Croceitalea litorea sp. nov., isolated from seashore sand

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Strain CBA3205^T is a Gram-stain-negative, non-motile and rod-shaped bacterium that was isolated from the seashore sand of Jeju Island in South Korea. Based on the phylogenetic analysis, the most closed related species was *Croceitalea eckloniae* DOKDO 025^T, with 94.8 % sequence similarity for the 16S rRNA gene. Strain CBA3205^T was observed to grow optimally at 25–30 °C and at pH 8.5 in the presence of 2–3 % (w/v) NaCl. The major fatty acids of strain CBA3205^T were iso-$C_{15:0}$, iso-$C_{15:1}$ G, and iso-$C_{17:0}$ 3-OH. The major respiratory quinone was MK-6 and the major polar lipids were two unidentified amino-group-containing phospholipids and an unidentified polar lipid. The G+C content of the genomic DNA of strain CBA3205^T was 62.5 mol%. Based on the phenotypic, genotypic and phylogenetic analyses, strain CBA3205^T was considered to be a novel species belonging to the genus *Croceitalea* within the family *Flavobacteriaceae*, for which the name *Croceitalea litorea* sp. nov. is proposed. The type strain is CBA3205^T (=KACC 17669^T=JCM 19531^T).

Species of the genus *Croceitalea* were isolated from the rhizosphere of the marine alga *Ecklonia kurome* (Lee *et al.*, 2008). The genus was included in the family *Flavobacteriaceae*, which is one of the main bacterial lineages of the phylum *Bacteroidetes* (Garrity & Holt, 2001; Bernardet & Nakagawa, 2006). The genus *Croceitalea* comprises Gram-negative, rod-shaped bacteria with yellow or orange colonies. The major respiratory quinone is MK-6 and the major fatty acids of the genus *Croceitalea* are iso-$C_{15:1}$ G and iso-$C_{15:0}$ (Lee *et al.*, 2008). The genomic G+C content ranges from 60–67 mol%. In this study, we found a novel strain, CBA3205^T, which was similar to species of the genus *Croceitalea*; it originated from the seashore sand of Jeju Island in South Korea. We investigated the exact taxonomic position by using a polyphasic approach that included the determination of phenotypic properties and a detailed phylogenetic investigation based on the 16S rRNA gene sequences. A novel species of the genus *Croceitalea* is proposed.

The seashore sand sample was collected from Jeju Island, Republic of Korea as described previously by Roh *et al.* (2013). The sample was serially diluted in marine broth (Difco) and aliquots were plated onto marine agar (MA) plates with 1.5 % (w/v) agar followed by incubation at 25 °C for 1 week. To obtain a pure colony, colonies were successively restreaked onto MA plates and the pure strains isolated were stored in 40 % (v/v) glycerol at −80 °C. Gram-staining was performed with a Gram-staining kit (bioMérieux) according to the manufacturer's instructions; cell morphology and size were determined with a phase-contrast microscope (Nikon Eclipse 80*i*). Motility was investigated on 0.5 % (w/v) semi-solid MA (Tittsler & Sandholzer, 1936) and gliding motility was assessed by the microscopic hanging drop technique (Agarwal *et al.*, 1997; Bernardet *et al.*, 2002). Growth at different temperatures was evaluated on MA at 5–50 °C with 5 °C intervals (with the addition of 37 °C) in the presence of 2 % (w/v) NaCl. Growth in the presence of various NaCl concentrations (0, 1, 2, 3, 4, 6, 8 and 10 %, w/v) was performed in MA with NaCl removed at 30 °C. The pH range for growth was determined from pH 5.0–10.5 with intervals

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One supplementary figure is available with the online Supplementary Material.

of 0.5 pH unit. Different biological buffers were used to adjust the pH: 10 mM 2-(*N*-morpholino) ethanesulphonic acid (pH 5.0–6.5), 10 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (pH 7.0–8.5), and 10 mM N-[tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid (pH 9.0–10.5). To observe growth under anaerobic conditions, strain CBA3205^T was incubated for 7 days on MA at 25 °C in an anaerobic chamber with a $N_2/CO_2/H_2$ (90 : 5:5, by vol.) atmosphere. The production of flexirubin-type pigments was assessed with a 20 % (w/v) KOH solution (Reichenbach, 1989; Bernard et *al.*, 2002).

Strain CBA3205^T formed smooth, opaque, circular colonies with yellow pigments and comprised Gram-stain-negative and rod-shaped (0.2–0.3 × 0.8–2.0 μm) cells with no gliding motility. Strain CBA3205^T grew in the presence of 2–5 % (w/v) NaCl, (optimum 2–3 %), at 15–37 °C (optimum 25–30 °C) and at pH 5.0–10.5 (optimum pH 8.5).

The biochemical analysis of strain CBA3205^T was performed after cultivation at 25 °C for 48 h on plates with MA as the basal medium. Oxidase and catalase activities were determined by indophenol blue production using 1 % (w/v) tetramethyl-*p*-phenylenediamine (Sigma) and bubble production in a 3 % (v/v) hydrogen peroxide solution, respectively. The tests for the hydrolysis of starch, gelatin, and Tweens 20, 40 and 80 were performed as described by Smibert & Krieg (1994) and González *et al.* (1978), respectively. The hydrolysis of L-tyrosine was tested as described by Roh *et al.* (2013). Enzymic activities and carbon source assimilation were determined utilizing API 20NE, API ZYM and API 50CH test kits (bioMérieux) according to the manufacturer's instructions.

Strain CBA3205^T was positive for catalase activity, but negative for oxidase and did not hydrolyse gelatin, starch, L-tyrosine or Tweens 20, 40 and 80. In the API 20NE gallery, strain CBA3205^T did not reduce nitrate or nitrite and was negative for indole production from tryptophan, urease, arginine dihydrolase, gelatinase, β-glucosidase and β-galactosidase activity. In the API ZYM panel for

Table 1. Differential characteristics of strain CBA3205^T and its closest phylogenetic relatives

Strains: 1, CBA3205^T; 2, *C. eckloniae* DOKDO 025^T; 3, *C. dokdonensis* DOKDO 023^T. Data from this study and Lee *et al.* (2008). +, Positive; −, negative; w, weakly positive.

Characteristic	1	2	3
Isolation source	Seashore sand	Marine alga	Marine alga
Cell size (μm)	0.2–0.3 × 0.8–2.0	0.3–0.5 × 1.0–2.8	0.4–0.6 × 1.4–3.1
Growth temperature range (optimum) (°C)	15–37 (25–30)	10–34 (29)	12–38 (35)
pH range for growth (optimum)	5–10.5 (8.5)	6.5–10 (8)	7–10 (8.5–9)
NaCl concn range for growth (optimum) (%, w/v)	2–5 (2–3)	0.4–5.4 (1.6)	0.8–5.4 (3.1)
Hydrolysis of gelatin	−	+	−
Enzyme activities:			
β-Glucosidase	−	−	+
β-Galactosidase	−	−	+
Assimilation of:			
D-Ribose	−	+	−
D-Galactose	+	+	−
Glycerol	+	−	−
L-Rhamnose	−	−	w
Gluconate	−	−	w
Methyl-α-D-glucoside	−	−	+
N-Acetylglucosamine	−	+	−
Amygdalin	−	w	−
Aesculin	w	+	−
Salicin	−	+	−
D-Cellobiose	−	+	+
D-Melibiose	−	+	+
D-Mannitol	+	w	−
D-Sucrose	−	+	+
D-Raffinose	−	−	+
Starch	+	−	−
Glycogen	−	+	w
Gentiobiose	−	−	+
D-Turanose	−	−	+
DNA G+C content (mol%)	62.5	59.5	66.5

enzyme activity, strain CBA3205^T had positive results for alkaline phosphatase, leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-chymotrypsin, α-glucosidase, esterase lipase (C8), valine arylamidase, and *N*-acetyl-β-glucosaminidase. In carbon source assimilation tests in the API 50CH test kit, strain CBA3205^T produced acid from D-glucose, glycerol, D-fructose, D-maltose, D-trehalose, D-mannose, D-galactose, D-mannitol, D-lactose and starch. The biochemical characteristics distinguishing strain CBA3205^T from the reference species are shown in Table 1.

The genomic DNA of strain CBA3205^T was extracted with a genomic DNA extraction kit (RBC) by following the manufacturer's instructions and the 16S rRNA gene was amplified as previously described (Roh *et al.*, 2008) using the AccPower PCR PreMix (Bioneer) and universal 16S rRNA gene primer set 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The sequence fragments of the 16S rRNA gene were assembled using the SeqMan software program (DNASTAR). Comparisons of the gene sequences to identify the nearest related species and to calculate the pairwise gene sequence similarities were performed with the EzTaxon-e server (*http://eztaxon-e.ezbiocloud.net/*) (Kim *et al.*, 2012). The 16S rRNA gene sequence alignments for strains CBA3205^T and related species were performed using the SILVA Incremental Aligner (Pruesse *et al.*, 2012). The phylogenetic tree based on the aligned 16S rRNA gene

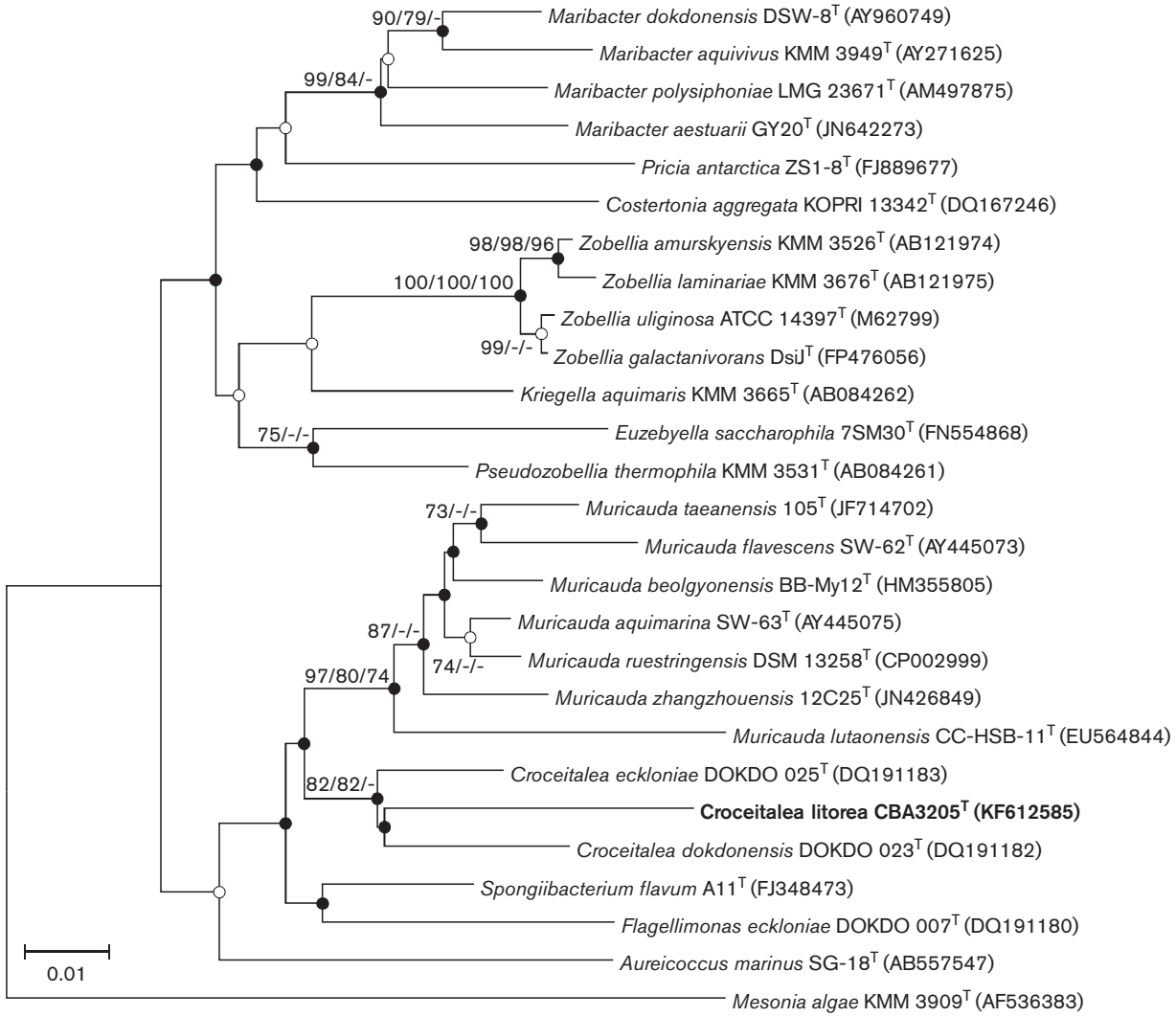

Fig. 1. Phylogenetic tree based on the 16S rRNA gene sequences showing the taxonomic position of strain CBA3205^T. The numbers at the nodes indicate the bootstrap values (>70 %) calculated using the neighbour-joining, maximum-likelihood (ML) and maximum-parsimony (MP) probabilities. The closed circles represent nodes recovered by both the MP and ML algorithms, while the open circles indicate nodes recovered with one of the algorithms. *Mesonia algae* KMM 3909^T served as an outgroup. Bar, 0.01 substitutions per nucleotide position.

sequences was reconstructed using MEGA 5 software (Tamura *et al.*, 2011) with the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge & Farris, 1969) algorithms. A bootstrap analysis was performed to obtain a consensus tree based on 1000 randomly generated trees.

The 16S rRNA gene sequence obtained for strain CBA3205^T was 1430 bp. A phylogenetic analysis revealed that it formed a distinct lineage within the family *Flavobacteriaceae*, belonging to a cluster containing the genera *Croceitalea*, *Aureicoccus*, *Muricauda*, *Flagellimonas* and *Spongiibacterium*. Strain CBA3205^T belonged to the genus *Croceitalea* (Fig. 1). The 16S rRNA gene sequence of strain CBA3205^T was most similar to *Croceitalea eckloniae* DOKDO 025^T and *Croceitalea dokdonensis* DOKDO 023^T with 94.8 % and 93.9 % identity, respectively.

The genomic DNA G+C content was determined by HPLC (Dionex UltiMate 3000), as described by Mesbah & Whitman (1989). For fatty acid analysis, cells were cultured on MA at 30 °C for 2 days. The cellular fatty acid composition was determined with the saponification, methylation and extraction steps described in the 'Sherlock Microbial Identification System' (MIDI, 1999) and detected by gas chromatography (Hewlett Packard 6890). The fatty acids were identified using the Microbial Identification software package (Sasser, 1990) based on the TSBA6 library. The isoprenoid quinones were extracted from freeze-dried cells with chloroform/methanol (2 : 1, v/v) (Collins & Jones, 1981a), purified with one-dimensional TLC on a silica gel 60 F_{254} plate (Merck) and identified by HPLC (UltiMate 3000; Dionex) (Collins & Jones, 1981b), coupled to a diode-array detector and a single quadrupole mass spectrometer (HCT Ion-Trap MS; Bruker). The polar lipid analysis was performed using two-dimensional TLC on silica gel 60 F_{254} plates, as described previously (Minnikin *et al.*, 1984).

The genomic DNA G+C content of strain CBA3205^T was 62.5 mol%, which is in the range of other species of the genus *Croceitalea*. The major fatty acids (>10 % of total fatty acids) of strain CBA3205^T were iso-$C_{15:0}$ (24.3 %), iso-$C_{15:1}$ G (19.8 %) and iso-$C_{17:0}$ 3-OH (26.1 %). The majority of the cellular fatty acid profile of the isolate was similar to those of the reference species, except for iso-$C_{14:0}$ (Table 2). The major isoprenoid quinone in strain CBA3205^T, as well as in the reference species, was MK-6. The polar lipid profile of strain CBA3205^T was similar to those of the reference species in showing two unidentified amino-group-containing phospholipids and an unidentified polar lipid (Fig. S1, available in the online Supplementary Material).

In conclusion, based on the 16S rRNA gene sequence similarities, strain CBA3205^T was most closely related to members of the genus *Croceitalea* in the family *Flavobacteriaceae*, and it is distinguishable from *Croceitalea eckloniae* DOKDO 025^T and *Croceitalea dokdonensis* DOKDO 023^T based on phylogenetic, genomic, phenotypic and chemotaxonomic analyses. Thus, strain CBA3205^T represents a novel species, for which the name *Croceitalea litorea* sp. nov. is proposed.

Table 2. Fatty acid composition of strain CBA3205^T and related species

Strains: 1, CBA3205^T; 2, *C. eckloniae* DOKDO 025^T; 3, *C. dokdonensis* DOKDO 023^T. All data are from this study. Values are percentages of the total fatty acids. Strains were cultivated under the same conditions. Fatty acids amounting to <1 % in all strains were omitted. TR, Trace amounts (<1.0 %); –, not detected.

Fatty acid	1	2	3
Straight-chain			
$C_{16:0}$	1.1	1.1	1.4
Branched			
iso-$C_{14:0}$	3.6	–	–
iso-$C_{15:0}$	24.3	20.1	28.6
Unsaturated			
$C_{15:1}$ $\omega6c$	1.4	1.4	2.5
iso-$C_{15:1}$ G	19.8	24.5	22.7
Hydroxy			
$C_{15:0}$ 3-OH	2.1	2.0	3.2
$C_{16:0}$ 3-OH	1.6	2.0	1.8
$C_{17:0}$ 3-OH	TR	1.2	TR
iso-$C_{15:0}$ 3-OH	6.6	5.5	7.4
iso-$C_{16:0}$ 3-OH	2.3	1.8	1.7
iso-$C_{17:0}$ 3-OH	26.1	26.9	21.3
Summed features*			
3	3.6	7.8	3.4
9	1.4	1.4	1.1

*A summed feature represents fatty acids that could not be separated by gas-liquid chromatography with the MIDI system. Summed feature 3 and 9 contain $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$ and 10-methyl $C_{16:0}$ and/or iso-$C_{17:1}\omega9c$, respectively.

Description of *Croceitalea litorea* sp. nov.

Croceitalea litorea (li.to're.a. L. fem. Adj. *litorea* inhabiting seashore).

Cells are non-motile, Gram-stain-negative, aerobic and rod-shaped, 0.2−0.3 μm wide and 0.8−2.0 μm long. Colonies are smooth, opaque, circular and raised with yellow pigmentation growing to a diameter of 1.0−2.0 mm after cultivation at 25 °C for 3 days on MA. Growth occurs at 15−37 °C, at pH 5.0−10.5 and in the presence of 2−5 % (w/v) NaCl, with optimal growth at 25−30 °C, pH 8.5 and with 2−3 % (w/v) NaCl present. Positive for catalase, but not for oxidase activity. Gelatin, starch, L-tyrosine, Tween 20, Tween 40 and Tween 80 are not hydrolysed. On the API 20NE gallery, positive for D-glucose, D-mannose, D-mannitol, D-maltose, capric acid, adipic acid, malic acid, trisodium citrate and phenylacetic acid. Cells produce acid from D-glucose, glycerol, D-fructose, D-maltose, D-trehalose, D-mannose, D-galactose, D-mannitol, D-lactose and starch.

The remaining utilization tests are negative. Aesculin, 5-ketogluconate and 5-ketogluconate are weakly assimilated. The API ZYM tests show positive results for alkaline phosphatase, leucine arylamidase, esterase lipase (C8), valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α–chymotrypsin, α–glucosidase and *N*-acetyl-β-glucosaminidase, but negative results for esterase (C4), lipase (C14), cysteine arylamidase, trypsin, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, α-mannosidase and α–fucosidase. The major fatty acids are iso-$C_{15:0}$, iso-$C_{15:1}$ G and iso-$C_{17:0}$ 3-OH. The major isoprenoid quinone is MK-6 and the major polar lipids are two unidentified amino-group-containing phospholipids and an unidentified polar lipid.

The type strain is CBA3205^T (=KACC 17669^T=JCM 19531^T), which was isolated from the seashore sand of Jeju Island in South Korea. The genomic DNA G+C content of the type strain is 62.5 mol%.

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