, AY594265 and AY594266, respectively.

mass of *E. acetylicum* KCTC 3255^T was obtained from trypticase soy agar (TSA; BBL) plates under the same conditions.

Cell morphology was examined by light microscopy (Nikon E600) and TEM. Flagellum type was examined by TEM using cells from exponentially growing cultures. The Gram reaction was determined using a Gram Stain kit (bioMérieux) according to the manufacturer's instructions. Growth at various temperatures, pH and NaCl concentrations was investigated on MA or in MB. Growth in the absence of NaCl was investigated in trypticase soy broth lacking NaCl. Catalase activity was tested by adding 3% (v/v) hydrogen peroxide solution to colonies grown on MA. Cytochrome oxidase activity was determined using Kovac's reagent. Anaerobic growth was determined after incubation in an anaerobic chamber with MA that had been prepared anaerobically. Nitrate reduction, urease activity and hydrolysis of casein, starch, aesculin, xanthine, hypoxanthine, tyrosine, gelatin and Tween 80 were analysed as described by Cowan & Steel (1965) and Lanyi (1987). Acid production from carbohydrates was determined as described by Leifson (1963). Preparation of cell wall peptidoglycan was carried out using the method described by Schleifer & Kandler (1972) and peptidoglycan structure was determined using an automated amino acid analyser (Hitachi model L-8500A). Menaquinones were extracted according to the method of Komagata & Suzuki (1987) and analysed using reversed-phase HPLC and a YMC ODS-A (250 × 4.6 mm) column. Fatty acid methyl esters were extracted and prepared according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990). Polar lipids were extracted using procedures described by Minnikin *et al.* (1984) and identified by two-dimensional TLC followed by spraying with the appropriate detection reagent (Komagata & Suzuki, 1987). Chromosomal DNA isolation and purification were carried out according to the method described by Yoon *et al.* (1996), with the exception that RNase T1 was applied in combination with RNase A. DNA G+C content was determined using the procedure of Tamaoka & Komagata (1984), with the modification that DNA was hydrolysed using nuclease P1 (Boehringer Mannheim) and the resultant nucleotides were analysed by reversed-phase HPLC.

DNA–DNA hybridization was performed fluorometrically by the method of Ezaki *et al.* (1989) using photobiotin-labelled DNA probes and microdilution wells. Hybridization was performed with five replications for each sample. Of the values obtained, the highest and lowest values in each sample were excluded. DNA–DNA relatedness values are the means of the remaining three values. Amplification of the 16S rRNA gene was carried out according to the method described previously using two universal primers (Yoon *et al.*, 1998). Sequencing of the amplified 16S rRNA gene was performed as described by Yoon *et al.* (2003). Alignment of sequences was carried out using the program

CLUSTAL_W (Thompson *et al.*, 1994) and gaps at the 5' and 3' ends of the alignment were omitted from further analysis. Evolutionary distances were calculated using the Kimura two-parameter correction with the CLUSTAL_W package (Thompson *et al.*, 1994). A phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987) on the basis of distance matrix data. The grouping reliability was assessed by 1000 bootstrap resamplings of the neighbour-joining dataset using the CLUSTAL_W package.

Strains TF-16^T and TF-19 had similar phenotypic characteristics. However, strain TF-80^T could be differentiated from strains TF-16^T and TF-19 by the following characteristics: flagellum type, colony colour, maximum growth temperature, NaCl tolerance and acid production from some substrates (Table 1). Other phenotypic characteristics are given in the species descriptions (see below). Some phenotypic characteristics that differentiated strains TF-16^T, TF-19 and TF-80^T from other *Exiguobacterium* species are indicated in Table 1. Cell wall analysis revealed that the peptidoglycan type in strains TF-16^T, TF-19 and TF-80^T was based on L-Lys–Gly, as described by Schleifer & Kandler (1972). The major menaquinone of strains TF-16^T, TF-19 and TF-80^T was MK-7. The three isolates and *E. aurantiacum* KCTC 9244^T contained iso-C_{17:0}, anteiso-C_{13:0}, iso-C_{13:0} and iso-C_{15:0} as the major fatty acids (Table 2). The cellular fatty acid profile of *E. acetylicum* KCTC 3255^T differed from the profiles of TF-16^T, TF-19, TF-80^T and *E. aurantiacum* KCTC 9244^T; this may be because of different cultivation conditions, e.g. growth media. *E. acetylicum* KCTC 3255^T contained large amounts of the unsaturated fatty acid C_{16:1ω11c} and the straight-chain fatty acids C_{14:0} and C_{16:0}, which were minor components in TF-16^T, TF-19, TF-80^T and *E. aurantiacum* KCTC 9244^T (Table 2). Polar lipids found in strains TF-16^T, TF-19 and TF-80^T were phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine and an unidentified phospholipid; ninhydrin-positive amino-group-containing lipids were also detected. The DNA G+C contents of strains TF-16^T, TF-19 and TF-80^T were 48.6, 48.4 and 48.0 mol%, respectively.

The 16S rRNA gene sequences of strains TF-16^T, TF-19 and TF-80^T determined in this study comprised 1517, 1517 and 1519 nt, respectively, representing approximately 96% of the *Escherichia coli* 16S rRNA sequence. The 16S rRNA gene sequences of strains TF-16^T and TF-19 were 99.9% similar (2 bp difference). 16S rRNA similarity values of strain TF-80^T with strains TF-16^T and TF-19 were 99.4 and 99.3%, respectively. As shown in the phylogenetic tree, strains TF-16^T, TF-19 and TF-80^T were phylogenetically affiliated to the genus *Exiguobacterium* (Fig. 1). Sequences from strains TF-16^T, TF-19 and TF-80^T exhibited highest similarity values to that of the type strain of *E. aurantiacum* (98.3, 98.3 and 98.1%, respectively). 16S rRNA gene sequence similarity levels between

Table 1. Differential phenotypic characteristics of *Exiguobacterium* species

Species: 1, *E. aestuarii* sp. nov.; 2, *E. marinum* sp. nov.; 3, *E. aurantiacum* (data from Gee *et al.*, 1980; Frühling *et al.*, 2002); 4, *E. acetylicum* (Jones & Keddie, 1986; Frühling *et al.*, 2002); 5, *E. undae* (Frühling *et al.*, 2002); 6, *E. antarcticum* (Frühling *et al.*, 2002); 7, *E. oxidotolerans* (Yumoto *et al.*, 2004). +, Positive; –, negative; W, weakly positive; ND, not determined; *n*, number of strains. All species are positive for catalase, hydrolysis of casein, aesculin and gelatin and acid production from D-fructose, D-glucose and maltose. All species are negative for hydrolysis of Tween 80.

Characteristic	1 (<i>n</i> =2)	2 (<i>n</i> =1)	3 (<i>n</i> =5)	4 (<i>n</i> =1)	5 (<i>n</i> =4)	6 (<i>n</i> =1)	7 (<i>n</i> =1)
Flagellum type	Peritrichous	Single polar	ND	Peritrichous	Peritrichous	Peritrichous	Peritrichous
Colour of colonies	Pale orange	Deep orange	Orange	Orange-yellow	Orange	Bright orange	Orange or yellow
Oxidase	–	–	–	+	+	+	+
Nitrate reduction	+	+	–	–	–	–	–
Growth at/in:							
44 °C	+	–	–	ND	ND	ND	–
17 % NaCl	+	–	–	ND	ND	ND	–
Hydrolysis of:							
Starch	–*	–*	+	+	ND	ND	+
Tyrosine	–	–	ND	+	ND	ND	ND
Xanthine	–	–	ND	+	ND	ND	ND
Acid production from:							
D-Ribose	+	+	–	–	+	+	+
D-Mannitol	+	+	+	+	+	–	W
Melibiose	+	+	–	–	+	–	+
D-Galactose	+	+	–	–	+	+	–
D-Mannose	+	+	–	+	+	+	+
D-Cellobiose	+	+	–	+	+	+	ND
Stachyose	–	+	ND	ND	ND	ND	ND
Sucrose	+	+	+	+	+	+	–
D-Trehalose	+	–	+	+	+	+	–
D-Raffinose	–	W	–	–	W	–	+
DNA G + C content (mol%)	48.4–48.6	48.0	53.2–55.8	46.6	ND	ND	46.7

*Poor growth observed on MA containing starch.

the three novel strains and the type strains of other *Exiguobacterium* species were 94.0–94.6 %. The relationship between the cluster comprising the three isolates and *E. aurantiacum* NCDO 2321^T and the cluster comprising other *Exiguobacterium* species was supported by a bootstrap confidence value of 100 % (Fig. 1). DNA–DNA hybridization was performed to determine the genetic relatedness between strains TF-16^T, TF-19 and TF-80^T and between these three novel isolates and *E. aurantiacum* KCTC 9244^T. The mean level of DNA–DNA relatedness between strains TF-16^T and TF-19 was 98 %. Strain TF-80^T showed mean DNA–DNA relatedness levels of 10–22 % to strains TF-16^T and TF-19. Strains TF-16^T and TF-19 and strain TF-80^T showed DNA–DNA relatedness levels of 3–11 and 12 % to *E. aurantiacum* KCTC 9244^T, respectively.

16S rRNA gene sequence analysis revealed that strains TF-16^T, TF-19 and TF-80^T fall within the evolutionary radiation enclosed by the genus *Exiguobacterium* (Fig. 1). The chemotaxonomic characteristics of the three strains, including cell wall peptidoglycan type and menaquinone and

fatty acid profiles, were similar to those of *Exiguobacterium* species (Collins *et al.*, 1983; Frühling *et al.*, 2002). The polar lipid profiles of the three strains were similar to those of *E. undae* and *E. antarcticum*, although they differed from those of *E. aurantiacum* and *E. acetylicum* in that phosphatidylinositol and phosphatidylserine were present (Collins *et al.*, 1983; Frühling *et al.*, 2002). Strains TF-16^T and TF-19 had morphological and physiological characteristics that differed from those of strain TF-80^T. Strains TF-16^T, TF-19 and TF-80^T could also be differentiated from other *Exiguobacterium* species by some phenotypic properties (Table 1). The phylogenetic and genetic distinctiveness indicated that strains TF-16^T and TF-19 and strain TF-80^T could be considered as members of two novel species that are separate from other *Exiguobacterium* species. Therefore, on the basis of the data presented, strains TF-16^T and TF-19 and strain TF-80^T should be classified in the genus *Exiguobacterium* as representatives of two distinct novel species, for which the names *Exiguobacterium aestuarii* sp. nov. and *Exiguobacterium marinum* sp. nov., respectively, are proposed.

Table 2. Cellular fatty acid compositions (%) of strains TF-16^T, TF-19 and TF-80^T and the type strains of two *Exiguobacterium* species

Strains: 1, TF-16^T; 2, TF-19; 3, TF-80^T; 4, *E. aurantiacum* KCTC 9244^T; 5, *E. acetylicum* KCTC 3255^T. —, Not detected. Fatty acids representing less than 0.5% in all strains were omitted.

Fatty acid	1	2	3	4	5
Straight chain					
C _{14:0}	—	—	—	—	10.6
C _{16:0}	5.3	5.4	4.3	4.4	14.3
C _{18:0}	1.7	1.6	0.8	0.7	0.8
Unsaturated					
C _{14:1} ω5c	—	—	—	—	1.4
C _{16:1} ω5c	—	—	—	—	1.7
C _{16:1} ω11c	—	0.6	—	1.3	15.8
Branched					
iso-C _{12:0}	1.7	1.7	2.6	3.4	0.6
iso-C _{13:0}	11.5	11.2	11.5	11.5	13.5
anteiso-C _{13:0}	15.6	15.5	18.1	19.5	8.5
iso-C _{14:0}	1.3	1.3	0.8	0.7	0.8
iso-C _{15:0}	13.1	13.2	10.4	10.4	19.7
anteiso-C _{15:0}	3.2	3.3	2.6	2.3	2.1
iso-C _{16:0}	7.1	7.3	5.0	4.0	—
iso-C _{17:0}	27.2	26.1	34.4	28.7	1.9
anteiso-C _{17:0}	8.2	8.2	7.1	8.3	—
iso-C _{17:1} ω10c	1.1	1.0	0.9	1.8	1.5
iso-C _{18:0}	2.2	2.0	1.2	0.6	—
Summed feature 3*	—	—	—	—	3.4

*Summed features represent groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 3 contains iso-C_{15:0}, 2-OH and/or C_{16:1}ω7c.

Description of *Exiguobacterium aestuarii* sp. nov.

Exiguobacterium aestuarii (aes.tu'a.ri.i. L. gen. n. *aestuarii* of a part of the sea-coast which, during the flood-tide, is overflowed, but at the ebb-tide is left covered with mud or slime).

Cells are Gram-variable and facultatively anaerobic; growth is better under aerobic conditions. Colonies are circular, glossy, raised and 3–4 mm in diameter after incubation for 2 days on MA at 30 °C under aerobic conditions. White-coloured colonies are formed under anaerobic conditions. Cells are short rods (in the early growth phase) or cocci (exponential and stationary growth phases). Growth occurs at 10 and 47 °C, with optimum growth at 30–37 °C; growth does not occur at 4 °C or above 48 °C. Growth does not occur in the presence of >19% NaCl. The optimal pH for growth is pH 6.5–8.5; growth is observed at pH 5.0 and 10.5, but not at pH 4.5 or 11.0. Hypoxanthine is not hydrolysed. Urease-negative. No acid is produced from D-sorbitol, *myo*-inositol, D-xylose, L-arabinose, D-melezitose, adonitol, L-rhamnose or lactose. The peptidoglycan type is

Fig. 1. Neighbour-joining phylogenetic tree showing the positions of strains TF-16^T, TF-19, TF-80^T and some other related taxa based on 16S rRNA gene sequences. The sequence of *Bacillus subtilis* NCDO 1769^T was used as an outgroup. Numbers on the branches indicate the bootstrap confidence values (%) of 1000 resamplings; only those greater than 50% are shown. Bar, 0.01 substitutions per nucleotide position.

L-Lys–Gly. The predominant menaquinone is MK-7. Major polar lipids are phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine. The major fatty acids are iso-C_{17:0}, anteiso-C_{13:0}, iso-C_{13:0} and iso-C_{15:0}. The DNA G+C content is 48.4–48.6 mol% (48.6 mol% for the type strain). Other phenotypic properties are given in Table 1.

The type strain, TF-16^T (= KCTC 19035^T = DSM 16306^T), was isolated from a tidal flat of Daepo Beach (Yellow Sea), near Mokpo City, Korea.

Description of *Exiguobacterium marinum* sp. nov.

Exiguobacterium marinum (ma.ri'num. L. neut. adj. *marinum* of the sea, marine).

Cells are Gram-variable and facultatively anaerobic; growth is better under aerobic conditions. Colonies are circular, glossy, raised and 3–4 mm in diameter after incubation for 2 days on MA at 30 °C under aerobic conditions. White-coloured colonies are formed under anaerobic conditions. Cells are short rods (in the early growth phase) or cocci (exponential and stationary growth phases). Growth occurs at 10 and 43 °C, with optimum growth at 30–37 °C; growth does not occur at 4 °C or above 44 °C. Growth does not occur in the presence of >17% NaCl. The optimal pH for growth is pH 6.5–8.5; growth is observed at pH 5.0 and 10.5, but not at pH 4.5 or 11.0. Hypoxanthine is not hydrolysed. Urease-negative. No acid is produced from D-sorbitol, *myo*-inositol, D-xylose, L-arabinose, D-melezitose, adonitol, L-rhamnose or lactose. The peptidoglycan type is

L-Lys–Gly. The predominant menaquinone is MK-7. Major polar lipids are phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine. The major fatty acids are iso-C_{17:0}, anteiso-C_{13:0}, iso-C_{13:0} and iso-C_{15:0}. The DNA G+C content of the type strain is 48.0 mol%. Other phenotypic properties are given in Table 1.

The type strain, TF-80^T (=KCTC 19036^T=DSM 16307^T), was isolated from a tidal flat of Daepo Beach (Yellow Sea), near Mokpo City, Korea.

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