Lishizhenia tianjinensis sp. nov., isolated from coastal seawater

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A Gram-negative, non-carbohydrate-utilizing, non-flagellated, motile, flexible, long rod-shaped, orange-pigmented bacterium, strain H6^T, was isolated from coastal seawater of Tianjin City, China, and its taxonomic position was investigated by using a polyphasic approach. Strain H6^T grew optimally at 30 °C, in the presence of 2.0 % (w/v) NaCl and at pH 7.6. Menaquinone-6 (MK-6) was the sole respiratory quinone and the major fatty acids were iso-C_{15:0} (36.5 % of the total), iso-C_{15:1} (27.3 %) and iso-C_{17:0} 3-OH (10.8 %). The DNA G + C content of strain H6^T was 34.6 mol%. Phylogenetic analysis based on 16S rRNA gene sequences demonstrated that strain H6^T was related most closely to *Lishizhenia caseinilytica* JCM 13821^T (97.8 % similarity). DNA–DNA relatedness between the two strains was 47.4 %. Strain H6^T could be further differentiated from *L. caseinilytica* JCM 13821^T based on activity of several enzymes, hydrolysis of casein and Tweens 40, 60 and 80 and production of H₂S. On the basis of phenotypic, chemotaxonomic, genomic and phylogenetic data, strain H6^T is considered to represent a novel species of the genus *Lishizhenia*, for which the name *Lishizhenia tianjinensis* sp. nov. is proposed. The type strain is H6^T (=CGMCC 1.7005^T =JCM 15141^T).

The genus *Lishizhenia* was established by Lau *et al.* (2006). At the time of writing, the genus comprises one recognized species, *Lishizhenia caseinilytica* (Lau *et al.*, 2006). The genus *Lishizhenia* is a member of the family *Cryomorphaceae* (Bowman *et al.*, 2003) in the class *Flavobacteria* (designation according to Cavalier-Smith, 2002) of the phylum *Bacteroidetes*. Members of the *Cryomorphaceae* are psychrotolerant, rod-shaped to filamentous and possess carotenoid pigments. They also require seawater salts and complex organic compounds for growth and are unable to utilize carbohydrates (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). In a study of the bacterial diversity in Tianjin coastal seawater, a non-carbohydrate-utilizing, mesophilic, orange-pigmented, rod-shaped, moderately halophilic bacterium, strain H6^T, was isolated and subsequently characterized by use of a polyphasic taxonomic study. On the

basis of the phenotypic, chemotaxonomic and phylogenetic data presented herein, strain H6^T is considered to represent a novel species of the genus *Lishizhenia*.

A coastal seawater sample was collected from Tianjin, China. The sample was diluted 10-fold with sterilized 2 % NaCl (w/v) solution, spread on low-organic seawater medium (LOSWM; containing 1.0 g peptone, 0.50 g yeast extract and 15 g agar per litre of seawater) and was incubated at 30 °C for 10 days. Individual colonies were picked, cultured in LOSWM and stored at –20 °C in 20 % (v/v) glycerol. Strain H6^T was obtained after several streakings and transfers on LOSWM plates; the organism was also able to grow in marine broth 2216 (MB; Difco) and on marine agar 2216 (MA; Difco).

Routine cultivation was conducted at 30 °C on MA. Cell morphology was examined by transmission electron microscopy (H600; Hitachi) and scanning electron microscopy (200; FEI Quanta). Gliding motility was investigated as described by Bowman (2000). The Gram reaction was determined by staining cells grown on MA at 30 °C for 24 h according to the method described by Gerhardt *et al.* (1994). Endospore formation was determined after malachite green staining (Dong & Cai, 2001) of cells grown on MA. Cells of strain H6^T were Gram-negative, non-spore-forming, non-flagellated, motile, flexible rods, 0.3–0.4 µm wide and 1.5–3.5 µm long (see Supplementary Fig. S1 in IJSEM Online).

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain H6^T is EU183317.

A scanning electron micrograph of cells of strain H6^T and cellular fatty acid profiles of strain H6^T and *Lishizhenia caseinilytica* JCM 13821^T are available as supplementary material with the online version of this paper.

The pH range for growth was determined in MB that was adjusted to various pH values (initial pH 7.0–10, at intervals of 0.2 pH units) with HCl or Na₂CO₃. Growth at various temperatures (0–40 °C) was measured on MA. The requirement for sodium ions and tolerance of NaCl were determined in MB with NaCl concentrations of 0–7.0 % (w/v, intervals of 0.5 % NaCl). Anaerobic growth was determined in MB in anaerobic test tubes with nitrogen gas. Catalase activity was determined based on the formation of bubbles after a 3 % H₂O₂ solution was dropped onto a fresh colony. Oxidase activity and hydrolysis of casein, starch and Tweens 20, 40, 60 and 80 were determined as described by Dong & Cai (2001) but using artificial seawater (ASW) instead of distilled water. The ASW contained (per litre distilled water) 23.6 g NaCl, 0.64 g KCl, 4.53 g MgCl₂·6H₂O, 5.94 g MgSO₄·7H₂O and 1.3 g CaCl₂·2H₂O (Bruns *et al.*, 2001). Acid production from carbohydrates was determined by using API 50CH strips (bioMérieux), with a medium comprising 50 % CHB/E medium (bioMérieux) with 0.075 % CaCl₂·2H₂O, 1.875 % NaCl and 0.375 % MgCl₂. Carbohydrate assimilation was determined by using API 50CH strips, with ASW supplemented with 0.05 % yeast extract for resuspension of cells, and the strips were incubated at 30 °C for 2 weeks. Additional biochemical tests were performed by using the API 20NE and API ZYM galleries (bioMérieux). The above tests were performed according to the manufacturer's instructions. Susceptibility to antibiotics was determined on MA plates by using filter-paper discs (Beijing Pharmaceutical Company) containing various antibiotics as specified in the species description. The phenotypic properties of strain H6^T are given in Table 1 and in the species description.

The absorption spectrum was examined by spectroscopy. Strain H6^T was grown in MB. Cells were harvested, resuspended in absolute ethanol and sonicated for 10 s. The sonicated cell material was then filtered through a 0.2-

µm Whatman filter and the absorption spectrum between 300 and 700 nm was determined with a UNICO UV-2802H spectrophotometer (Shanghai Optical Company). The bathochromic shift test for flexirubin was performed by addition of 20 % KOH (Fautz & Reichenbach, 1980). Strain H6^T contained carotenoid pigments with major absorption peaks at 445, 471 and 503 nm, as found for *L. caseinilytica* JCM 13821^T (Lau *et al.*, 2006).

The cellular fatty acid profile of strain H6^T was determined via the MIDI Sherlock Microbial Identification System (Microbial ID) with cells grown on MA at 30 °C for 3 days. Isoprenoid quinone analysis was performed by the HPLC method (Collins, 1994), with menaquinones extracted from *L. caseinilytica* JCM 13821^T (Lau *et al.*, 2006) as reference. Strain H6^T contained iso-C_{15:0} (36.5 % of the total), iso-C_{15:1} (27.3 %) and iso-C_{17:0} 3-OH (10.8 %) as major fatty acids, a similar profile to that of *L. caseinilytica* JCM 13821^T. Strain H6^T differed from *L. caseinilytica* JCM 13821^T in that C_{15:0}, C_{15:0} 2-OH and C_{16:0} 2-OH were present at low levels (1.1–2.1 %), while C_{16:0} 3-OH was not detected (Supplementary Table S1). The sole respiratory quinone was menaquinone-6 (MK-6).

The G + C content of the genomic DNA was determined by thermal denaturation (Marmur & Doty, 1962) with DNA from *Escherichia coli* K-12 as a standard for calibration of the *T_m* value. The G + C content of strain H6^T was 34.6 mol%.

The 16S rRNA gene of strain H6^T was amplified by using two bacterial universal primers, 27F and 1492R (Lane, 1991), and was sequenced as described by Zhang *et al.* (2003). Searches for similar sequences were made via the BLAST program (Altschul *et al.*, 1990) on the NCBI database. Strain H6^T showed 97.8 % 16S rRNA gene sequence similarity to *L. caseinilytica* JCM 13821^T, but less than 93 % similarity to other members of the family *Cryomorphaceae*. A phylogenetic tree of 16S rRNA gene

Table 1. Differential characteristics between strain H6^T and the type strain of *Lishizhenia caseinilytica*

Strains: 1, H6^T; 2, *L. caseinilytica* JCM 13821^T (data from Lau *et al.*, 2006). The two strains were positive for motility, requirement of sodium ions for growth, oxidase activity and hydrolysis of gelatin and Tween 20 and negative for utilization of and acid production from carbohydrates, nitrate reduction, indole production, activity of α-chymotrypsin, α-galactosidase and arginine dihydrolase, and hydrolysis of starch and agar. +, Positive; –, negative; w, weakly positive.

Characteristic	1	2
Enzyme activity		
Catalase	–	+
Esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, acid phosphatase, β-galactosidase, N-acetyl-β-glucosaminidase	+	–
Naphthol-AS-BI-phosphohydrolase	w	–
Hydrolysis of:		
Casein	–	+
Tweens 40, 60 and 80	+	–
Production of H ₂ S	+	–
DNA G + C content (mol%)	34.6	35.8 ± 0.5

sequences was constructed by the neighbour-joining method (Saitou & Nei, 1987) with MEGA version 3.1 (Kumar *et al.*, 2004). Strain H6^T formed a distinct phylogenetic lineage within the genus *Lishizhenia* (Fig. 1).

Differential characteristics between strain H6^T and *L. caseinilytica* JCM 13821^T are given in Table 1. Strain E6^T could be differentiated on the basis of several phenotypic features, including activity of some enzymes, hydrolysis of casein and Tweens 40, 60 and 80 and production of H₂S. In order further to distinguish strain H6^T from *L. caseinilytica* JCM 13821^T, DNA–DNA hybridization was carried out by the thermal denaturation and renaturation method (De Ley *et al.*, 1970) as modified by Huß *et al.* (1983). The level of DNA–DNA relatedness between strain H6^T and *L. caseinilytica* JCM 13821^T was 47.4 %.

On the basis of phenotypic, chemotaxonomic, genomic and phylogenetic characteristics, we suggest that strain H6^T represents a novel species of the genus *Lishizhenia*, for which the name *Lishizhenia tianjinensis* sp. nov. is proposed.

Description of *Lishizhenia tianjinensis* sp. nov.

Lishizhenia tianjinensis (tian.ji.nen'sis. N.L. fem. adj. *tianjinensis* pertaining to Tianjin, from where the type strain was isolated).

Strictly aerobic and heterotrophic. Cells are Gram-negative, non-spore-forming, non-flagellated, motile, flexible rods, 0.3–0.4 µm wide and 1.5–3.5 µm long. Colonies are 1–2 mm in diameter, orange-pigmented, smooth and with a mucoid consistency after cultivation on MA at 30 °C for 24 h. Growth is observed in the presence of 0.5–6.5 % NaCl (w/v), at 4–36 °C and at pH 7.2–9.6, with optimum growth in 2 % NaCl, at 30 °C and at pH 7.6. In the API ZYM system, shows activity for esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosidase and *N*-acetyl-β-glucosaminidase, but not for alkaline phosphatase, α-galactosidase, lipase (C14), α-chymotrypsin, β-glucuronidase, α-glucosidase, β-glucosidase, α-mannosidase or β-fucosidase. Resistant to (µg per disc) streptomycin (10) and neomycin

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequence analysis showing the position of strain H6^T and related species. Numbers at nodes represent percentage bootstrap support based on an analysis of 1000 resampled datasets. Bar, 2 % sequence divergence.

(30), but sensitive to benzylpenicillin (10), norfloxacin (10) carbenicillin (100), polymyxin B (300), ampicillin (10), vancomycin (30), ciprofloxacin (5), rifampicin (5), chloramphenicol (30), kanamycin (30), erythromycin (15), tetracycline (30), gentamicin (10) and novobiocin (5). Cells possess carotenoid pigments. The sole respiratory quinone is MK-6. Major fatty acids are iso-C_{15:0}, iso-C_{15:1} and iso-C_{17:0} 3-OH. The DNA G + C content of the type strain is 34.6 mol%. Additional characteristics are given in Table 1.

The type strain, H6^T (=CGMCC 1.7005^T =JCM 15141^T), was isolated from coastal seawater of Tianjin, China.

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