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Costertonia aggregata gen. nov., sp. nov., a mesophilic marine bacterium of the family *Flavobacteriaceae*, isolated from a mature biofilm

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A marine bacterium, strain KOPRI 13342^T, was isolated from a mature marine biofilm, including various marine algae, covering a rock-bed of the East Sea, Korea (also known as the Sea of Japan). Colonies of the isolate were orange-coloured on marine agar 2216. The isolate showed relatively high 16S rRNA gene sequence similarities to members of the genera *Maribacter* (91·2–92·4 % similarity), *Zobellia* (90·7–91·5 %) and *Muricauda* (90·7–91·4 %). Phylogenetic analysis based on the nearly complete 16S rRNA gene sequence revealed that the isolate formed a phyletic lineage with members of the genus *Muricauda*. Cells were aerobic, motile, Gram-negative rods and they produced non-diffusible carotenoid pigments. Optimal growth was observed at pH 7·5–8·0 and 26–32 °C and required the presence of 3 % (w/v) sea salt. The strain required Ca^{2+} and K^+ ions in addition to NaCl for growth. The dominant fatty acids were i-15 : 0, i-15 : 1ω10, 15 : 0 and 16 : 1ω9. The major respiratory quinone was MK-6. The DNA G + C content was 35·8 mol%. On the basis of this polyphasic taxonomic evidence, strain KOPRI 13342^T should be classified as a representative of a novel species in a new genus in the family *Flavobacteriaceae*; the name *Costertonia aggregata* gen. nov., sp. nov. is proposed. The type strain of *Costertonia aggregata* is KOPRI 13342^T (=KCCM 42265^T=JCM 13411^T).

Bacteria in nature often exist as sessile multispecies communities called biofilms (Costerton *et al.*, 1995). In oligotrophic marine environments, bacterial colonization on surfaces is regarded as a microbial survival strategy that provides micro-organisms with important advantages, including increased access to nutrients, protection against toxins and antibiotics and retention of signal molecules (Jefferson, 2004; Pasmore & Costerton, 2003). Direct observations of a wide variety of natural ecosystems have established that the vast majority of bacteria in most aquatic environments grow within matrix-enclosed biofilms (Costerton *et al.*, 1994). Therefore, biofilms could serve as a source of diverse micro-organisms and many novel bacterial strains have been reported from marine biofilm matrices including algal surfaces (Gillan *et al.*, 1998; Golyshin *et al.*, 2002; Matsuo *et al.*, 2003; Patel *et al.*, 2003; Nedashkovskaya *et al.*, 2004a, b, 2005; Bowman & Nichols, 2005; Lau *et al.*, 2004). Of these, up to 30 % could be affiliated with the phylum *Bacteroidetes* (Webster *et al.*, 2004). We have also isolated many bacteria belonging to the family *Flavobacteriaceae* from mature biofilms; taxonomic analysis of a novel strain, KOPRI 13342^T, is described herein.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain KOPRI 13342^T is DQ167246.

Approximately 10 cm^3 biofilm consisting of diverse algal species on a rock-bed was harvested using a razor blade and dispersed in 30 ml sterilized seawater. The dispersed biofilm was spread on marine agar 2216 (MA; Difco) after serial dilution with sterilized seawater and cultivated at 25 °C for a week. Among the distinct colonies that grew on MA, a tiny, orange-coloured colony was isolated, strain KOPRI 13342^T, and preserved in 20 % glycerol solution at −80 °C. The isolate was further cultivated on MA for morphological and biochemical characterization.

Unless otherwise stated, methods used for physiological and morphological characterization were as described previously (Sohn *et al.*, 2004b; Kwon *et al.*, 2005). The degradation of starch and casein by strain KOPRI 13342^T was tested according to Smibert & Krieg (1994). Physiological, biochemical and morphological characteristics of strain KOPRI 13342^T are given in the genus and species descriptions and in Table 1.

NaCl, Mg^{2+} and/or Ca^{2+} requirements were tested according to Sohn *et al.* (2004a). However, no growth was observed in the presence of NaCl alone or in the presence of Mg^{2+} and Ca^{2+} ions, so other components found in seawater were tested. Tested elements and their concentrations were described by Parsons *et al.* (1984). Combinations of four

Table 1. Phenotypic characteristics that differentiate strain KOPRI 13342^T from closely related members of the family *Flavobacteriaceae*

Strains/genera: 1, KOPRI 13342^T; 2, *Muricauda*; 3, *Maribacter*; 4, *Zobellia*; 5, *Arenibacter* (characteristics for all strains of these genera are shown); 6, *Pibocella ponti* KMM 6031^T; 7, *Robiginitalea biformata* HTCC2501^T. Data for reference taxa were taken from Barbeyron *et al.* (2001), Bruns *et al.* (2001), Ivanova *et al.* (2001), Cho & Giovannoni (2004), Nedashkovskaya *et al.* (2003, 2004a, b, c, 2005) and Yoon *et al.* (2005a, b). All taxa are positive for catalase, require oxygen for growth and have MK-6 as major respiratory quinone; none of the strains requires specific growth factors or produces indole. ND, Not determined; V, variable.

Characteristic	1	2	3	4	5	6	7
Cell size (μm)	0·35–0·41 × 0·50–4·25	0·2–0·6 × 1·1–6·0	0·4–0·5 × 2·0–10·0	0·3–0·5 × 1·2–8·0	0·4–0·7 × 3·0–5·0	0·4–0·5 × 0·6–2·3	0·3–0·7 × 1·6–5·6
Gliding motility	−	+/ND	+	+	−	+	−
Tolerance of:*							
Temperature (°C)	10–35 (26–32)	8–44 (20–30, 30–37)†	4–33 (21–24)	4–45 (21–35)	4–42 (28–30)	4–33 (21–24)	10–44 (30)
pH	6·5–9·0 (7·5–8·0)	6·0–8·0 (6·5–7·5)	5·5–10·0 (7·5–8·5)	ND	5·5–10 (7·5–8·5)	ND	6–9 (8–8·5)
Salt concentration (%)	1·5–12·0 (3·0)	0·5–9·0 (2·0–3·0)	1·0–7·0 (1·5–2·0)	0·5–10·0 (2·0–3·0)	1–10	1–13	0·25–10 (2·5)
Seawater requirement‡	+	−	−	−	−	−	−
Oxidase activity	+	V	+	+	+	+	+
Nitrate reduction	+	−	V	+	+	−	−
Production of:							
Urease	−	−	−	−	V	−	−
H_2S	−	−	−	−	V	−	−
Acid from carbohydrate	−	V	V	V	V	+	−
Hydrolysis of:							
Agar	−	−	V	+	−	−	ND
Casein	−	−	−	−	−	+	−
Gelatin	+	−	V	+	V	+	−
Starch	−	−	V	V	−	+	+
Major fatty acids§							
i-15 : 0	39·7	14·7–23·8	10·6–20·5	16·8–22·5	8·5–17·3	8·7	24–28
i-15 : 1ω10	22·4	19·5–21·6	10·1–18·9	8·8–14·9	14·3–19·3	11·7	14–21
a-15 : 0					6·6–8·6	5·4	3–4
15 : 0	7·8	5·1–13·2	3·5–14·5	7·5–14·4	13·3–29·0	4·2	5–6
i-16 : 0						12·1	
i-16 : 1						6·2	
16 : 1ω9	4·6				<5–11·0		
i-17 : 1			2·0–4·0	2·4–5·1		5·2	
i-15 : 0 3-OH		4·6–5·5	2·9–5·4	4·6–8·3			4·3
i-16 : 0 3-OH		1·7–4·6				5·9	
i-17 : 0 3-OH		17·3–20·9	11·6–29·2	15·1–25·9	<5–6·1	5·6	25–27
Summed feature 3‖		2·3–4·2	5·8–12·9	9·9–15·5		11·4	
Unknown		7·0–8·8	2·7–10·3			5·5	
DNA G+C content (mol%)	35·8	41–45·4	35–39	36–43	37·5–40	35·5	55–56

*Ranges with optima shown in parentheses.

†The genus *Muricauda* contains two different groups with different optimal growth temperature ranges.

‡A seawater requirement indicates that Na^+ alone does not support growth and additional cations that are present in seawater, such as Mg^{2+}, Ca^{2+} and/or K^+, are required for growth.

§Fatty acids present at less than 3 % of the total fatty acids in all strains were not included.

‖Contains 16 : 1ω7*c* and/or i-15 : 0 2-OH.

major components ($CaCl_2.2H_2O$, KCl, $MgCl_2.6H_2O$ and Na_2SO_4) and a mixture of five trace components (H_3BO_3, KBr, Na_2CO_3, NaF and $SrCl_2.6H_2O$) were supplied to the DW substituted (including 3 % NaCl) ZoBell 2216e medium. Growth was observed in the presence of Ca^{2+} and K^+ ions in addition to NaCl.

The profile of cellular fatty acid methyl esters was determined according to Sohn *et al.* (2004b). The dominant fatty acids of KOPRI 13342^T were i-15 : 0 (39·7 %), i-15 : 1ω10 (22·4 %), 15 : 0 (7·8 %) and 16 : 1ω9 (4·6 %). The strain also contained small amounts of 16 : 0 (2·7 %), C_{18} polyunsaturated fatty acids (2·5 %), 18 : 0 (2·2 %), 13 : 0 (2·0 %), 10-methyl 16 : 0 (1·6 %), 18 : 1ω9 (1·5 %) and 14 : 0 (1·2 %). The isolate contained a relatively large amount of i-15 : 0 compared with that found in members of closely related genera in the family *Flavobacteriaceae* (Table 1).

The major respiratory quinone was determined to be menaquinone by the reverse-phase-TLC method described by Kim *et al.* (2000) and confirmed to be MK-6 by HPLC analysis according to the method described by Collins (1985). The DNA G+C content was 35·8 mol%, as determined by the thermal denaturation method (Kim *et al.*, 2000).

Genomic DNA extraction and amplification and sequencing of the 16S rRNA gene were carried out according to Sohn *et al.* (2004b). A phylogenetic tree including strain KOPRI 13342^T and members of closely related genera was generated based on the maximum-likelihood distance model and the neighbour-joining method. 16S rRNA gene sequences of *Bacteroides fragilis* ATCC 25285^T (GenBank accession no. NC_003228) and *Sphingobacterium spiritivorum* DSM 2582^T (AJ459411) served as outgroups. A total of 1331 unambiguously aligned positions was compared. The closest neighbour was *Maribacter dokdonensis* DSW-8^T (92·4 % sequence similarity), followed by *Maribacter sedimenticola* KMM 3903^T (92·1 %) and *Maribacter orientalis* KMM 3947^T (92·0 %). Members of the genera *Zobellia* and *Muricauda* showed a similar range of similarities (90·7–91·5 and 90·7–91·4 %, respectively) to strain KOPRI 13342^T. Phylogenetic analysis of 16S rRNA gene sequences from organisms with validly published names revealed that strain KOPRI 13342^T shared a phyletic line with members of the genus *Muricauda*. This small clade lies within a larger clade containing the genera *Arenibacter*, *Maribacter*, *Pibocella* and *Zobellia* (Fig. 1).

Strain KOPRI 13342^T shared many characteristics, including major respiratory quinone type, oxygen requirement and temperature range, pH and salt concentration for growth, with closely related members of the family *Flavobacteriaceae*. However, the strain required an additional seawater component in addition to NaCl (Table 1) and contained a larger amount of i-15 : 0 fatty acid, enabling it to be differentiated from other members of the family *Flavobacteriaceae*. Hence, it is proposed that strain KOPRI 13342^T should be identified as a representative of a novel species in a new genus in the family *Flavobacteriaceae*, *Costertonia aggregata* gen. nov., sp. nov.

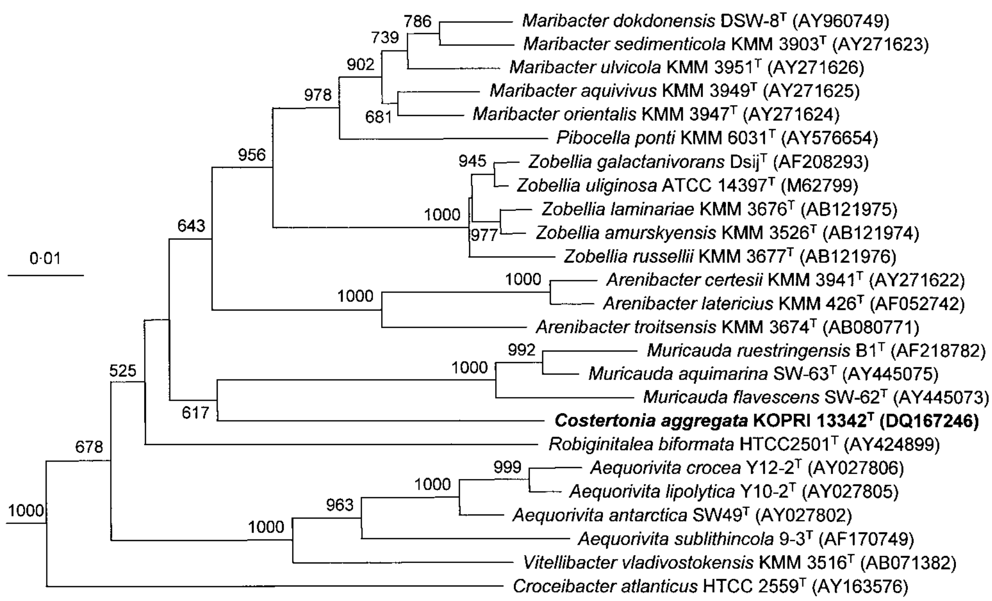

Fig. 1. Phylogenetic tree based on nearly complete 16S rRNA gene sequences (1321 unambiguously aligned base pairs) showing the relationship between strain KOPRI 13342^T and other members of the family *Flavobacteriaceae*. The tree is based on the maximum-likelihood distances model and the neighbour-joining method. Bootstrap values >50 % of 1000 resampled are shown. Bar, 0·01 substitutions per nucleotide position.

Description of *Costertonia* gen. nov.

Costertonia (Cos.ter.ton′i.a. N.L. fem. n. *Costertonia* honouring J. W. Costerton, a famous American biofilm microbiologist).

Cells are aerobic, motile, Gram-negative rods. Gliding motility is absent. Orange-coloured colonies form on MA. Produce non-diffusible carotenoid pigments, but flexirubin-type pigments are absent. The major respiratory quinone is MK-6. The major cellular fatty acids are i-C15 : 0, i-C15 : 1 and 15 : 0. Oxidase- and catalase-positive. As determined by 16S rRNA gene sequence analysis, the genus *Costertonia* is a member of the family *Flavobacteriaceae*, phylum *Bacteroidetes*. The type species is *Costertonia aggregata*.

Description of *Costertonia aggregata* sp. nov.

Costertonia aggregata (ag.gre.ga′ta. L. fem. adj. *aggregata* joined together, referring to the formation of aggregates during cultivation in liquid medium).

Cells are 0·50–0·57 μm in length and 0·35–0·41 μm in diameter. However, rods can sometimes be longer than 4 μm. Properties are as described for the genus in addition to the following. Growth occurs at 10–35 °C, pH 6·5–9·0 and with 1·5–12·0 % sea salts. Cells form irregular aggregates during growth in liquid medium. Obligately requires NaCl, Ca^{2+} and K^{+} for growth. Optimal growth is observed at pH 7·5–8·0 and 26–32 °C and requires the presence of 3 % (w/v) sea salts. Reduces nitrate to nitrogen gas in API 20 E test strip. Positive for β-glucosidase, β-galactosidase, urease, arginine dihydrolase and protease. Degrades dextrin, glycogen, Tweens 40 and 80, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, adonitol, L-arabinose, D-arabitol, cellobiose, L-erythritol, D-fructose, L-fucose, D-galactose, gentiobiose, α-D-glucose, *myo*-inositol, α-D-lactose, lactulose, maltose, D-mannitol, D-mannose, D-melibiose, methyl β-D-glucoside, D-raffinose, L-rhamnose, D-sorbitol, sucrose, D-trehalose, turanose, xylitol, methyl pyruvate, acetic acid, *cis*-aconitic acid, citric acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucuronic acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, α-ketoglutaric acid, α-ketovaleric acid, DL-lactic acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, succinic acid, bromosuccinic acid, glucuronamide, alaninamide, D-alanine, L-alanine, L-alanyl–glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-proline, L-pyroglutamic acid, D-serine, L-serine, L-threonine, γ-aminobutyric acid, urocanic acid, inosine, uridine, phenylethylamine, putrescine, 2-aminoethanol, glycerol, DL-α-glycerol phosphate, glucose 1-phosphate and glucose 6-phosphate as sole carbon sources. The dominant fatty acids are i-15 : 0 (39·7 %), i-15 : 1ω10 (22·4 %), 15 : 0 (7·8 %) and 16 : 1ω9 (4·6 %).

The type strain is KOPRI 13342^{T} (=KCCM 42265^{T}=JCM 13411^{T}), isolated from a mature marine biofilm formed on a rock-bed in Jungdongjin, Korea. The DNA G+C content of the type strain is 35·8 mol%.

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