Lishizhenia caseinilytica gen. nov., sp. nov., a marine bacterium of the phylum *Bacteroidetes*

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A light-orange, aerobic bacterium, strain UST040201-001^T, that degrades casein, gelatin and Tween 20, was isolated in February 2004 from a sand-filtered seawater sample from Port Shelter, Hong Kong SAR, China. The strain possessed menaquinone-6 and its 16S rRNA gene sequence shared only 90.1 % similarity with that of *Brumimicrobium glaciale* IC156^T. Phylogenetic analysis showed that UST040201-001^T formed a distinct lineage within the family *Cryomorphaceae*. Its ecophysiological and biochemical characteristics suggest that this strain represents a novel genus and species within the phylum *Bacteroidetes*. The name *Lishizhenia caseinilytica* gen. nov., sp. nov. is proposed. The type strain of *Lishizhenia caseinilytica* is UST040201-001^T (=NRRL B-41434^T=JCM 13821^T).

Bowman *et al.* (2003) established the family *Cryomorphaceae* in the phylum *Bacteroidetes* based on a polyphasic taxonomic approach. The *Cryomorphaceae* encompasses four marine genera, *Brumimicrobium*, *Cryomorpha*, *Crocinitomix* and *Owenweeksia*, and a recently described freshwater genus, *Fluviicola*, which form a distinct clade in the class *Flavobacteria*, branching between the families *Flavobacteriaceae* and *Bacteroidaceae* (Bowman *et al.*, 2003; Lau *et al.*, 2005; O'Sullivan *et al.*, 2005). Members of the *Cryomorphaceae* cannot utilize carbohydrates and require complex organic compounds for growth (Bowman *et al.*, 2003; Lau *et al.*, 2005; O'Sullivan *et al.*, 2005). Molecular phylogenetic studies have found that phylotypes related to the *Cryomorphaceae* are associated with phytoplankton blooms (Pinhassi *et al.*, 2004; Grossart *et al.*, 2005).

A seawater sample was collected in February 2004 from the outlet of a tank storing sand-filtered seawater that was pumped from 5 m depth adjacent to the Coastal Marine Laboratory of Hong Kong University of Science and Technology. Aliquots of 100 µl were spread onto YPS-SW agar (Lau *et al.*, 2005) and incubated at 30 °C for 3 days. Pigmented colonies were selected and then purified by repeatedly restreaking on YPS-SW agar. These isolates were grown in marine broth 2216 (MB; Difco) and stored in MB

supplemented with 50 % (v/v) glycerol at –80 °C. The isolates were subjected to partial 16S rRNA gene sequencing and one isolate, UST040201-001^T, shared only 90 % sequence similarity with its nearest relative, *Crocinitomix catalasitica* NCIMB 1418^T. We further characterized this novel bacterium and determined its taxonomic position by using the polyphasic approach and propose that strain UST040201-001^T represents a novel species of a new genus in the family *Cryomorphaceae*.

We observed colony morphology on plates of marine agar 2216 (MA; Difco) that had been incubated at 30 °C for 5 days. Cell size and gliding motility were examined using a Zeiss MC100 Spot microscope at ×1000 magnification. To study surface morphology, we collected cells from an overnight culture that were then washed in PBS, fixed overnight in 2.5 % glutaraldehyde, dehydrated in ethanol, dried in a critical-point drier and coated with gold before being examined under a scanning electron microscope (JOEL JSM 6300F). The Gram-reaction was assessed according to Collins *et al.* (1989). Growth under different conditions was monitored by measuring the optical density at 600 nm with a spectrophotometer and the range giving the highest cell yield was taken as the optimum. Growth was evaluated at various temperatures (4, 16, 20, 25, 30, 33, 37, 40 and 42 °C) in artificial seawater (ASW; Lewin & Lounsbury, 1969) containing 0.4 % yeast extract (ASWY). Growth at various pH values (pH 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 or 10.0) was evaluated in YP-SW medium (Lau *et al.*, 2005) adjusted with 1 M HCl or NaOH. Salinity tolerance was determined in YP-SW prepared with 0, 5, 15, 20, 40, 75

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain UST040201-001^T is AB176674.

A photograph showing colony morphology and phase-contrast and scanning electron micrographs of strain UST040201-001^T are available as supplementary material in IJSEM Online.

or 100 % filtered seawater. Salt requirement and tolerance were tested in ASWY with NaCl added at 0, 1, 2, 3, 4, 5, 7.5, 10 or 15 % (w/v). Anaerobic growth was examined in the Oxoid anaerobic system on YP-SW agar supplemented with 0.1 % NaNO₃, 0.1 % glucose or 0.1 % meat extract. Acid production from carbohydrates was determined using API 50CH strips (bioMérieux), using a medium composed of 50 % CHB/E medium (bioMérieux) with 0.075 % CaCl₂·2H₂O, 1.875 % NaCl and 0.375 % MgCl₂. Carbohydrate assimilation was determined using API 50CH strips, using ASW supplemented with 0.05 % yeast extract for resuspension of cells, and the strips were incubated at 30 °C for 2 weeks. Tests for fermentation of D(+) -glucose, D(−) -mannitol and sucrose and hydrolysis of alginate, chitin and Tweens 20, 40, 60 and 80 were carried out according to Baumann & Baumann (1981). The activities of catalase, oxidase, lecithinase and nitrate reductase, indole production, H₂S generation from cysteine or thiosulfate and hydrolysis of casein, cellulose, starch and gelatin were examined according to Smibert & Krieg (1994). β -Galactosidase activity was examined according to Gosink *et al.* (1998). DNA hydrolysis was determined according to Lau *et al.* (2005). Haemolytic activity was investigated using defibrinated rabbit blood (5 %, v/v) prepared with blood agar base (BBL) using filtered seawater or double-distilled water. Degradation of dead yeast cells was tested on VY/2 agar (Reichenbach, 1989) prepared with 100 % filtered seawater.

The absorption spectrum was determined by extraction of a 48 h YP-SW culture with absolute ethanol and scanning the extract from 300 to 700 nm with a Beckman DU650 spectrophotometer. The bathochromic shift test for flexirubin was performed by addition of 20 % KOH (Fautz & Reichenbach, 1980). Isoprenoid quinone analysis was performed by the HPLC method (Collins, 1994), using menaquinones extracted from *Cytophaga lytica* (Nakagawa & Yamasato, 1993) as the MK-6 reference. Fatty acid methyl ester analysis was determined by the MIDI Sherlock Microbial Identification System (Microbial ID) with cells grown on MA in 12 °C for 5 days. Genomic DNA was extracted using the TaKaRa MiniBEST bacterial genomic DNA extraction kit and the DNA base composition was determined by the HPLC method (Mesbah *et al.*, 1989). The 16S rRNA gene was amplified by using the primer pair 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1525R (5'-AAGGAGTGTCCARCC-3') (Lane, 1991) with Vent DNA polymerase (NEB) and sequenced using an Applied Biosystems 3100 automated DNA sequencer. Related 16S rRNA gene sequences were retrieved from the NCBI nucleotide database after MEGABLAST search (Zhang *et al.*, 2000). Sequences of strain UST040201-001^T and related strains were aligned by using CLUSTAL X (Thompson *et al.*, 1997) and the alignment was edited by using the BioEdit sequence alignment editor version 5.0.9 (Hall, 1999; <http://www.mbio.ncsu.edu/BioEdit/bioedit.html>). Evolutionary distances were computed using Kimura's two-parameter model (Kimura, 1980) and phylogenetic trees were

Table 1. Phenotypic features of strain UST040201-001^T

The strain did not form acid from or utilize the following carbohydrates: D-glucose, dextran, glycerol, erythritol, D- and L-arabinose, D-ribose, D- and L-xylose, D-adonitol, methyl β -D-xylopyranoside, D-galactose, D-fructose, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl α -D-mannopyranoside, methyl α -D-glucopyranoside, N-acetylglucosamine, amygdalin, arbutin, aesculin, ferric citrate, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, sucrose, D-trehalose, D-melezitose, D-raffinose, glycogen, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, D- and L-fucose, D- and L-arabitol, potassium gluconate, potassium 2-ketogluconate and potassium 5-ketogluconate. It was unable to ferment glucose, sucrose and mannitol.

Characteristic	Result
Salinity range (% seawater)	15–100
Oxidase	+
Catalase	+
Nitrate reduction	–
Alkaline phosphatase	+
Arginine dihydrolase	–
β -Galactosidase	–
Lecithinase	–
Utilization of:	
Tween 20	+
Gelatin	+
Agar	–
Casein	+
Cellulose	–
Chitin	–
DNA	–
Starch	–
Production of:	
H ₂ S	–
Indole	–
Hydrolysis of dead yeast cells	–
Haemolysis in rabbit blood agar	–

generated by MEGA version 2.1 (Kumar *et al.*, 2001) using the neighbour-joining method (Saitou & Nei, 1987) or the maximum-parsimony algorithm and evaluated by bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings.

Cells of strain UST040201-001^T were rod-shaped (0.3–0.5 × 0.5–3.8 μ m) and non-flagellated. Enlarged cells and filamentous cells were occasionally seen in stationary phase in broth culture. Colonies were 1.0–2.4 mm in diameter, light orange, circular, convex, smooth, glistening and translucent, with entire margins in MA after 5 days incubation at 30 °C. A photomicrograph showing colony morphology and phase-contrast and scanning electron micrographs are available as Supplementary Fig. S1 in IJSEM Online. Cells were motile by gliding in liquid culture but did not swarm on MA or YP-SW agar. UST040201-001^T was mesophilic, growing between 4 and 37 °C with optimum growth at 27–30 °C. Growth occurred between pH 5.0 and

Table 2. Whole-cell fatty acid profiles of UST040201-001^T and members of the family *Cryomorphaceae*

Strains: 1, UST040201-001^T; 2, *Owenweeksia hongkongensis* UST20020801^T; 3, *Brumimicrobium glaciale* IC156^T; 4, *Cryomorpha ignava* 1-22^T; 5, *Crocinitomix catalasitica* NCIMB 1418^T; 6, *Fluviicola taffensis* RW262^T. Data are from Bowman *et al.* (2003), Lau *et al.* (2005), O'Sullivan *et al.* (2005) and this study. UST040201-001^T and *O. hongkongensis* UST20020801^T were grown on MA at 12 °C for 5 days, *Brumimicrobium glaciale* IC156^T, *Cryomorpha ignava* 1-22^T and *Crocinitomix catalasitica* NCIMB 1418^T were grown on MA at 10–12 °C for 3–5 days (Bowman *et al.*, 2003) and *Fluviicola taffensis* RW262^T was grown on MA (minus NaCl) at 20 °C for 3 days (O'Sullivan *et al.*, 2005). Values given are percentages of total fatty acids. Fatty acids are designated as follows: total number of carbon atoms:number of double bonds, followed by the position of the double bond from the aliphatic end of the molecule. Prefixes i, a and OH represent iso-branched, anteiso-branched and hydroxy fatty acids, respectively. The suffix *c* represents a *cis* isomer. ECL, Equivalent chain length; NA, not applicable (component of summed feature); tr, trace (less than 0.1 %); –, not detected.

Fatty acid	1	2	3	4	5	6
13:0	0.1	–	0.5	–	–	0.1
14:0	1.6	–	1.5	–	0.3	3.2
15:0	–	–	2.9	0.8	29.3	7.5
16:0	0.5	0.3	1.9	0.9	1.8	3.0
17:0	–	–	–	–	–	0.2
18:0	–	–	0.3	–	–	–
i10:0	–	tr	–	–	–	–
i11:0	tr	0.3	–	–	–	–
i13:0	0.3	–	5.6	–	–	–
a13:0	–	–	tr	–	–	–
13:1 at 12–13	0.1	–	–	–	–	–
i14:0	–	–	0.8	13.8	–	–
i15:0	44.0	18.7	36.4	7.8	36.3	44.2
i15:1	34.8	28.0	–	–	–	–
a15:0	–	–	tr	5.8	–	0.5
i16:0	tr	tr	0.6	7.8	0.4	0.6
a16:0	–	0.2	–	–	–	–
i17:0	–	–	–	–	–	0.1
i14:1 ω 9 <i>c</i>	–	–	0.4	6.8	0.7	1.0
i15:1 ω 10 <i>c</i>	–	–	–	7.1	10.9	11.8
a15:1 ω 10 <i>c</i>	–	–	45.0	8.2	–	–
i16:1 ω 6 <i>c</i>	–	–	0.5	–	–	–
i16:1 ω 11 <i>c</i>	–	–	–	2.9	0.3	0.7
i17:1 ω 5 <i>c</i>	0.5	–	–	–	–	–
i17:1 ω 9 <i>c</i>	–	7.3	–	–	–	–
i17:1 ω 12 <i>c</i>	–	–	–	–	0.7	–
i17:1 ω 13 <i>c</i>	–	–	–	–	0.2	–
a17:1 ω 13 <i>c</i>	–	–	–	–	tr	–
15:1 ω 4 <i>c</i>	–	–	–	–	tr	–
15:1 ω 6 <i>c</i>	–	1.3	–	–	–	–
15:1 ω 8 <i>c</i>	–	–	1.4	–	–	–
15:1 ω 11 <i>c</i>	–	–	–	–	8.3	1.2
16:1 ω 5 <i>c</i>	–	–	–	–	tr	–
16:1 ω 7 <i>c</i>	NA	NA	1.5	–	–	–
16:1 ω 12 <i>c</i>	–	–	–	–	4.4	4.9

Table 2. cont.

Fatty acid	1	2	3	4	5	6
17:1 ω 13 <i>c</i>	–	–	–	–	2.1	0.7
18:1 ω 5 <i>c</i>	–	0.3	–	–	–	–
18:1 ω 7 <i>c</i>	–	–	0.3	–	–	–
18:1 ω 9 <i>c</i>	–	–	0.4	–	–	–
2-OH i14:0	–	–	–	6.7	–	–
2-OH 15:0	–	3.0	–	–	–	–
2-OH i15:0	NA	NA	–	1.6	–	3.5
2-OH a15:0	–	–	–	18.4	–	–
3-OH i14:0	tr	–	–	–	–	–
3-OH 15:0	–	1.0	–	–	tr	–
3-OH i15:0	2.5	4.9	–	–	1.0	0.5
3-OH 16:0	0.5	–	–	2.9	–	–
3-OH i16:0	0.4	0.2	–	6.8	tr	4.2
3-OH 17:0	–	1.2	–	–	tr	–
3-OH i17:0	9.3	18.1	–	–	3.3	12.3
Sum (2-OH i15:0/16:1 ω 7 <i>c</i>)	2.0	10.0	–	–	–	–
Sum (i16:1/3-OH 14:0)	tr	–	–	–	–	–
Unknown ECL 11:543	0.3	0.4	–	–	–	–
Unknown ECL 13:565	2.2	4.5	–	–	–	–
Unknown ECL 16:582	0.8	0.2	–	–	–	–

9:0 with optimum growth at around pH 7.0. The strain was halophilic, growing between 1.0 and 7.5 % (w/v) NaCl, with optimum growth at 1–3 %. Cells contained carotenoid pigments with major absorption peaks at 446, 471 and 502 nm. The isoprenoid quinone of UST040201-001^T was MK-6, which matches the major respiratory quinone of members of the family *Flavobacteriaceae* (Bernardet *et al.*, 2002). The DNA G + C content of UST040201-001^T was 35.8 ± 0.5 mol%, which is within the range of values for members of the family *Cryomorphaceae* (35–40 mol%). Table 1 lists the phenotypic features analysed.

The fatty acid profile of UST040201-001^T and those of phylogenetically related members of the family *Cryomorphaceae* are given in Table 2. Strain UST040201-001^T contained three major fatty acids: i15:0 (44.0 %), i15:1 (34.8 %) and 3-OH i17:0 (9.3 %), which accounted for 89 % of the total fatty acids. Similar to other members of the family *Cryomorphaceae* (Bowman *et al.*, 2003; O'Sullivan *et al.*, 2005), UST040201-001^T possessed high levels of C₁₃–C₁₇ branched-chain fatty acids (79.7 %). The fatty acid profile of UST040201-001^T showed more similarity to that of *Owenweeksia hongkongensis* UST20020801^T, but differed in the content of 14:0, i15:0, 3-OH i15:0 and 3-OH i17:0. In summary, the fatty acid profile of strain UST040201-001^T differed significantly from those of other members of the family *Cryomorphaceae* (Table 2).

The 16S rRNA gene sequence of strain UST040201-001^T (1504 nt) shared 89.2 % similarity with that of *Crocinitomix catalasitica* NCIMB 1418^T (Bowman *et al.*, 2003). Phylogenetic analysis showed that strain UST20020801^T formed a distinct lineage within the family *Cryomorphaceae*

Table 3. Characteristics that differentiate strain UST040201-001^T from the type species of other genera of the family Cryomorphaceae

Strains: 1, UST040201-001^T; 2, *Owenweeksia hongkongensis* UST20020801^T; 3, *Brumimicrobium glaciale* IC156^T; 4, *Cryomorpha ignava* 1-22^T; 5, *Crocinitomix catalasitica* NCIMB 1418^T; 6, *Fluviicola taffensis* RW262^T. Data are from Bowman *et al.* (2003), Lau *et al.* (2005), O'Sullivan *et al.* (2005) and this study. +, Positive; –, negative; v, variable; w, weak; ND, data not available. All strains are positive for catalase.

Characteristic	1	2	3	4	5	6
Habitat	Marine	Marine	Marine	Marine	Marine	Freshwater
Length of cells (µm)	0.5–3.8	0.5–4	1–3	0.5–3	3–100	1.5–5.7
Colour of cell mass*	O	O	O	O	Y	O–Y
Growth on/at:						
25 °C	+	+	v/w	–	+	+
35 °C	+	+	–	–	–	–
5 % NaCl	+	+	+	–	+	–
7.5 % NaCl	+	+	–	–	–	–
Metabolism†	A	A	F	A	A	A
Oxidase	+	+	–	–	–	–
Flexirubin reaction	–	–	–	–	–	+
Glucose utilization	–	–	+	–	–	–
Degradation of:						
Gelatin	+	+	–	–	–	W
Casein	+	–	–	–	–	ND
DNase	–	–	v	–	–	+
Phosphatase	+	+	+	–	+	ND
Nitrate reductase	–	–	+	–	–	–
DNA G + C content (mol%)	35.8 ± 0.5	39–40	38–40	36–37	35	37.2

*O, Orange; Y, yellow.

†A, Strictly aerobic; F, facultatively anaerobic.

Fig. 1. Phylogenetic relationship of strain UST040201-001^T and related taxa within the phylum *Bacteroidetes* based on 16S rRNA gene sequences. The tree was created by using the neighbour-joining method and percentages at nodes represent levels of bootstrap support from 1000 resampled datasets. *Escherichia coli* ATCC 11775^T was used as the outgroup. Bar, 0.05 nucleotide substitutions per position.

and linked to *Brumimicrobium glaciale* IC156^T with bootstrap support of 91 % by the neighbour-joining algorithm (Fig. 1) and 80 % by the maximum-parsimony analysis (data not shown). Sequence similarities to other members of the family *Cryomorphaceae* were less than 91 % (*Brumimicrobium glaciale* IC156^T, 90.1 %; *Fluviicola taffensis* RW262^T, 89.6 %; *Owenweeksia hongkongensis* UST20020801^T, 87.3 %; *Cryomorpha ignava* 1-22^T, 86.3 %), indicating that strain UST040201-001^T is distantly related to them at the genus level.

Strain UST040201-001^T displayed some common phenotypic traits of the *Cryomorphaceae*: rod- to filamentous-shaped cells, possession of carotenoid or flexirubin pigments, ability to perform aerobic respiration, requirement for complex organic growth factors like yeast extract or peptone and inability to utilize carbohydrates, arginine, agar and starch. UST040201-001^T could be differentiated from other members of the family *Cryomorphaceae* by several phenotypic tests (Table 3): from *Owenweeksia hongkongensis* UST20020801^T by its caseinase activity and its lower DNA G+C content; from *Fluviicola taffensis* RW262^T by its requirement for Na⁺ ions, the presence of oxidase, absence of DNase and susceptibility to chloramphenicol; and from *Brumimicrobium glaciale* IC156^T, *Crocinitomix catalasitica* NCIMB 1418^T and *Cryomorpha ignava* 1-22^T by its ability to grow at 35 °C, tolerance of 7.5 % NaCl and presence of oxidase.

On the basis of the characteristics described above, we propose that the strain UST040201-001^T should be placed in a new genus as a representative of a novel species, *Lishizhenia caseinilytica* gen. nov., sp. nov.

Description of *Lishizhenia* gen. nov.

Lishizhenia [Li.shi.zhe'ni.a. N.L. fem. n. *Lishizhenia* after Li Shizhen (1518–1593), the famous Chinese naturalist].

Cells are Gram-negative, halophilic, heterotrophic, non-flagellated and motile flexible short rods, which divide by binary fission. Cells do not form spores, are strictly aerobic and require Na⁺, sea salts and organic growth factors such as yeast extract or peptone for growth. Cells do not utilize carbohydrates. Cells contain oxidase, catalase and alkaline phosphatase. Major respiratory quinone is MK-6. Predominant fatty acids are i15:0, i15:1, 3-OH i17:0 and 2-OH i15:0/16:1 ω 7c. Phylogenetically, *Lishizhenia* is a member of the family *Cryomorphaceae* in the phylum *Bacteroidetes*. The type species is *Lishizhenia caseinilytica*.

Description of *Lishizhenia caseinilytica* sp. nov.

Lishizhenia caseinilytica (ca.sei.ni.ly'ti.ca. N.L. n. *caseinum* casein; N.L. fem. adj. *lytica* dissolving from Gr. v. *lyein* to dissolve; N.L. fem. adj. *caseinilytica* casein-dissolving).

Exhibits the following properties in addition to those given in the genus description. Cells are about 0.3–0.5 μ m in

diameter and 0.5–3.8 μ m in length. Enlarged cells and filamentous cells are seen occasionally in stationary phase in broth culture. Colonies are light orange, circular, convex, smooth, glistening and translucent with an entire margin. Cells are motile by gliding but do not swarm on MA. Mesophilic, growing between 4 and 37 °C with optimum growth at 27–30 °C. Growth occurs between pH 5.0 and 9.0 with optimum growth at around pH 7.0. Halophilic, growing between 1.0 and 7.5 % (w/v) NaCl with optimum growth at 1–3 %. Cells possess carotenoid pigments. Other physiological and biochemical properties are listed in Table 1. The fatty acid profile of the type strain is given in Table 2. Sensitive to ampicillin (10 μ g), chloramphenicol (30 μ g), penicillin G (2 U), rifampicin (10 μ g), tetracycline (30 μ g) and polymyxin B (300 U) but resistant to kanamycin (10 μ g), gentamicin sulfate (10 μ g) and spectinomycin (10 μ g). The DNA G+C content of the type strain is 35.8 $\pm$ 0.5 mol%.

The type strain is UST040201-001^T (=NRRL B-41434^T=JCM 13821^T), which was isolated from a sample of sand-filtered seawater collected at Port Shelter, adjacent to the Coastal Marine Laboratory, Hong Kong University of Science and Technology.

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