Flaviflexus huanghaiensis gen. nov., sp. nov., an actinobacterium of the family Actinomycetaceae

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Strain H5T was isolated from a sediment sample collected from the coastal area of Qingdao, China. The cells were Gram-stain-positive, non-motile, straight or curved rods. The temperature range for growth was 20–37 °C and the pH for growth ranged from 6.5 to 9.0, with optimum growth occurring in the temperature range 28–30 °C and pH range 7.5–8.0. Growth occurred in the presence of 0–6 % (w/v) NaCl (optimum, 0–2 %). Strain H5T had MK-9, MK-9(H2) and MK-9(H4) as the major menaquinones and C18:1ω9c, C16:0ω0, C14:0ω0, C18:0ω0 and C16:1ω9c as major fatty acids. The cell-wall peptidoglycan type was A5α-L-Lys-L-Ala-L-Lys-D-Glu. The major polar lipids were phosphatidylglycerol (PG), an unknown phospholipid (PL1) and two unknown phosphoglycolipids (PGL1, PGL2). An unknown phospholipid (PL2) and two unknown glycolipids (GL1, GL2) were present in moderate to minor amounts in the polar lipid profile. The genomic DNA G+C content was 61.8 mol%. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain H5T represents a novel lineage in the family Actinomycetaceae. On the basis of the phenotypic, physiological and molecular characteristics, it is proposed that the novel isolate should be classified as a novel species in a new genus: Flaviflexus huanghaiensis. The strain H5T (=DSM 24315T = CICC 10486T) is the type strain of the type species.

At the time of writing, the family Actinomycetaceae includes six genera. Many members of this family were isolated from clinical specimens. Recently, Arcanobacterium phocae was identified from seals collected from coastal waters around Scotland (Ramos et al., 1997) and Actinomyces marinimammalium was recovered from marine mammals (Hoyles et al., 2001). In this study, we report the characterization of a novel actinobacterium isolated from a marine sediment sample collected from the coastal area of Qingdao, China (120°19’14”E, 36°03’31”N).

The sediment sample (approximately 1 g) was diluted with 9 ml sterile seawater and spread onto 2216 Marine Agar (MA; Difco). The plates were incubated at 28 °C for 5 days. Strain H5T was obtained in pure culture after three successive transfers to fresh MA plates and stored at −80 °C in 20 % (v/v) glycerol. The isolate was grown aerobically at 28 °C on tryptone soya agar (TSA; Hopebio) and maintained at room temperature. General taxonomic characterization of strain H5T was performed as described by Jordan et al. (2007). The cell morphology and flagella of strain H5T were observed by transmission electron microscopy. The colony morphology was observed on TSA after 5 days of incubation at 28 °C and cells were resuspended in saline for use as an inoculum. The effects of different growth temperatures were assessed on TSA plates with incubation at 4, 10, 15, 20, 26, 28, 30, 32, 37, 40 and 42 °C. The plates were incubated for 4 weeks and growth was indicated by visible colonies. The pH range for growth was determined by adding MES (pH 6.0), PIPES (pH 6.5 and 7.0), HEPES (pH 7.5 and 8.0), Tricine (pH 8.5) or N-cyclohexyl-2-hydroxy-3-aminopropanesulfonic acid (CAPSO) (pH 9.0 and 9.5) to TSB medium at concentrations of 20 mM. The pH of the medium was adjusted by addition of 1 M HCl or NaOH before autoclaving. Tolerance of 0, 1, 2, 4, 6 and 8 % (w/v) NaCl was assessed on plate count agar (PCA; Oxoid). Antibiotic sensitivity was assessed as described by CLSI (2012): a cell suspension (McFarland standard 0.5) was swabbed over the surface of Iso-Sensitest agar (Oxoid) plates to create a uniform lawn before aseptic placement of antibiotic discs onto the agar surface. Inoculated plates were incubated at 28 °C for up to 7 days.

Abbreviation: CAPSO, N-cyclohexyl-2-hydroxy-3-aminopropanesulfonic acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of Flaviflexus huanghaiensis H5T is JN815236.

Two supplementary figures are available with the online version of this paper.
Oxidase and catalase activities were determined by using standard methods. Routine tests, like Gram staining and agarase, amylase, urease, catalase, gelatinase and oxidase activities, were carried out as described by Smibert & Krieg (1994). The culture was characterized biochemically using the API 20E, API 20NE and API ZYM systems according to the manufacturer’s instructions (bioMérieux). Acid production from carbohydrates was determined with API 20E, API 20NE and API 50CH strips (bioMérieux). The API 20E, API 20NE and API 50CH strips were read after 5 days incubation at 28 °C. The isolates were further tested for their ability to oxidize different carbon sources using GEN III MicroPlates (Biolog) according to the manufacturer’s instructions. Data were analysed using the software package provided by Biolog. All of the tests were performed in duplicate.

Cellular fatty acids were determined on a 5 day old culture (end of the exponential phase) grown on TSA plates after incubation at 28 °C. The fatty acids were extracted, methylated and analysed using the standard MIDI (Microbial Identification) system (Sasser, 1990). Analysis of respiratory quinones and polar lipids was carried out for the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany. Respiratory quinones were extracted and purified according to the protocol of Collins (1985) and were analysed by HPLC (Wu et al., 1989). Polar lipids were extracted and examined by two-dimensional TLC and identified using previously described procedures (Minnikin et al., 1984). Cell wall peptidoglycan was isolated and analysed by the methods of Schleifer & Kandler (1972), Schleifer (1985), MacKenzie (1987) and Groth et al. (1996). The G+C content of the DNA was determined directly by HPLC according to a method described previously (Tamaoka & Komagata, 1984; Mesbah et al., 1989).

DNA was extracted and purified using a bacteria genomic DNA Mini kit (TaKaRa Bio) following the manufacturer’s protocol. The 16S rRNA gene was amplified by PCR with two universal primers 27f and 1492r (Jordan et al., 2007). The purified PCR product was ligated to the vector pMD 18-T (TaKaRa Bio) and cloned according to the manufacturer’s instructions. Sequencing reactions were carried out using an ABI BigDye 3.1 Sequencing kit (Applied BioSystems) and an automated DNA sequencer (ABI3730; Applied BioSystems).

The nearly complete 16S rRNA gene sequence (1497 bp) of strain H5T was submitted to GenBank and EMBL to search for similar sequences using the BLAST algorithm. Multiple sequence alignment was carried out using the CLUSTAL_X 2.0 program and the sequence alignment was carefully edited manually (Thompson et al., 1997). A phylogenetic dendrogram of strain H5T and some closely related members of the family Actinomycetaceae based on 16S rRNA gene sequences, was constructed using the neighbour-joining method in the MEGA software package version 4 (Tamura et al., 2007). The resultant tree topologies were evaluated by bootstrap analysis based on 1000 replicates. Phylogenetic analysis (Fig. 1) including representative members of all six genera of the family Actinomycetaceae and some related genera showed that strain H5T grouped with members of the genera Arcanobacterium and Trueperella. This separate phylogenetic position indicated that strain H5T deserved the status of a novel genus. This topology was also supported by results obtained using maximum-parsimony and maximum-likelihood algorithms.

![Fig. 1. Neighbour-joining tree constructed with 16S rRNA gene sequences, showing the phylogenetic position of strain H5T within the family Actinomycetaceae. The tree was constructed using the neighbour-joining algorithm. Numbers at nodes represent bootstrap values (% of 1000 replicates); only values ≥ 50% are shown. GenBank accession numbers of 16S rRNA gene sequences are given in parentheses. Bar, 1 % sequence divergence.](image-url)
are C18:1 phosphoglycolipids (PGL1, PGL2). The major fatty acids of strain H5T were straight or curved rods, and flagella were not observed. A representative transmission electron micrograph of strain H5T is shown in Fig. S1, available in IJSEM Online. Strain H5T was non-motile and non-endospore-forming. Even so, the isolate was catalase- and oxidase-negative. Strain H5T contained MK-9 (14%), MK-9(H2) (9%) and MK-9(H4) (67%) as the major menaquinones. The major polar lipids were phosphatidylglycerol (PG), an unknown phospholipid (PL1) and two unknown phosphoglycolipids (PGL1 and PGL2). An unknown phospholipid (PL2) and two unknown glycolipids (GL1, GL2) were present in moderate to minor amounts in the polar lipid profile (Fig. S2). The cell-wall peptidoglycan type was A5z L-Lys-L-Ala-L-Lys-d-Glu. The amino acids found in the cell wall hydrolysate were lysine, alanine and glutamic acid in a molar ratio of 4.2 Ala:2.3 Glu:1.0 Lys. The predominant cellular fatty acids of strain H5T were C18:1ω9c (52.82%), C16:0 (31.57%), C14:0 (5.51%), C18:0 (4.75%) and C16:1ω9c (3.08%). The genomic DNA G+C content was 61.8 mol%.

The complete morphological, physiological and biochemical characteristics for strain H5T are given in the species description. The characteristics that differentiate strain H5T from the other six genera of the family Actinomycetaceae are shown in Table 1. Based on the phenotypic and phylogenetic evidence, strain H5T cannot be placed in any of the six genera of the family Actinomycetaceae and we propose to create a new genus, named Flaviflexus gen. nov. Strain H5T represents the type strain of the type species, Flaviflexus huanghaiensis sp. nov.

**Description of Flaviflexus gen. nov.**

Flaviflexus (Fla.vi.flex'us. L. adj. flavus yellow; L. masc. n. flexus bend, curve; N.L. masc. n. Flaviflexus a yellow-coloured bend).

Cells are Gram-stain-positive, non-motile, straight or slightly curved rods and facultatively anaerobic. After 72–96 h of incubation on TSA agar, colonies are 0.5–1.5 mm in diameter, yellow, convex and circular with entire edges. The cell-wall peptidoglycan type is A5z L-Lys-L-Ala-L-Lys-d-Glu. The major polar lipids are phosphatidylglycerol (PG), an unknown phospholipid (PL1) and two unknown phosphoglycolipids (PGL1, PGL2). The major fatty acids are C18:1ω9c, C16:0, C14:0, C18:0 and C16:1ω9c. The principal menaquinone is MK-9(H4). The type species is Flaviflexus huanghaiensis.

**Description of Flaviflexus huanghaiensis sp. nov.**

Flaviflexus huanghaiensis (hu.ang.hai.en’sis. N.L. masc. adj. huanghaiensis pertaining to Huanghai, the Chinese name for the Yellow Sea, the geographical origin of the type strain).

Displays the following characteristics in addition to those in the genus description. Cells are 0.4–0.5 µm x 1.8–2.2 µm in size. Colonies are yellow, smooth, circular and convex with entire margins after 4 days at 28 °C on TSA agar. Catalase and oxidase are not produced. Growth occurs at 20–37 °C and pH 6.5–9.0, with optimum growth occurring in the temperature range 28–30 °C and pH range 7.5–8.0. Growth occurs in the presence of 0–6% (w/v) NaCl, with optimum growth occurring in the presence of 0–2% (w/v) NaCl. Starch and gelatin are hydrolysed, but urea and cellulose are not. Negative for indole production and positive for the Voges–Proskauer reaction. Cells are positive for the activities of esterase lipase (C8), Leucine arylamidase and naphthol-AS-BI-phosphohydrolase. Alkaline phosphatase, esterase, esterase lipase (C4), lipase (C14), valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, acid phosphatase, z-galactosidase, b-galactosidase, b-glucuronidase, z-glucosidase, b-glucosidase, N-acetyl-b-glucosaminidase, z-mannosidase, z-fucosidase, lysine decarboxylase and ornithine decarboxylase are not produced. The following results for carbon source assimilation were positive according to Biolog GEN III: dextrin, sucrose, turanose, z-D-glucose, D-fructose, inosine, fusidic acid, D-sorbitol, D-arabitol, glycerol, pectin, tetrazolium violet, tetrazolium blue, p-hydroxyphenylacetic acid, Tween 40, \( \gamma \)-aminobutyric acid, acetoacetic acid, propionic acid, acetic acid and sodium butyrate. Acid is produced from d-ribose, aesculin ferric citrate, sucrose, xylitol, D-lyxose, D-tagatose, potassium 2-keto-D-gluconate and potassium 5-keto-D-glucuronate in API 50CH strips. The type strain is resistant to nalidixic acid (30 µg), nitrofurantoin (30 µg) and cotrimoxazole (25 µg) but sensitive to tetracycline (30 µg), penicillin G (1 µg), ampicillin (10 µg), chloramphenicol (30 µg), gentamicin (10 µg), kanamycin (30 µg), carbenicillin (100 µg), acetylsalicylic acid (30 µg), tobramycin (10 µg), midecamycin (30 µg) and streptomycin (10 µg), as determined by antibiotic discs. The major polar lipids were phosphatidylglycerol (PG), an unknown phospholipid (PL1) and two unknown phosphoglycolipids (PGL1 and PGL2). An unknown phospholipid (PL2) and two unknown glycolipids (GL1 and GL2) are present in moderate to minor amounts in the polar lipid profile. The major menaquinones are MK-9(H4), MK-9 and MK-9(H4).

The type strain, H5T (=DSM 24315=CCIC 10486T), was isolated from a sediment sample collected from the coastal area of Qingdao, China. The genomic DNA G+C content of the type strain is 61.8 mol%.

**Acknowledgements**

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**Table 1.** Differential characteristics of strain HS\(^T\) and related genera of the family Actinomycetaceae

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
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<th>5</th>
<th>6</th>
<th>7</th>
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<tbody>
<tr>
<td>Cell morphology</td>
<td></td>
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<tr>
<td>Shape</td>
<td>Straight to slightly curved rods</td>
<td>Straight to slightly curved rods</td>
<td>Straight or slightly curved rods with or without clubbed ends</td>
<td>Slender, irregular rods or granular, segmented cocci</td>
<td>Curved rods with tapered ends</td>
<td>Coccobacilli and short rods</td>
<td>Short, straight or curved diphteroid rods</td>
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<td>Filaments</td>
<td>-</td>
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<td>+</td>
<td>-</td>
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<tr>
<td>O(_2) metabolism</td>
<td>An/F</td>
<td>An/F</td>
<td>An/F</td>
<td>F</td>
<td>AN</td>
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<tr>
<td>Optimum temperature for growth (°C)</td>
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<td>30–37</td>
<td>37</td>
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<td>Acid production from glucose</td>
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<td>V</td>
<td>V</td>
<td>+</td>
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<td>+</td>
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<td>Catalase</td>
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<td>V</td>
<td>+</td>
<td>ND</td>
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<td>V</td>
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<td>Major polar lipids</td>
<td>PG, PL1, PGL1, PGL2</td>
<td>DPG, PG, PI, PIM</td>
<td>DPG, PC, PI, PIM</td>
<td>DPG, PC, PI, PIM</td>
<td>DPG, PG, PI, PIM</td>
<td>DPG, PG, PI, PIM</td>
<td>DPG, PG, PI, PIM</td>
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<tr>
<td>Respiratory quinones</td>
<td>MK-9(H(_4))(^*)</td>
<td>MK-10(H(_4))(^*)</td>
<td>MK-10(^*)</td>
<td>MK-9(H(_4))(^*)</td>
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<td>MK-10(H(_4))(^*)</td>
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<tr>
<td>DNA G+C content (mol%)</td>
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<td>55–57</td>
<td>54–70</td>
<td>50–57</td>
<td>49–54</td>
<td>56–66</td>
<td>51.7</td>
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\(^{*}\) Major component of respiratory quinones.
References


