Flavobacterium ponti sp. nov., isolated from seawater

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A Gram-stain-negative, non-flagellated, non-gliding, yellow-pigmented and rod-shaped bacterial strain, designated GSW-R14^T, was isolated from seawater of Geoje Island in the South Sea, Korea. Strain GSW-R14^T grew optimally at 25 °C, at pH 7.0–8.0 and in the presence of 2% (w/v) NaCl. Phylogenetic analyses based on 16S rRNA gene sequences showed that strain GSW-R14^T belonged to the genus *Flavobacterium*, joining *Flavobacterium gelidilacus* LMG 21477^T by a bootstrap resampling value of 100%. Strain GSW-R14^T exhibited 97.6% 16S rRNA gene sequence similarity to *F. gelidilacus* LMG 21477^T and similarities of 91.2–95.2% to other members of the genus *Flavobacterium*. Strain GSW-R14^T contained MK-6 as the predominant menaquinone. The fatty acid profile of strain GSW-R14^T was similar to that of *F. gelidilacus* LMG 21477^T. The DNA G+C content of strain GSW-R14^T was 31.4 mol% and its DNA–DNA relatedness with *F. gelidilacus* LMG 21477^T was 31%. Strain GSW-R14^T could be distinguished from *F. gelidilacus* and the other species of the genus *Flavobacterium* by its phylogenetic and genetic distinctiveness and by several phenotypic properties. On the basis of these data, strain GSW-R14^T is considered to represent a novel species of the genus *Flavobacterium*, for which the name *Flavobacterium ponti* sp. nov. is proposed; the type strain is GSW-R14^T (=KCTC 22802^T =CCUG 58402^T).

The genus *Flavobacterium* was created by Bergey *et al.* (1923). Phylogenetically, it constitutes a distinct evolutionary lineage within the family *Flavobacteriaceae* of the phylum 'Bacteroidetes' (Bernardet & Nakagawa, 2006). Recently, continuous descriptions of novel species, e.g. eight species in 2007, four species in 2008 and eight species in 2009, have increased considerably the number of species belonging to the genus *Flavobacterium*. At the time of writing, the genus *Flavobacterium* comprised at least 62 species with validly published names (Euzéby, 2009; <http://www.bacterio.cict.fr>). In this study, a bacterial strain, GSW-R14^T, which was isolated from seawater of Geoje Island in the South Sea, Korea, is described. Comparative 16S rRNA gene sequence analysis indicated that strain GSW-R14^T is a member of the genus *Flavobacterium*. The aim of the present work was to determine the exact taxonomic position of strain GSW-R14^T by using a polyphasic characterization that included the determination of phenotypic properties, a

detailed phylogenetic investigation based on 16S rRNA gene sequences and genetic analysis.

The standard dilution plating technique was used for the isolation of bacterial strains from seawater samples. Strain GSW-R14^T was isolated on marine agar 2216 (MA; Difco) at 25 °C and cultivated routinely under these isolation conditions. For short-term preservation, serial transfer from agar slants to an appropriate medium was used and the agar slants were stored at 4 °C. For long-term preservation, the strain was preserved at –80 °C in a deep freeze or in liquid nitrogen as a suspension in 20% (w/v) glycerol. *Flavobacterium gelidilacus* LMG 21477^T was used as a reference strain for DNA–DNA hybridization, phenotypic characterization, and isoprenoid quinone and fatty acid analyses. Cell morphology was examined by using light microscopy (E600; Nikon) and transmission electron microscopy (CM-20; Philips). The presence of flagella was assessed by using transmission electron microscopy of cells from an exponentially growing culture. For this purpose, cells were negatively stained with 1% (w/v) phosphotungstic acid and the grids were examined after being

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain GSW-R14^T is GQ370387.

air-dried. Gliding motility was investigated as described by Bowman (2000). The Gram reaction was determined using the bioMérieux Gram staining kit according to the manufacturer's instructions. Growth at 4, 15, 20, 25, 30, 35, 37 and 40 °C was measured on MA. The pH range for growth was investigated in marine broth 2216 (MB; Difco) adjusted to pH 4.5–10.0 in increments of 0.5 pH units by using sodium acetate/acetic acid and Na₂CO₃ buffers. Growth in the absence of NaCl and in the presence of 0.5, 1.0, 2.0 and 3.0 % (w/v) NaCl was investigated in trypticase soy broth prepared according to the formula of the Difco medium except that NaCl was excluded and that 0.45 % (w/v) MgCl₂·6H₂O or 0.06 % (w/v) KCl was added. Growth in the presence of final concentrations of NaCl of 2.0–8.0 % (w/v) (in increments of 1.0 %) was investigated in MB. Growth under anaerobic conditions was investigated in a Forma anaerobic chamber on MA and on MA supplemented with 0.1 % (w/v) potassium nitrate, both of which had been prepared anaerobically under a nitrogen atmosphere. Catalase and oxidase activities were determined as described by Cowan & Steel (1965). Hydrolysis of casein, hypoxanthine, starch, xanthine, tyrosine, and Tweens 20, 40, 60 and 80 was performed on MA using the substrate concentrations described previously (Cowan & Steel, 1965). Nitrate reduction and hydrolysis of aesculin, gelatin and urea were performed as described by Lányi (1987) with the modification that artificial seawater (23.6 g NaCl, 0.64 g KCl, 4.53 g MgCl₂·6H₂O, 5.94 g MgSO₄·7H₂O and 1.3 g CaCl₂·2H₂O per litre of distilled water; Bruns *et al.*, 2001) was used for preparation of media. Degradation of CM-cellulose (Sigma) was investigated on MA containing 0.5 % CM-cellulose and detected according to the method of Teather & Wood (1982). DNase activity was examined by using DNase test agar with methyl green (Difco) with the modification that artificial seawater was used for preparation of the medium. The presence of flexirubin-type pigments was investigated as described by Reichenbach (1992). Acid production from carbohydrates was assessed as described by Leifson (1963). Susceptibility to antibiotics was tested on MA plates using antibiotic discs (BBL) containing the following: ampicillin (10 µg), carbenicillin (100 µg), cephalothin (30 µg), chloramphenicol (100 µg), gentamicin (30 µg), kanamycin (30 µg), lincomycin (15 µg), neomycin (30 µg), novobiocin (5 µg), oleandomycin (15 µg), penicillin G (20 U), polymyxin B (100 U), streptomycin (50 µg) and tetracycline (30 µg). Enzyme activities and additional biochemical tests were performed by using the API ZYM and API 20E systems (bioMérieux) incubated at 25 °C according to the instructions of the manufacturer. Morphological, cultural, physiological and biochemical properties of strain GSW-R14^T are given in the species description and in Table 1. Strain GSW-R14^T grew anaerobically on MA and on MA supplemented with nitrate. Some species of the genus *Flavobacterium* have been known to grow anaerobically through the reduction of nitrate, including *Flavobacterium denitrificans*, which has been reported to use nitrate as an electron acceptor (Horn *et al.*, 2005; Bernardet & Bowman, 2006).

Table 1. Differential phenotypic characteristics of strain GSW-R14^T and *Flavobacterium gelidilacus* LMG 21477^T

Strains: 1, *Flavobacterium ponti* sp. nov. GSW-R14^T; 2, *F. gelidilacus* LMG 21477^T. Data are from this study except where marked. +, Positive reaction; –, negative reaction; w, weakly positive reaction. Both strains are positive for: growth at 30 °C; hydrolysis of casein, gelatin, starch, and Tweens 20, 40, 60 and 80; acid production from D-mannose and maltose; catalase, oxidase, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase (weak for GSW-R14^T), cystine arylamidase (weak for both strains) and α-glucosidase (weak for LMG 21477^T) activities; and susceptibility to chloramphenicol, lincomycin and oleandomycin. Both strains are negative for: Gram staining, spore formation, gliding motility, production of flexirubin-type pigments and nitrate reduction [data for these characteristics for *F. gelidilacus* LMG 21477^T are from Van Trappen *et al.* (2003)]; hydrolysis of agar, aesculin, hypoxanthine and xanthine; production of acetoin, H₂S and indole; acid production from L-arabinose, cellobiose, D-galactose, D-fructose, myo-inositol, lactose, D-mannitol, melezitose, melibiose, raffinose, L-rhamnose, D-ribose, D-sorbitol, sucrose, trehalose and D-xylose; urease, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase, lipase (C14), α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase activities; and susceptibility to ampicillin, cephalothin, gentamicin, kanamycin, neomycin, penicillin G and polymyxin B.

Characteristic	1	2
Growth at 35 °C	+	–
Anaerobic growth with nitrate	+	–*
Hydrolysis of L-tyrosine	+	–
Enzyme activity (API ZYM):		
Trypsin	–	w
Acid phosphatase	–	w
Naphthol-AS-BI-phosphohydrolase	–	w
Acid production from D-glucose	+	–
Susceptibility to:		
Carbenicillin	+	w
Novobiocin	–	+
Streptomycin	+	w
Tetracycline	–	+

*Data from Van Trappen *et al.* (2003).

Cell biomass for DNA extraction and isoprenoid quinone analysis was obtained from cultures grown for 3 days in MB at 25 °C. Chromosomal DNA was extracted and purified according to the method described by Yoon *et al.* (1996), with the modification that RNase T1 was used in combination with RNase A to minimize contamination by RNA. The 16S rRNA gene amplification was performed using two universal primers as described previously (Yoon *et al.*, 1998) and PCR products were purified by using a QIAquick PCR purification kit (Qiagen). Sequencing of the amplified 16S rRNA gene and phylogenetic analysis were performed as described by Yoon *et al.* (2003). The

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic position of *Flavobacterium ponti* GSW-R14^T and other related taxa. Bootstrap values (expressed as percentages of 1000 replications) >70% are shown at branching points. Filled circles indicate that the corresponding nodes were also recovered in trees generated with the maximum-likelihood and maximum-parsimony algorithms. *Capnocytophaga granulosa* LMG 12119 (GenBank accession no. U41348) was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

almost-complete 16S rRNA gene sequence of strain GSW-R14^T determined in this study comprised 1438 bp, representing approximately 96 % of the *Escherichia coli* 16S rRNA gene sequence. In the phylogenetic tree reconstructed using the neighbour-joining algorithm, strain GSW-R14^T fell within the clade comprising species of the genus *Flavobacterium*, joining *F. gelidilacus* LMG 21477^T with a bootstrap confidence value of 100 % (Fig. 1). The relationship between strain GSW-R14^T and *F. gelidilacus* LMG 21477^T was also maintained in phylogenetic trees reconstructed using the maximum-likelihood and maximum-parsimony algorithms (Fig. 1). Strain GSW-R14^T exhibited 97.6 % 16S rRNA gene sequence similarity to *F. gelidilacus* LMG 21477^T and 91.2–95.2 % to the type strains of the other species of the genus *Flavobacterium*.

Isoprenoid quinones were analysed as described by Komagata & Suzuki (1987) using reversed-phase HPLC and a YMC ODS-A (250 × 4.6 mm) column. The predominant isoprenoid quinone detected in strain GSW-R14^T was MK-6, which is in line with all members of the family *Flavobacteriaceae* (Bernardet & Nakagawa, 2006). For cellular fatty acid analysis, cell masses of strain GSW-R14^T and *F. gelidilacus* LMG 21477^T were harvested from MA plates after cultivation for 3 days at 25 °C. Fatty acids were extracted and fatty acid methyl esters were prepared according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990). The cellular fatty acid profiles of strain GSW-R14^T and *F. gelidilacus* LMG 21477^T analysed in this study are compared in Table 2. The fatty acid profiles of the two strains were similar, although there were differences in the proportions of some fatty acids (Table 2). The DNA G + C content was determined by the method of Tamaoka & Komagata (1984) with the modification that DNA was hydrolysed using nuclease P1 (Sigma) and the resultant nucleotides were analysed by reversed-phase HPLC. The DNA G + C content of strain GSW-R14^T was 31.4 mol%, which is in the range of values determined for species of the genus *Flavobacterium* (Bernardet & Bowman, 2006) and similar to that (30.0–30.4 mol%) reported for *F. gelidilacus* (Van Trappen *et al.*, 2003).

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 at 45 °C with five replications for each sample. The highest and lowest values obtained for each sample were excluded and the means of the remaining three values were quoted as DNA–DNA relatedness values. The mean DNA–DNA relatedness value between strain GSW-R14^T and *F. gelidilacus* LMG 21477^T was 31 %, indicating that they belong to two different species (Wayne *et al.*, 1987). Strain GSW-R14^T can also be differentiated from *F. gelidilacus* LMG 21477^T by differences in several phenotypic characteristics, activity of several enzymes and susceptibility to several antibiotics (Table 1). Accordingly, the phylogenetic and genetic distinctiveness and differential phenotypic properties of

strain GSW-R14^T warrant its description as a representative of a novel species of the genus *Flavobacterium*, for which the name *Flavobacterium ponti* sp. nov. is proposed.

Description of *Flavobacterium ponti* sp. nov.

Flavobacterium ponti [pon'ti. L. gen. n. *ponti* (poet. for *maris*) of the sea, referring to the place that the type strain was isolated].

Cells are Gram-stain-negative, non-flagellated, non-gliding, non-endospore-forming and rod-shaped, 0.3–0.8 µm in diameter and 0.8–5.0 µm in length. Colonies on MA are circular with regular edges, convex, glistening, smooth, yellow in colour and 0.5–1.0 mm in diameter after incubation for 3 days at 25 °C. Flexirubin-type pigments are not produced. Anaerobic growth occurs on MA and on MA supplemented with nitrate. Optimal growth temperature is 25 °C; growth occurs at 10 and 35 °C, but not at 4 or 37 °C. Optimal pH for growth is between 7.0 and 8.0; growth occurs at pH 5.0, but not at pH 4.5. Growth occurs in the presence of 0–6 % (w/v) NaCl, with optimum growth in approximately 2.0 % (w/v) NaCl. Mg²⁺ ions are

Table 2. Cellular fatty acid composition (%) of strain GSW-R14^T and *Flavobacterium gelidilacus* LMG 21477^T

Strains: 1, *Flavobacterium ponti* sp. nov. GSW-R14^T; 2, *F. gelidilacus* LMG 21477^T. All data are from this study. Fatty acids representing <1.0 % in both strains were omitted. tr, Trace (<1.0 %).

Fatty acid	1	2
Straight-chain:		
C _{15:0}	12.9	13.2
Unsaturated:		
C _{15:1ω6c}	5.4	8.7
C _{17:1ω6c}	2.3	5.6
Branched:		
iso-C _{13:0}	1.1	tr
iso-C _{14:0}	1.0	2.6
iso-C _{15:0}	18.5	9.4
iso-C _{15:1}	12.2	7.2
anteiso-C _{15:0}	2.1	4.5
iso-C _{16:0}	1.8	6.3
iso-C _{16:1}	tr	3.4
iso-C _{17:1ω9c}	2.8	1.1
Hydroxy:		
C _{15:0} 3-OH	1.6	2.2
iso-C _{15:0} 3-OH	8.3	5.8
C _{16:0} 3-OH	tr	1.1
iso-C _{16:0} 3-OH	2.7	11.5
C _{17:0} 3-OH	tr	1.1
iso-C _{17:0} 3-OH	13.4	7.3
Summed feature 3*	5.0	2.2

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

required for growth. Catalase- and oxidase-positive. Nitrate is not reduced to nitrite. Casein, DNA, gelatin, starch, tyrosine, and Tweens 20, 40, 60 and 80 are hydrolysed, but aesculin, agar, CM-cellulose, hypoxanthine, urea and xanthine are not. Acetoin, H₂S and indole are not produced. The predominant menaquinone is MK-6. The major cellular fatty acids (>10 % of the total fatty acids) are iso-C_{15:0}, iso-C_{17:0}, 3-OH, C_{15:0} and iso-C_{15:1}. Additional phenotypic characteristics are given in Table 1.

The type strain is GSW-R14^T (=KCTC 22802^T =CCUG 58402^T), isolated from seawater of Geoje Island in the South Sea, Korea. The DNA G+C content of the type strain is 31.4 mol%.

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