Cohaesibacter marisflavi sp. nov., isolated from sediment of a seawater pond used for sea cucumber culture, and emended description of the genus *Cohaesibacter*

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A Gram-negative, catalase-negative, oxidase-positive, rod-shaped bacterium, strain DQHS21^T, was isolated from sediment of a seawater pond used for sea cucumber culture at Jimo in Qingdao province on the east coast of China. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain DQHS21^T belonged to the genus *Cohaesibacter*, sharing the highest sequence similarity (96.1 %) with *Cohaesibacter gelatinilyticus* CL-GR15^T, while the similarity to other strains was below 93.0 %. The cellular fatty acids consisted mainly of $C_{18:1}\omega7c$ (60.7 %), $C_{18:0}$ (17.8 %), $C_{16:0}$ (8.5 %) and summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH; 6.0 %), which together accounted for 93 % of the total fatty acids. Ubiquinone 10 was the major quinone. The G+C content of the chromosomal DNA of strain DQHS21^T was 55.2 mol%. The combined genotypic and phenotypic data showed that strain DQHS21^T represents a novel species of the genus *Cohaesibacter*, for which the name *Cohaesibacter marisflavi* sp. nov. is proposed, with the type strain DQHS21^T (=CGMCC 1.9157^T =NCCB 100300^T).

In recent years, much attention has been paid to marine bacteria because of their important role in marine ecology. As a result, many bacterial strains have been isolated and characterized taxonomically from coastal marine environments. Hwang & Cho (2008) isolated two Gram-negative bacterial strains, CL-GR15^T and CL-GR35, from coastal seawater of Korea, and proposed that these strains belong to a novel genus, *Cohaesibacter*. During a screen of bacteria that might be applied to the bioremediation of coastal marine environments of the Yellow Sea, strain DQHS21^T was isolated and was considered to represent a novel species of the genus *Cohaesibacter*. In this paper, it was subjected to a polyphasic taxonomic investigation by genotypic and classical phenotypic characterization.

The sediment was sampled from a seawater pond for sea cucumber culture at Jimo in Qingdao province, China (depth 10 m, 36.50° N 120.82° E), on the west coast of the Yellow Sea, in March 2009. The sediment sample was incubated in a tube containing an enrichment medium (per litre Yellow Sea seawater: 0.2 g $MgSO_4.7H_2O$, 1 g NH_4Cl, 5 g $NaHCO_3$, 0.5 g K_2HPO_4, 1.5 g peptone, pH 7.2) covered with a layer of paraffin (Li *et al.*, 2006). This medium was used for the purpose of enriching photosynthetic bacteria. Enrichment was conducted at room temperature for about 2 weeks. Sequential transfers were performed three times at intervals of 2 weeks. Bacteria were then isolated using the plate screening method on 2216E agar medium (per litre Yellow Sea seawater: 5 g tryptone, 1 g yeast extract, 15 g agar, pH 7.5) for 3 days (Hou *et al.*, 2008). The 2216E medium was used for all experiments in this paper unless noted otherwise. Strain DQHS21^T was isolated and subsequently purified five times on 2216E agar medium at 25 °C. The strain was maintained on 2216E agar medium at 25 °C and stored frozen in 2216E medium supplemented with 30 % (v/v) glycerol at −80 °C.

Genomic DNA was prepared following the method of Hiraishi (1992) and the 16S rRNA gene was amplified using PCR with universal primers 27F and 1492R (Hiraishi, 1992; Lane, 1991). PCR products were purified using the Wizard SV Gel and PCR Clean-Up system (Promega), and direct sequence determination of PCR-amplified DNA was carried out in both directions with universal primers 27F and 1492R on a model 3730XL automated DNA Analyzer

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain DQHS21^T is GQ200200.

Two supplementary figures are available with the online version of this paper.

(Applied Biosystems). The almost-complete 16S rRNA gene sequence of strain DQHS21^T (1446 bp) was obtained and investigated for similarity in the GenBank DNA database using BLASTN searches (Altschul *et al.*, 1990). Sequences of related taxa were then obtained from the GenBank database (Benson *et al.*, 2009). A phylogenetic analysis was performed using MEGA version 4 (Tamura *et al.*, 2007), after multiple alignment of the data with BioEdit version 7.0.9 (Hall, 1999). A phylogenetic tree was reconstructed by the neighbour-joining method of Saitou & Nei (1987) (distance options according to Kimura's two-parameter model) and the topology of the phylogenetic tree was evaluated by using bootstrap percentages based on 1000 replications. As shown in Fig. 1, the phylogenetic tree based on 16S rRNA gene sequences indicated that strain DQHS21^T and *Cohaesibacter gelatinilyticus* CL-GR15^T formed an independent monophyletic cluster, with high bootstrap support (100 %). A tree was also reconstructed using the minimum-evolution method (Rzhetsky & Nei, 1992) (shown in Supplementary Fig. S1, available in IJSEM Online), and it was similar to the neighbour-joining tree. Strain DQHS21^T and *C. gelatinilyticus* CL-GR15^T shared 96.1 % 16S rRNA gene sequence similarity. Strain DQHS21^T was also relatively closely related to members of the genera *Daeguia*, *Ensifer*, *Ochrobactrum*, *Sinorhizobium* and *Phyllobacterium* in the order *Rhizobiales* (91–92.7 % similarity), but similarities between DQHS21^T and other related strains were low (<93 %). The high similarity confirms that strain DQHS21^T belongs to the genus *Cohaesibacter* (the only genus of the family *Cohaesibacteraceae* of the order *Rhizobiales*). Three 16S rRNA gene signature nucleotides for the genus *Cohaesibacter* were reported by Hwang & Cho (2008). Strain DQHS21^T contains all the three signature nucleotides, which are two compensatory transversion mutations (positions 678A and 712T) and a single transversion mutation (position 194T). In general, a 16S rRNA gene sequence divergence of more than 3 % is accepted as a criterion for delineating different species (Stackebrandt & Goebel, 1994). The 16S rRNA gene sequence divergence between strain DQHS21^T and *C. gelatinilyticus* CL-GR15^T was 3.9 %, and thus the data support the view that strain DQHS21^T represents a novel species.

General cell morphological and physiological tests were performed as follows. Gram-staining and catalase and oxidase activities were examined according to standard methods (Dong & Cai, 2001). Cellular morphology and the presence of flagella were observed using transmission electron microscopy (H-7000; Hitachi). Anaerobic growth was checked on 2216E agar medium using the GasPak anaerobic system (BBL). Poly-β-hydroxybutyrate granules were observed by epifluorescence microscopy (CKX41; Olympus) after harvested cells were stained using Nile blue A (Dong & Cai, 2001; Ostle & Holt, 1982). The temperature range for growth was determined in 2216E incubated at 5, 10, 15, 20, 25, 30, 35, 38, 40, 42 and 45 °C. Tolerance of NaCl was tested by using Luria–Bertani medium (Sambrook *et al.*, 1989) supplemented with NaCl at 0, 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12, 15, 20, 25 and 30 % (w/v). Growth was also determined in 2216E over the pH range 3–10 at intervals of 1 pH unit; the pH was adjusted using appropriate biological buffers described by Gomori (1955). Other biochemical tests were carried out using API 20NE and API ZYM strips (bioMérieux) and the Biolog GN2 MicroPlate test panel according to the manufacturers' instructions, with the NaCl concentration set to 3.0 %. For comparison, the same tests were also performed on *C. gelatinilyticus* CL-GR15^T at the same time. These results are given in the species description and in Table 1.

Fatty acids and isoprenoid quinones were obtained from whole cells grown on marine agar 2216 (BD 212185) at 28 °C for 48 h. For fatty acid analysis, the harvested cells were first freeze-dried and the fatty acids were then extracted, saponified and esterified (Sasser, 1990). GC analysis of the fatty acid methyl esters was then performed according to the instructions of the MIDI System (Sasser, 1990) at the Marine Culture Collection of China (Xiamen,

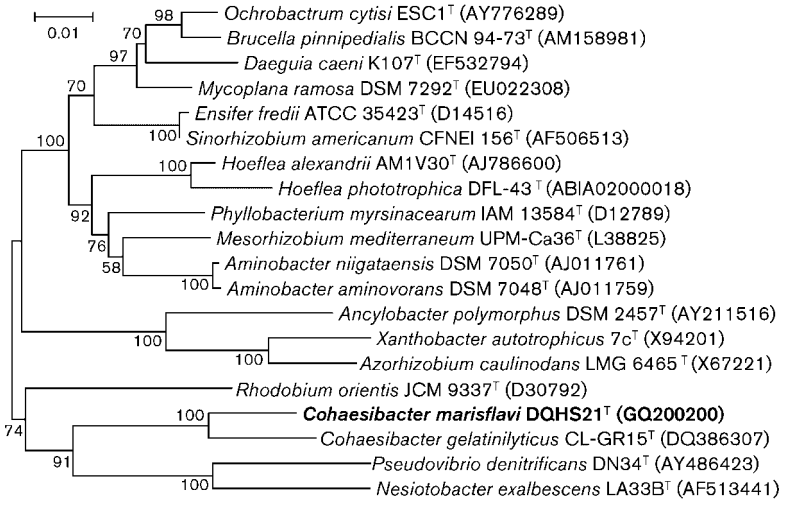

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences reconstructed using the neighbour-joining method (Saitou & Nei, 1987), showing the positions of strain DQHS21^T and type strains of related taxa within the order *Rhizobiales*. Bootstrap values (expressed as percentages of 1000 replications) are shown at branch points. Bar, 0.01 nucleotide substitution rate (K_{nuc}) units.

Table 1. Differential phenotypic characteristics of strain DQHS21^T and *C. gelatinilyticus* CL-GR15^T

Strains: 1, DQHS21^T; 2, *C. gelatinilyticus* CL-GR15^T. Data were obtained in this study under the same conditions unless indicated. Both strains are positive for oxidase (data for *C. gelatinilyticus* CL-GR15^T from Hwang & Cho, 2008). In the API 20NE test, both strains were positive for nitrate reduction and negative for D-glucose fermentation, arginine dihydrolase activity and utilization of D-mannitol, potassium gluconate, capric acid, adipic acid and phenylacetic acid. In the API ZYM test, both strains were positive for esterase (C4), naphthol-AS-BI-phosphohydrolase and trypsin and negative for lipase (C14), *N*-acetyl-β-glucosaminidase, α-chymotrypsin, α-fucosidase, α- and β-glucosidase, α-mannosidase, α- and β-galactosidase and β-glucuronidase. In the Biolog GN2 test, both strains were positive for utilization of L-arabinose and dextrin and negative for utilization of other carbon sources not included in the table. +, Positive; W, weak; −, negative.

Characteristic	1	2
Cell width (μm)	0.6–0.7	0.2–0.4*
Cell length (μm)	1.8–2.0	1.0–3.0*
Catalase	−	+*
Temperature range (°C)	10–38	15–31*
pH range	4–9	6–9*
NaCl concentration (%, w/v)	0.5–15	2–5*
API 20NE results		
Indole production, urease, β-glucosidase (aesculin hydrolysis)	+	−
β-Galactosidase	W	−
Denitrification and gelatin hydrolysis	−	+
Utilization of D-glucose, D-mannose, *N*-acetylglucosamine and malic acid	+	−
Utilization of trisodium citrate	+	W
Utilization of maltose and L-arabinose	W	−
API ZYM results		
Acid phosphatase, alkaline phosphatase, esterase lipase (C8) and leucine aminopeptidase	W	+
Cystine aminopeptidase and valine aminopeptidase	−	W
Susceptibility to antimicrobial agents (per disc)		
Nalidixic acid (30 μg), penicillin G (10 IU)	+	−
Vancomycin (30 μg), lincomycin (2 μg)	−	+
Biolog GN2 utilization results		
N-Acetyl-D-glucosamine	+	W
D-Fructose, glycerol, raffinose and sucrose	+	−
L-Fucose, acetic acid and thymidine	−	+
Glycogen	−	W
DNA G+C content (mol%)	55.2	53.0*

*Data from Hwang & Cho (2008).

China). The fatty acid profile of *C. gelatinilyticus* CL-GR15^T was also determined in parallel with strain DQHS21^T. Results for both strains are shown in Table 2. The major fatty acid in both strains was $C_{18:1}\omega7c$ (60.7 and 56.3 %, respectively, for strain DQHS21^T and *C. gelatinilyticus* CL-GR15^T). The fatty acid profile of *C. gelatinilyticus* CL-GR15^T determined in this study is very similar to that reported by Hwang & Cho (2008). However, the two strains showed large differences (>7 %) in the content of summed feature 3 (iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$), $C_{16:0}$, $C_{18:0}$ and $C_{20:1}\omega7c$. As the fatty acid profiles of strain DQHS21^T and *C. gelatinilyticus* CL-GR15^T were determined under the same conditions, these differences could be used to distinguish the two strains. The isoprenoid quinone of strain DQHS21^T was determined to be ubiquinone 10 (Q-10) by HPLC analysis according to Komagata & Suzuki (1987). This trait was in accordance with the properties of the genus *Cohaesibacter*.

Antibiotic susceptibility tests were performed on strain DQHS21^T and *C. gelatinilyticus* CL-GR15^T by the disc diffusion method (Lai & Shao, 2008). The results for strain DQHS21^T are given in the species description. The two strains could be differentiated by their susceptibility to four antibiotics (Table 1). The G+C content of the chromosomal DNA was determined according to the method described by Mesbah & Whitman (1989) using reversed-phase HPLC. The DNA G+C content of strain DQHS21^T was 55.2 mol%, which is slightly higher than that reported for *C. gelatinilyticus* CL-GR15^T (53.0 mol%).

Cells of strain DQHS21^T were facultatively anaerobic rods that were motile by means of single polar flagella (Supplementary Fig. S2). The strain was Gram-negative, catalase-negative and oxidase-positive and accumulated intracellular poly-β-hydroxybutyrate granules. Phylogenetic analysis based on 16S rRNA gene sequences strongly suggests that strain DQHS21^T belongs to the genus

Table 2. Cellular fatty acid contents of strain DQHS21^T and *C. gelatinilyticus* CL-GR15^T

Values are percentages of total fatty acids; tr, minor fatty acids (<1 %); ND, not detected/not reported. Data from Hwang & Cho (2008) are ranges for *C. gelatinilyticus* strains CL-GR15^T and CL-GR35.

Peak name	Strain DQHS21^T	*C. gelatinilyticus* CL-GR15^T	
		This study	Hwang & Cho (2008)
$C_{9:0}$	ND	ND	tr
$C_{12:0}$ aldehyde	ND	tr	ND
iso-$C_{13:0}$	tr	ND	ND
$C_{14:1}\omega5c$	ND	ND	tr
$C_{14:0}$	tr	tr	tr
Summed feature 2*	1.27	2.10	3.0
Summed feature 3*	5.99	18.41	19.2–20.4
$C_{16:0}$	8.47	1.24	1.1
$C_{17:1}\omega8c$	ND	tr	1.1–1.5
$C_{17:0}$	1.23	tr	tr
$C_{16:0}$ 3-OH	ND	tr	tr
iso-$C_{17:0}$ 3-OH	ND	ND	tr
$C_{18:1}\omega7c$	60.68	56.25	54.3–55.1
$C_{18:0}$	17.76	5.00	3.1–3.3
11-Methyl $C_{18:1}\omega7c$	1.98	ND	ND
Summed feature 7*	tr	tr	ND
$C_{19:0}$ cyclo $\omega8c$	ND	1.04	tr
$C_{19:0}$	ND	tr	tr
10-Methyl $C_{19:0}$	ND	tr	tr
$C_{18:0}$ 3-OH	ND	tr	1.9–2.0
$C_{20:1}\omega7c$	ND	12.26	9.6–11.1
$C_{20:0}$	ND	tr	tr

*Summed features are groups of two or three fatty acids that could not be separated by GLC with the MIDI System. Summed feature 2 contains $C_{14:0}$ 3-OH and/or iso-$C_{16:1}$ I; summed feature 3 contains iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$; summed feature 7 contains an unknown fatty acid with ECL 18.846 and/or $C_{19:1}\omega6c$.

Cohaesibacter (Fig. 1 and Supplementary Fig. S1). Strain DQHS21^T is clearly different from *C. gelatinilyticus* CL-GR15^T in its physiological, biochemical and chemotaxonomic characteristics (Tables 1 and 2). On the basis of data described above, strain DQHS21^T should be classified as representing a novel species within the genus *Cohaesibacter*, for which the name *Cohaesibacter marisflavi* sp. nov. is proposed.

Emended description of the genus *Cohaesibacter* Hwang and Cho 2008

The characteristics of the genus are as described previously by Hwang & Cho (2008), with the following amendments. Activity of catalase is variable. The principal fatty acids are $C_{16:1}\omega7c$/iso-$C_{15:0}$ 2-OH, $C_{18:1}\omega7c$ and $C_{18:0}$. The DNA G+C content ranges from 53.0 to 55.2 mol%. The type species is *Cohaesibacter gelatinilyticus*.

Description of *Cohaesibacter marisflavi* sp. nov.

Cohaesibacter marisflavi (ma.ris.fla′vi. L. neut. n. *mare -is* the sea; L. adj. *flavus -a -um* yellow; N.L. gen. n. *marisflavi* of the Yellow Sea, referring to the isolation of the type strain).

Cells are Gram-negative, catalase-negative, oxidase-positive, facultatively anaerobic rods, motile by means of single polar flagella, 0.6–0.7 μm wide and 1.8–2.0 μm long. Reproduces by binary fission or asymmetrical division. Intracellular poly-β-hydroxybutyrate granules are accumulated. Colonies on 2216E agar medium are white, smooth, circular, lightly transparent and 1.0–2.0 mm in diameter after 3–5 days of cultivation at 30 °C. Grows at 10–38 °C (optimum 25–30 °C), at pH 4–9 (optimum pH 7–8) and in 0.5–15 % (w/v) NaCl (optimum 3 %). Sensitive (μg per disc, unless otherwise indicated) to ampicillin (10), carbenicillin (100), cefalexin (30), chloramphenicol (30), ciprofloxacin (5), doxycycline (30), erythromycin (15), kanamycin (30), nalidixic acid (30), neomycin (30), novobiocin (30), penicillin G (10 IU), rifampicin (5), streptomycin (10) and tetracycline (30) and resistant to clindamycin (2), lincomycin (2), oxacillin (1), polymyxin B (300 IU) and vancomycin (30). In API 20NE tests, positive for indole production, urease, β-glucosidase (aesculin hydrolysis), nitrate reduction and utilization of D-glucose,

D-mannose, malic acid, *N*-acetylglucosamine and trisodium citrate and weakly positive for utilization of β-galactosidase, maltose and L-arabinose; negative for gelatin hydrolysis, D-glucose fermentation, arginine dihydrolase and utilization of D-mannitol, potassium gluconate, capric acid, adipic acid and phenylacetic acid. In the API ZYM test strip, positive for esterase (C4), naphthol-AS-BI-phosphohydrolase and trypsin, weakly positive for acid phosphatase, alkaline phosphatase, esterase lipase (C8) and leucine aminopeptidase and negative for cystine aminopeptidase, lipase (C14), *N*-acetyl-β-glucosaminidase, valine aminopeptidase, α-chymotrypsin, α-fucosidase, α-galactosidase, α-glucosidase, α-mannosidase, β-galactosidase, β-glucosidase and β-glucuronidase. Of the 95 substrates in the Biolog GN2 system, positive for utilization of dextrin, D-fructose, raffinose, glycerol, L-arabinose, *N*-acetyl-D-glucosamine and sucrose. Principal fatty acids are $C_{18:1}\omega7c$, $C_{18:0}$, $C_{16:0}$ and $C_{16:1}\omega7c$/iso-$C_{15:0}$ 2-OH. Ubiquinone 10 is the major quinone. The DNA G+C content of the type strain is 55.2 mol%. Table 1 shows the characteristics that can be used to distinguish the type strain from *C. gelatinilyticus* CL-GR15^T.

The type strain, DQHS21^T (=CGMCC 1.9157^T =NCCB 100300^T), was isolated from sediment sampled from a seawater pond used for sea cucumber culture at Jimo, Qingdao province, China, on the west coast of the Yellow Sea.

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