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Tamlana crocina gen. nov., sp. nov., a marine bacterium of the family *Flavobacteriaceae*, isolated from beach sediment in Korea

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A Gram-negative, aerobic, saffron-coloured marine bacterium, designated HST1-43^T, was isolated from beach sediment on the coast in Jeju, Korea, and its taxonomic status was established in a polyphasic study. 16S rRNA gene sequence analyses revealed that the isolate belonged to the family *Flavobacteriaceae*. The closest phylogenetic neighbours of strain HST1-43^T were members of the genera *Algibacter*, *Gaetbulibacter* and *Yeosuana*, with levels of sequence similarity in the range 96.3–96.5 %. The isolate was non-motile and required sea salts or natural seawater for growth. The optimum temperature and pH ranges for growth were 25–30 °C and pH 6.1–8.1, respectively. MK-6 was the major menaquinone. The dominant cellular fatty acids were iso-$C_{15:0}$, $C_{18:0}$, $C_{16:0}$, iso-$C_{15:1}$ and iso-$C_{17:0}$ 3-OH. The DNA G+C content was 36.2 ± 0.4 mol%. On the basis of phylogenetic distance and phenotypic characteristics, the isolate is considered to represent a novel genus and species in the family *Flavobacteriaceae*, for which the name *Tamlana crocina* gen. nov., sp. nov. is proposed. The type strain is HST1-43^T (=KCTC 12721^T=JCM 14021^T).

During investigations of the biodiversity of marine bacteria, a novel Gram-negative bacterium with an obligate requirement for seawater was isolated from a sediment sample collected at Hwasun beach in Jeju, Republic of Korea. A 16S rRNA gene sequence analysis showed that the organism belonged to the family *Flavobacteriaceae* and was phylogenetically related to *Gaetbulibacter saemankumensis* (Jung *et al.*, 2005) and *Yeosuana aromativorans* (Kwon *et al.*, 2006), two species that were also isolated recently from sediment samples in subtropical regions of Korea, and to *Algibacter lectus* (Nedashkovskaya *et al.*, 2004), isolated from the surface of green algae collected in the Sea of Japan. A polyphasic approach that included morphological, physiological, biochemical and chemotaxonomic characterization, as well as phylogenetic analysis, was used to classify this non-motile, saffron-coloured, rod-shaped bacterium.

The family *Flavobacteriaceae* (Jooste, 1985; Reichenbach, 1989; Bernardet *et al.*, 2002) is one of the major evolutionary lineages of descent within the phylum *Bacteroidetes* (Garrity & Holt, 2001) and contains rod-shaped bacteria that are non-motile or motile by gliding, Gram-negative and chemoheterotrophic, with MK-6 as the major respiratory quinone (Bernardet *et al.*, 1996, 2002). Marine members of the family form a well-defined 'marine clade'. They have been isolated from a wide range of marine substrates in polar and temperate ecosystems, including sea ice and water, quartz stone, subliths, marine sediment and algae (Bowman *et al.*, 1997; Bowman & Nichols, 2005; Ivanova *et al.*, 2004; Nedashkovskaya *et al.*, 2004, 2005a, b; Jung *et al.*, 2005; Kwon *et al.*, 2006; Nichols *et al.*, 2005).

For the isolation of marine bacteria, a wet sediment sample was aseptically air-dried for 24 h under laminar flow and then stamped directly onto starch-casein agar (Küster & Williams, 1964) supplemented with 60 % (v/v) sterilized natural seawater (Lee, 2006), using a sterile rubber stopper. The isolation medium (SC-SW agar) consisted of 1 % soluble starch, 0.03 % casein, 0.2 % KNO_3, 0.2 % NaCl, 0.002 % $CaCO_3$, 0.005 % $MgSO_4.7H_2O$, 0.001 % $FeSO_4.7H_2O$ and 1.8 % agar in a mixture of 60 % natural seawater and 40 % distilled water (pH 7.2). Following incubation at 30 °C for 7 days, colonies were collected and streaked on yeast extract-malt extract agar (Shirling & Gottlieb, 1966) supplemented with 60 % (v/v) sterilized natural seawater (YE-SW agar: 0.4 % yeast extract, 1.0 % malt extract, 0.4 % glucose and 1.8 % agar in a mixture of 60 % natural seawater and 40 % distilled water; pH 7.2). After the purity of the culture had been verified, strain HST1-43^T was stored at −20 and −80 °C in 60 % (v/v) natural seawater supplemented with 20 % (v/v) glycerol.

DNA isolation and PCR amplification of the 16S rRNA gene were performed as described elsewhere (Lee, 2006). The PCR product was purified using the Wizard PCR Preps DNA purification system (Promega). 16 rRNA gene

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain HST1-43^T is AM286230.

A transmission electron micrograph of cells of strain HST1-43^T is available as a supplementary figure in IJSEM Online.

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sequencing was carried out using an ABI PRISM BigDye Terminator cycle sequencing kit (Applied Biosystems) and an automatic DNA sequencer (model 3730xl; Applied Biosystems). An almost-complete (1426 bp) 16S rRNA gene sequence of strain HST1-43^T was determined, and a preliminary BLAST search against GenBank showed that the isolate was related to members of the family *Flavobacteriaceae*. Alignment of sequences was carried out using CLUSTAL X software (Thompson *et al.*, 1997). Phylogenetic analyses were performed using the neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) methods. Evolutionary distances for the neighbour-joining method were calculated using the method of Jukes & Cantor (1969). *Borrelia anserina* ES-1 (GenBank accession no. U42284) was used as the outgroup in the construction of the phylogenetic tree. In total, 1306 unambiguously aligned nucleotides were used for the tree inference. A bootstrap analysis (Felsenstein, 1985) was performed for estimating the tree topology, with 1000 resamplings of the dataset.

The neighbour-joining tree (Fig. 1) showed that strain HST1-43^T formed a distinct cluster with the genera *Algibacter*, *Gaetbulibacter* and *Yeosuana*, albeit supported by a low bootstrap percentage (35 %). This branching pattern was also recovered in the maximum-likelihood tree (data not shown). Sequence similarity calculations obtained after a neighbour-joining analysis revealed that the closest neighbours of strain HST1-43^T were *A. lectus* (96.5 %), *Gaetbulibacter saemankumensis* (96.3 %) and *Y. aromativorans* (96.3 %). The levels of 16S rRNA gene sequence similarity between the isolate and members of the genera *Winogradskyella*, *Gelidibacter*, *Bizionia*, *Formosa*, *Subsaxibacter*, *Subsaximicrobium* and *Lacinutrix* were in the range 95.2–96.2 %.

For chemotaxonomic analyses, biomass was obtained from cultures in marine broth 2216 (MB; Difco) grown for 3 days at 30 °C in an orbital shaker. Isoprenoid quinones were extracted by using the method of Minnikin *et al.* (1984) and identified by HPLC as described by Kroppenstedt (1985). Cellular fatty acids were analysed according to the instructions of the Sherlock Microbial Identification System (MIDI, version 6). Fatty acid methyl esters were extracted from cells grown on marine agar 2216 (MA; Difco) for 3 days at 30 °C. The G+C content of the DNA was determined by using HPLC, as described by Mesbah *et al.* (1989).

The fatty acid profile of strain HST1-43^T was dominated by branched and saturated straight-chain fatty acids; there were minor proportions of unsaturated fatty acids. The major cellular fatty acids were iso-$C_{15:0}$ (13.7 %), $C_{18:0}$ (12.7 %), $C_{16:0}$ (8.5 %), iso-$C_{15:1}$ (8.2 %) and iso-$C_{17:0}$ 3-OH (8.0 %). Fatty acids amounting to at least 1 % were as follows: $C_{12:0}$ (6.0 %), $C_{15:0}$ (5.0 %), iso-$C_{15:0}$ 3-OH (5.0 %), anteiso-$C_{15:0}$ (4.4 %), iso-$C_{16:0}$ 3-OH (3.2 %), $C_{15:1}\omega6c$ (2.4 %), $C_{15:0}$ 2-OH (1.6 %), iso-$C_{16:0}$ 2-OH (1.3 %), $C_{18:1}\omega9c$ (1.2 %), $C_{14:0}$ (1.0 %), $C_{15:0}$ 3-OH (1.0 %), $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH (5.0 %), $C_{14:1}$ *trans*9 and/or *cis*9 (1.1 %) and two unknown fatty acids with equivalent chain-lengths of 9.521 and 13.566 (1.2 and 4.3 %, respectively). The predominant menaquinone was MK-6. The DNA G+C content was 36.2 ± 0.4 mol%, a value that is intermediate between the values of the phylogenetic neighbours, as shown in Table 1.

The cell morphology was determined by using light microscopy on cultures grown on MA at 30 °C for 3 days. Cells were suspended in sterile distilled water and stained using the bioMérieux Gram stain kit according to the manufacturer's instructions. Cell motility was observed under an Olympus light microscope equipped with phase-contrast optics (magnification ×400), and the presence of flagella was examined using transmission electron microscopy. Gliding motility was tested using the hanging drop technique after growing the cells in 0.1 × MB for 24 h at 30 °C (Bernardet *et al.*, 2002). Colony morphology and pigmentation were observed after 5 days incubation at 30 °C on MA. The presence of flexirubin pigments was investigated by noting whether a colour shift occurred when a mass of bacteria collected on agar was flooded with 20 % KOH (Bernardet *et al.*, 2002). Carotenoid pigments were extracted from the biomass with acetone/methanol (7 : 2, v/v), purified with diethyl ether as described by Schmidt *et al.* (1994) and then analysed using a spectrophotometer (UVmini-1240; Shimadzu). The temperature range for growth was tested on YE-SW agar at 4, 10, 20, 30, 37 and 42 °C. The pH range for growth was determined in MB for which the pH had been adjusted, after sterilization, from 4.1 to 12.1 (using increments of 1 pH unit) with 1 M NaOH and 1 M HCl. The requirement of strain HST1-43^T for natural seawater was evaluated on yeast extract-malt extract agar (YE agar), tryptic soy agar (Difco) and nutrient agar (Difco) with and without the addition of 60 % natural seawater. Tolerance of sea salts was determined on YE agar supplemented with sea salts (Sigma) at 0, 1, 3, 5, 7, 10 and 15 % (w/v). Growth with NaCl as the only salt was studied on YE agar supplemented with 0–9 % (w/v) NaCl. Tolerance of various NaCl concentrations (1–9 %, w/v) was tested on MA. Catalase and oxidase activities were tested using 3 % (v/v) H_2O_2 and 1 % (w/v) *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine solutions, respectively. Growth under anaerobic conditions was tested on MA supplemented with sodium thioglycolate and then incubated in an anaerobic chamber. Protease, amylase, lipase and tyrosinase activities were determined on MA supplemented with 1 % (w/v) casein, 1 % (w/v) soluble starch, 1 % (v/v) Tween 80 or 0.5 % (w/v) tyrosine, respectively. The ability to utilize 95 individual substrates as sole carbon sources was tested using GN2 MicroPlates (Biolog) according to the manufacturer's instructions. Cells grown on MA at 30 °C for 3 days were suspended in a 2 % (w/v) sea salts solution, inoculated into the microplate and incubated for 48 h at 30 °C. Other physiological and biochemical properties were tested using API 20E, API 20NE and API ZYM strips (bioMérieux) according to the manufacturer's instructions. The strips were inoculated in the same way as the GN2 MicroPlates. The API 20E and API 20NE strips were incubated at 30 °C for 48 h, whereas the API ZYM strip was incubated at 37 °C

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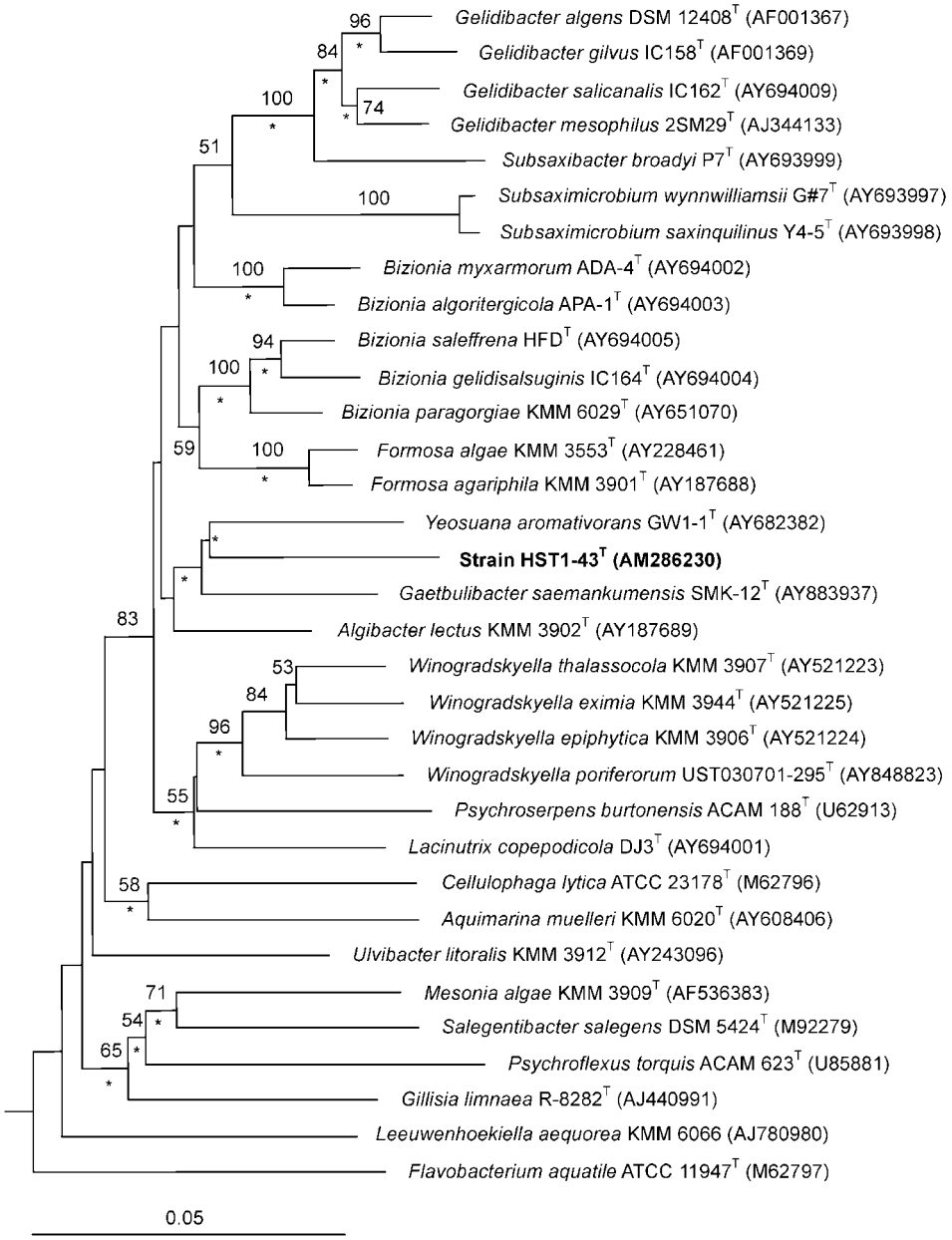

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the position of strain HST1-43^T within the family *Flavobacteriaceae*. Evolutionary distances, calculated using the Jukes–Cantor coefficient, are based on 1306 unambiguously aligned nucleotides. *Borrelia anserina* (GenBank accession no. U42284) was used as an outgroup (not shown). Only those bootstrap values above 50 % are shown at the branching points. Asterisks indicate branches that are also recovered in the maximum-likelihood phylogenetic tree. Bar, 0.05 substitutions per nucleotide position.

for 4 h. The results of the physiological and biochemical tests are shown in Table 1 and are described in the genus and species descriptions. The morphology of the cells of strain HST1-43^T is shown in Supplementary Fig. S1, available in IJSEM Online. Characteristics that can be used to distinguish the isolate from its phylogenetic relatives are given in Table 1.

On the basis of the physiological and chemotaxonomic data and the phylogenetic distance for the isolate, strain HST1-43^T represents a novel genus and species in the family *Flavobacteriaceae*, for which the name *Tamlana crocina* gen. nov., sp. nov. is proposed.

Description of *Tamlana* gen. nov.

Tamlana (Tam.la′na. N.L. fem. n. *Tamlana* named after Tamla, the old name for Jeju Island, referring to the region where the bacterium was isolated).

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Table 1. Differential characteristics of strain HST1-43^T and related taxa

Taxa: 1, strain HST1-43^T; 2, *A. lectus* (data from Nedashkovskaya *et al.*, 2004); 3, *Gaetbulibacter saemankumensis* (Jung *et al.*, 2005); 4, *Y. aromativorans* (Kwon *et al.*, 2006). +, Positive; −, negative; V, variable; ND, not determined. Values in parentheses represent optimal ranges.

Characteristic	1	2	3	4
Pigment production	Saffron	Orange	Yellow	Yellowish-brown
Cell size (μm)	0.3–0.5 × 0.7–1.1	0.4–0.5 × 2–3	0.4–0.5 × 3.0–4.5	0.2–0.3 × 0.7–1.7
Colony size (mm)	1.0–1.5	3–4	ND	1–1.5
Gliding motility	−	+	+	−
NaCl range for growth (%)	2–4 (2–3)	1–6 (2–3)	0–7 (2–3)	0.5–4.0 (2.0)
Temperature range for growth (°C)	20–37 (25–30)	4–35 (21–23)	13–40 (25–30)	23–39 (33–36)
pH range for growth	6.1–10.1 (6.1–8.1)	ND	5.5–ND (7.0–8.0)	5–8 (7)
Requirement for:				
Oxygen	+	−	−	+
Sea salts	+	−	−	+
Production of:				
Oxidase	+	+	+	−
Nitrate reductase	+	−	+	−
Acid from carbohydrates	−	+	+	−
Hydrolysis of:				
Aesculin	+	ND	−	ND
Agar	−	+	ND	−
DNA	+	−	ND	ND
Gelatin	−	+	−	+
Starch	−	V/+	+	−
DNA G+C content (mol%)	36.2±0.4	31–33	34.7–34.9	51.4
Major menaquinone	MK-6	MK-6	MK-6	MK-5, MK-6

Cells are Gram-negative, aerobic, non-motile rods. Catalase- and oxidase-positive. Endospores are not formed. Chemoheterotrophic. Gliding motility is not observed. Cells produce non-diffusible carotenoid pigments (absorption maxima at 446 nm) but no flexirubin pigments. The cellular fatty acid composition is dominated by branched and saturated straight-chain fatty acids, with minor amounts of unsaturated and hydroxylated fatty acids. The major menaquinone is MK-6. As shown by 16S rRNA gene sequence analysis, the genus belongs to the family *Flavobacteriaceae*. The type species is *Tamlana crocina*.

Description of *Tamlana crocina* sp. nov.

Tamlana crocina (cro.ci′na. L. fem. adj. *crocina* saffron-coloured).

Displays the following properties in addition to those given in the genus description. Cells are very short rods, 0.7–1.1 μm long and 0.3–0.5 μm wide. On MA, colonies are opaque, convex, circular and saffron-coloured. Grows at 20–37 °C (optimum, 25–30 °C). Grows at pH 6.1–10.1 (optimum, pH 6.1–8.1). Na^+ alone does not support growth. Has an obligate requirement for natural seawater or artificial sea salts (1–3 %, w/v). Grows on MA supplemented with 0–2 % (w/v) NaCl. Does not grow on tryptic soy agar, nutrient agar or YE agar. DNA and aesculin are degraded but casein, starch, Tween 80 and DL-tyrosine are not. In API 20NE tests, nitrate is reduced to nitrite, but indole production, urease activity, arginine dihydrolase activity, glucose fermentation and gelatin hydrolysis are negative. In API 20E tests, production of arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, H_2S and tryptophan deaminase, utilization of citrate and Voges–Proskauer reaction are negative, and acid is not produced from glucose, mannose, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin or arabinose. In API ZYM tests, alkaline phosphatase, leucine arylamidase and acid phosphatase are positive; esterase lipase (C8) and naphthol-AS-BI-phosphohydrolase are weakly positive; and esterase (C4), lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are negative. In the GN2 MicroPlate test, the following substrates are utilized as sole carbon and energy sources: dextrin, glycogen, Tween 40, adonitol, D-arabitol, D-cellobiose, i-erythritol, D-fructose, L-fucose, D-galactose, gentiobiose, α-D-glucose, α-D-lactose, lactulose, maltose, D-mannose, D-melibiose, methyl β-D-glucoside, D-psicose, D-raffinose, L-rhamnose, D-sorbitol, sucrose, D-trehalose, turanose, xylitol, methyl pyruvate, monomethyl succinate, acetic acid, *cis*-aconitic acid, citric acid, D-glucosaminic acid, D-glucuronic acid, α-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, itaconic acid, α-ketobutyric acid, α-ketoglutaric acid, α-ketovaleric

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acid, DL-lactic acid, quinic acid, D-saccharic acid, succinamic acid, glucuronamide, L-alaninamide, L-alanine, L-alanyl glycine, L-glutamic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-histidine, hydroxy-L-proline, L-ornithine, L-proline, L-pyroglutamic acid, L-threonine, DL-carnitine, urocanic acid, inosine, uridine, thymidine and α-D-glucose 1-phosphate. The following substrates are not utilized: α-cyclodextrin, Tween 80, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, L-arabinose, *myo*-inositol, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, β-hydroxybutyric acid, malonic acid, propionic acid, sebacic acid, succinic acid, bromosuccinic acid, D-alanine, L-asparagine, L-aspartic acid, L-leucine, L-phenylalanine, D-serine, L-serine, γ-aminobutyric acid, 2-aminoethanol, glycerol, DL-α-glycerol phosphate and α-D-glucose 6-phosphate. Utilization of D-mannitol, γ-hydroxybutyric acid, phenylethylamine, putrescine and 2,3-butanediol as sole carbon sources is weakly positive. Major cellular fatty acids are iso-$C_{15:0}$, $C_{18:0}$, $C_{16:0}$, iso-$C_{15:1}$ and iso-$C_{17:0}$ 3-OH. The DNA G+C content of the type strain is 36.2± 0.4 mol%.

The type strain, HST1-43^T (=KCTC 12721^T=JCM 14021^T), was isolated from a beach sediment in Jeju, Republic of Korea.

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