Tamlana agarivorans sp. nov., isolated from seawater off Jeju Island in Korea

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A Gram-negative, non-motile, rod-shaped, agarolytic bacterial strain, designated JW-26^T, was isolated from seawater off Jeju Island in Korea, and was subjected to a polyphasic taxonomic investigation. Strain JW-26^T grew optimally at pH 7.0–8.0, at 30 °C and in the presence of approximately 2% (w/v) NaCl. Phylogenetic analyses based on 16S rRNA gene sequences revealed that strain JW-26^T joins *Tamlana crocina* HST1-43^T, with which it exhibited a 16S rRNA gene sequence similarity value of 95.5%. Strain JW-26^T contained MK-6 as the predominant menaquinone and iso-C_{15:0}, C_{15:0} and iso-C_{15:1} as the major fatty acids. The DNA G+C content was 36.8 mol%. There were no distinct phenotypic and, particularly, chemotaxonomic properties to differentiate strain JW-26^T from the genus *Tamlana*. DNA–DNA relatedness data and differential phenotypic properties, together with the phylogenetic distinctiveness, demonstrated that strain JW-26^T is distinguishable from the type strain of *T. crocina*. On the basis of phenotypic, phylogenetic and genetic data, therefore, strain JW-26^T represents a novel species within the genus *Tamlana*, for which the name *Tamlana agarivorans* sp. nov. is proposed. The type strain is JW-26^T (=KCTC 22176^T =CCUG 55327^T).

In the course of the screening of micro-organisms from seawater off Jeju Island in Korea, many bacterial strains belonging to the phylum *Bacteroidetes* have been isolated. One of these isolates, strain JW-26^T, which shows agar-degrading activity, is the subject of this study. A comparative 16S rRNA gene sequence analysis showed that strain JW-26^T is phylogenetically related to the genera *Tamlana* and *Algibacter*. The aim of the present work was to determine the exact taxonomic position of strain JW-26^T by using a polyphasic characterization that included determination of the phenotypic properties and a detailed phylogenetic investigation based on 16S rRNA gene sequences.

Strain JW-26^T was isolated by means of the standard dilution plating technique at 30 °C on marine agar 2216 (MA; Difco). *Tamlana crocina* KCTC 12721^T was used as a reference strain for phenotypic characterization and DNA–DNA hybridization. The morphological, physiological and biochemical characteristics of strain JW-26^T were investigated using routine cultivation on MA at 30 °C. The cell morphology was examined by using light microscopy (E600; Nikon) and transmission electron microscopy (CM-20; Philips). Flagellation was determined by using transmission electron microscopy on cells from an exponentially growing culture negatively stained with 1% (w/v) phosphotungstic acid. Grids were examined after being

air-dried. The Gram reaction was determined by using the bioMérieux Gram stain kit according to the manufacturer's instructions. Gliding motility was investigated as described by Bowman (2000). Growth under anaerobic conditions was determined after incubation in a Forma anaerobic chamber on MA and on MA supplemented with potassium nitrate (0.1%, w/v), both of which had been prepared anaerobically under a nitrogen atmosphere. Growth in the absence of NaCl was investigated using trypticase soy broth prepared according to the formula of the Difco medium except that NaCl was excluded. Growth at various NaCl concentrations (0.5%, w/v, and 1.0–10.0%, w/v, in increments of 1.0%) was investigated in marine broth 2216 (MB; Difco) and trypticase soy broth (Difco). Growth at various temperatures (4, 10, 15, 20, 22, 25 and 28 °C and 30–40 °C in 1 °C increments) was measured on MA. The pH range for growth was determined in MB adjusted to various pH values (pH 4.5–9.5, in increments of 0.5 pH units) by the addition of HCl or Na₂CO₃. Catalase and oxidase activities were determined as described by Cowan & Steel (1965). Hydrolysis of casein, starch, Tweens 20, 40, 60 and 80, hypoxanthine, tyrosine and xanthine was tested on MA, using the substrate concentrations described by Cowan & Steel (1965). Hydrolysis of aesculin, gelatin and urea and reduction of nitrate were investigated as described previously (Lányi, 1987) with the modification that artificial seawater was used for the preparation of media. The artificial seawater contained (l⁻¹ distilled water) 23.6 g

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain JW-26^T is EU221275.

NaCl, 0.64 g KCl, 4.53 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 5.94 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 1.3 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (Bruns *et al.*, 2001). H_2S production was tested as described previously (Bruns *et al.*, 2001). The presence of flexirubin-type pigments was investigated as described by Reichenbach (1992). For pigment-absorption spectrum analysis, strain JW-26^T was cultivated aerobically in the dark at 30 °C in MB. The culture was centrifuged, washed twice using a MOPS buffer (MOPS/NaOH, 0.01 M; KCl, 0.1 M; MgCl_2 , 0.001 M; pH 7.5) and disrupted by sonication with a Branson 450 sonifier. After removal of cell debris by centrifugation, the supernatant was examined on a Beckman Coulter DU800 spectrophotometer to determine the absorption spectrum. Susceptibility to various antibiotics was investigated on MA plates by using antibiotic discs containing the following: polymyxin B, 100 U; streptomycin, 50 µg; penicillin G, 20 U; chloramphenicol, 100 µg; ampicillin, 10 µg; cephalothin, 30 µg; gentamicin, 30 µg; novobiocin, 5 µg; tetracycline, 30 µg; kanamycin, 30 µg; lincomycin, 15 µg; oleandomycin, 15 µg; neomycin, 30 µg; and carbenicillin, 100 µg. The utilization of various substrates for growth was determined as described by Baumann & Baumann (1981), using supplementation with 2 % (v/v) Hutner's mineral salts solution (Cohen-Bazire *et al.*, 1957) and 1 % (v/v) vitamin solution (Staley, 1968). Enzyme activities were determined after incubation for 8 h at 30 °C by using the API ZYM system (bioMérieux).

Cell biomass for DNA extraction and for isoprenoid quinone analysis was obtained from a culture grown for 2 days in MB at 30 °C. Chromosomal DNA was isolated and purified according to the method described by Yoon *et al.* (1996), with the exception that RNase T1 was used in combination with RNase A to minimize contamination by RNA. The 16S rRNA gene was amplified by means of a

PCR using two universal primers, as described previously (Yoon *et al.*, 1998). The sequencing of the amplified 16S rRNA gene and phylogenetic analysis were performed as described by Yoon *et al.* (2003). Isoprenoid quinones were analysed as described by Komagata & Suzuki (1987) using reversed-phase HPLC. For the cellular fatty acid analysis, cell mass of strain JW-26^T and *T. crocina* KCTC 12721^T was harvested from MA plates after cultivation for 3 days at 30 °C. The fatty acids were extracted and fatty acid methyl esters prepared according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990). The DNA G+C content was determined using the method of Tamaoka & Komagata (1984), with the modification that the DNA was hydrolysed using nuclease P1 (Sigma) and the resultant nucleotides were analysed by reversed-phase HPLC. DNA–DNA hybridization was performed fluorometrically using the method of Ezaki *et al.* (1989) with photobiotin-labelled DNA probes and microdilution wells. Hybridization was performed with five replications for each sample. The highest and lowest values obtained in each sample were excluded and the means of the remaining three values are quoted as DNA–DNA relatedness values.

Morphological, cultural, physiological and biochemical characteristics of strain JW-26^T are given in the species description (see below) and in Table 1. Sonicated cell extracts of strain JW-26^T showed an absorption maximum at 446 nm, indicating the presence of carotenoids. Flexirubin-type pigments were also present, as shown by the positive KOH test. The almost-complete 16S rRNA gene sequence of strain JW-26^T comprised 1474 nt, representing approximately 96 % of the *Escherichia coli* 16S rRNA gene sequence. In the phylogenetic tree constructed using the neighbour-joining algorithm, strain

Table 1. Differential phenotypic characteristics of strain JW-26^T and the type strain of *T. crocina*

Data for *T. crocina* are for strain HST1-43^T from Lee (2007) unless indicated. The two strains are aerobic, rod-shaped and positive for the following: catalase and oxidase activities, a requirement for sea salts, production of carotenoid pigments, nitrate reduction, hydrolysis of aesculin, utilization of D-glucose, D-galactose, cellobiose and D-mannose, susceptibility to cephalothin (weak for JW-26^T), chloramphenicol, novobiocin, carbenicillin, lincomycin and oleandomycin and activities of alkaline phosphatase, esterase lipase (C8) (weak for *T. crocina* HST1-43^T), leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase (weak for *T. crocina* HST1-43^T). The two strains are negative for the following: Gram stain, gliding motility, urease activity, hydrolysis of casein, gelatin, starch and Tween 80, utilization of trehalose, L-arabinose, maltose, acetate, citrate, succinate, benzoate, L-malate, pyruvate, salicin, formate and L-glutamate, susceptibility to polymyxin B, streptomycin, gentamicin, tetracycline, kanamycin and neomycin and activities of lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α -galactosidase, α -glucuronidase, α -glucosidase, β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase.

Characteristic	Strain JW-26 ^T	<i>T. crocina</i> HST1-43 ^T
Cell size (µm)	0.6–1.0 × 1.0–3.0	0.3–0.5 × 0.7–1.1
Growth at 10 and 38 °C	+	–
Flexirubin-type pigments	+	–
Hydrolysis of agar and tyrosine	+	–
Utilization of D-fructose, D-xylose and sucrose*	–	+
Susceptibility to penicillin G and ampicillin*	–	+
API ZYM tests for esterase (C4), α -chymotrypsin and β -galactosidase	+	–

*Data for *T. crocina* KCTC 12721^T from this study.

JW-26^T joined the type strain of *T. crocina* with a bootstrap resampling value of 82.7 % (Fig. 1). The two strains exhibited 95.5 % 16S rRNA gene sequence similarity. The relationship between strain JW-26^T and *T. crocina* HST1-43^T was also maintained in the trees constructed using the maximum-likelihood and maximum-parsimony algorithms (Fig. 1).

The predominant isoprenoid quinone detected in strain JW-26^T was MK-6, at a peak area ratio of approximately 96 %. The cellular fatty acid profile of strain JW-26^T is shown in Table 2, together with that of *T. crocina* KCTC 12721^T, which was also analysed in this study. The major fatty acids (constituting >10 % of total fatty acids) detected in strain JW-26^T were iso-C_{15:0} (25.9 %), C_{15:0} (12.1 %) and iso-C_{15:1} (11.8 %). This fatty acid profile was similar to that of *T. crocina* KCTC 12721^T, although there were differences in the proportions of some fatty acids (Table 2). Hence, no distinct chemotaxonomic properties served to differentiate strain JW-26^T from *T. crocina* (Lee, 2007; Table 2). The DNA G+C content of strain JW-26^T was 36.8 mol%, which was in line with that of *T. crocina* HST1-43^T (36.2 ± 0.4 mol%; Lee, 2007). Accordingly, on the basis of the phylogenetic and chemotaxonomic data, it is appropriate to classify strain JW-26^T within the genus *Tamlana*.

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the positions of strain JW-26^T, *T. crocina* HST1-43^T and some other related taxa. Bootstrap percentages (based on 1000 replications) >50 % 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. *Bacteroides fragilis* ATCC 25285^T was used as an outgroup. Bar, 0.05 substitutions per nucleotide position.

Table 2. Cellular fatty acid compositions (%) of strain JW-26^T and *T. crocina* KCTC 12721^T

Data are from this study. Fatty acids that represented <0.5 % in both strains were omitted. —, Not detected.

Fatty acid	Strain JW-26 ^T	<i>T. crocina</i> KCTC 12721 ^T
Straight-chain		
C _{13:0}	0.6	0.2
C _{14:0}	1.4	—
C _{15:0}	12.1	9.9
C _{16:0}	3.4	0.9
Unsaturated		
C _{15:1} ω6c	3.0	3.4
C _{17:1} ω6c	0.9	1.4
Branched		
iso-C _{13:0}	1.0	0.4
iso-C _{14:0}	1.4	1.6
iso-C _{15:1}	11.8	12.4
anteiso-C _{15:1}	0.7	1.3
iso-C _{15:0}	25.9	18.7
anteiso-C _{15:0}	3.2	5.0
iso-C _{16:1}	—	0.8
iso-C _{16:0}	2.0	1.2
iso-C _{17:1} ω9c	0.5	0.5
Hydroxy		
iso-C _{15:0} 3-OH	3.5	5.1
C _{15:0} 2-OH	—	1.4
C _{15:0} 3-OH	1.9	1.5
iso-C _{16:0} 3-OH	2.1	7.4
C _{16:0} 3-OH	5.2	0.5
iso-C _{17:0} 3-OH	9.9	14.3
C _{17:0} 2-OH	0.6	2.4
C _{17:0} 3-OH	0.9	0.7
Summed feature 3*	5.0	4.8
Unknown†		
ECL 13.565	1.4	1.8
ECL 16.582	1.0	1.5

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

†ECL, Equivalent chain length.

The mean value for DNA–DNA relatedness between strain JW-26^T and *T. crocina* KCTC 12721^T was 9 %, when their DNAs were used individually as labelled DNA probes for cross-hybridization, indicating that strain JW-26^T represents a genomic species that is different from *T. crocina* (Wayne *et al.*, 1987). Strain JW-26^T was distinguishable from *T. crocina* by differences in terms of several phenotypic characteristics, as listed in Table 1. The phylogenetic and genetic distinctiveness and differential phenotypic properties of strain JW-26^T are sufficient to show that strain JW-26^T is separate from *T. crocina* (Stackebrandt & Goebel, 1994). Therefore, on the basis of the data presented, strain JW-26^T represents a novel species

of the genus *Tamlana*, for which the name *Tamlana agarivorans* sp. nov. is proposed.

Description of *Tamlana agarivorans* sp. nov.

Tamlana agarivorans (a.ga.ri.vo'rans. N.L. n. *agarum* agar, algal polysaccharide; L. v. *vorare* to devour, to digest; N.L. part. adj. *agarivorans* agar-devouring).

Cells are Gram-negative, non-flagellated rods, 0.6–1.0 µm in diameter and 1.0–3.0 µm long. Colonies on MA are circular with regular margins, flat to raised, smooth, glistening, yellow in colour and 1.0–2.0 mm in diameter after 3 days incubation at 30 °C. Growth occurs at 10 and 38 °C, but not at 4 or 39 °C. The 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 0.5–6 % (w/v) NaCl. The optimal NaCl concentration for growth is approximately 2 % (w/v). Anaerobic growth does not occur on MA or on MA supplemented with nitrate. Hypoxanthine, xanthine and Tweens 20, 40 and 60 are not hydrolysed. H₂S is not produced. Carotenoid and flexirubin pigments are produced. The predominant menaquinone is MK-6. The major fatty acids (>10 % of total fatty acids) are iso-C_{15:0}, C_{15:0} and iso-C_{15:1}. The DNA G+C content of the type strain is 36.8 mol% (determined by HPLC). Other phenotypic characteristics are given in Table 1.

The type strain, JW-26^T (=KCTC 22176^T =CCUG 55327^T), was isolated from seawater off Jeju Island in Korea.

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