Actinoalloteichus hymeniacidonis sp. nov., an actinomycete isolated from the marine sponge *Hymeniacidon perleve*

Haitao Zhang,^{1,2} Wen Zheng,³ Jianyu Huang,^{1,2} Hongli Luo,³ Yan Jin,¹ Wei Zhang,^{1,4} Zhiheng Liu³ and Ying Huang³

Correspondence

Wei Zhang

weizhang@dicp.ac.cn

Ying Huang

huangy@im.ac.cn

¹Marine Bioproducts Engineering Group, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China

²Graduate School of the Chinese Academy of Sciences, Chinese Academy of Sciences, Beijing 100049, China

³State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100080, China

⁴Department of Medical Biotechnology, School of Medicine, Flinders University, Adelaide, SA 5042, Australia

A polyphasic study was undertaken to establish the taxonomic status of a Gram-positive, aerobic actinomycete, strain HPA177^T, isolated from a marine sponge, *Hymeniacidon perleve*. The organism formed branching, non-fragmenting vegetative hyphae and produced black pigment. Chemotaxonomic characteristics were consistent with its assignment to the genus *Actinoalloteichus*. Analysis of the 16S rRNA gene sequence showed that strain HPA177^T formed a robust clade with type strains of the genus *Actinoalloteichus*, but was distinct from them. A number of phenotypic characteristics also readily distinguished strain HPA177^T from species of the genus *Actinoalloteichus* with validly published names. On the basis of the above data, it is proposed that strain HPA177^T represents a novel species, *Actinoalloteichus hymeniacidonis* sp. nov. The type strain of *Actinoalloteichus hymeniacidonis* is HPA177^T (= CGMCC 4.2500^T = JCM 13436^T).

The genus *Actinoalloteichus* was established by Tamura *et al.* (2000) after several initial taxonomic studies on its phenotypic and genotypic properties by Liu *et al.* (1984), Itoh *et al.* (1987) and Tamura & Hatano (1998). The genus currently comprises only two species, *Actinoalloteichus cyanogriseus* (Tamura *et al.*, 2000) and *Actinoalloteichus spitiensis* (Singla *et al.*, 2005). During the investigation of actinomycete diversity of an intertidal marine sponge, *Hymeniacidon perleve*, a novel actinomycete, strain HPA177^T, was isolated. The aim of the present study was to determine the taxonomic status of this strain using a polyphasic approach.

Strain HPA177^T was isolated by the following procedure. The marine sponge was collected at the inter-tidal beach of Dalian, on the Chinese Yellow Sea, located in northern China (38° 52' N 121° 41' E). Freshly collected sponge specimens were rinsed five times in sterile seawater to remove transient and loosely attached bacteria and then thoroughly homogenized in a sterile mortar. A 10-fold

dilution series of sponge homogenate was made and plated in triplicate on modified arginine/glycerol agar (ISP medium 5) plates (6 ml 100 % glycerol, 1 g arginine, 1 g K₂HPO₄, 0.5 g MgSO₄, 18 g agar and 1 l natural seawater). After incubating the plates at 28 °C for 4 weeks, an isolate, designated HPA177^T, was picked and subcultured on TSA plates (Difco) until a pure culture was obtained.

The arrangement of hyphae and spore chains of strain HPA177^T were observed on yeast extract/malt extract agar (ISP medium 2) and oatmeal agar (ISP medium 3) after 2–3 weeks at 28 °C using light and scanning electron microscopes. Cultural characteristics were observed on a number of standard media (Table 1) after 2 weeks at 28 °C. The strain was examined further for a range of physiological properties using established procedures described by Gordon *et al.* (1974) and Yokota *et al.* (1993). Biomass for chemical studies was prepared by growing the strain in shake flasks of TSB at 28 °C for 7 days. Cells were harvested by centrifugation, washed with distilled water and freeze-dried. The isomers of diaminopimelic acid (A₂pm) and whole-organism sugars were analysed according to the procedures developed by Hasegawa *et al.* (1983) and Lechevalier

Abbreviation: A₂pm, diaminopimelic acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain HPA177^T is DQ144222.

Table 1. Growth and cultural characteristics of strain HPA177^T

Agar medium	Growth	Aerial mycelium	Substrate mycelium	Diffusile pigment
Yeast extract/malt extract (ISP 2)	Abundant	Brownish-purplish-grey; abundant	Brownish-black	Black
Oatmeal (ISP 3)	Abundant	Pale grey; abundant	Grey	Smoky grey
Inorganic salts/starch (ISP 4)	Poor	Colourless; poor	Pale grey	None
Glycerol/asparagine (ISP 5)	Poor	Pale greyish-white; moderate	Light olive grey	Light purplish-grey
Peptone/yeast extract/iron (ISP 6)	Moderate	Greyish-white; moderate	Greyish-brown	Black
Tyrosine (ISP 7)	Poor	Greyish-white; poor	Light greyish-brown	Greyish-brown
Modified Bennett's	Abundant	Brownish-grey; abundant	Olive black	Black

& Lechevalier (1980). Polar lipids were examined by two-dimensional TLC and identified using the method of Minnikin *et al.* (1984). Menaquinones were extracted from freeze-dried biomass and purified according to Collins (1985); the purified preparation was analysed using an HPLC procedure (Wu *et al.*, 1989). Mycolic acids were checked by the acid methanolysis method as described previously (Minnikin *et al.*, 1980). Fatty acids were extracted, methylated and analysed by GC using the standard Sherlock MIDI (Microbial Identification) system (Sasser, 1990; Kämpfer & Kroppenstedt, 1996).

The morphological characteristics of strain HPA177^T were similar to those of *A. cyanogriseus*, the type species of the genus. It formed grey to black, branched vegetative hyphae that tended to fragment after 3 weeks of cultivation, aerial hyphae with straight spore chains (Fig. 1) and a greyish aerial spore mass on several standard media (Table 1). The assignment of strain HPA177^T to the genus *Actinoalloteichus* is also supported by its chemotaxonomic profile. This strain contained *meso*-A₂pm as the cell wall diamino acid, galactose, glucose, mannose and ribose as the diagnostic sugars in whole-organism hydrolysates and tetrahydrogenated menaquinone with nine isoprene units [MK-9(H₄)] (64 %) as the

predominant isoprenoid quinone, with smaller amounts of MK-9(H₆) (23 %) and MK-9(H₈) (12 %). It also contained phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannoside and some phospholipids of unknown structure containing glucosamine as diagnostic polar lipids, but lacked mycolic acids (data not shown). The predominant cellular fatty acids were 15:0 anteiso (20 %), 17:1 ω 8c (19 %), 16:0 iso (16 %) and 17:0 (11 %). Strain HPA177^T differed significantly from *A. cyanogriseus* (Tamura *et al.*, 2000) and *A. spitiensis* (Singla *et al.*, 2005) in having larger amounts of 17:1 ω 8c and 17:0. The complete fatty acid profile and differences from those of *A. cyanogriseus* and *A. spitiensis* are shown in Table 2.

Table 2. Chemotaxonomic characteristics that distinguish strain HPA177^T from *A. cyanogriseus* IFO 14455^T and *A. spitiensis* DSM 44848^T

Strains: 1, HPA177^T; 2, *A. cyanogriseus* IFO 14455^T (data from Tamura *et al.*, 2000); 3, *A. spitiensis* DSM 44848^T (data from Singla *et al.*, 2005). Values less than 2 % are not shown. —, Not detected.

Characteristic	1	2	3
Cellular fatty acids (%)			
14:0 iso	4	5	6
15:0 iso	6	15	17
15:0 anteiso	20	10	7
15:0	6	—	—
16:1 iso	—	8	6
16:1	—	2	2
16:0 iso G	6	—	—
16:0 iso	16	19	33
16:0	—	2	2
17:0 iso	—	3	3
17:0 anteiso	4	20	8
17:1 ω 8c	19	—	—
17:0	11	—	—
Menaquinones (%)			
MK-8(H ₄)	—	10	5
MK-9(H ₂)	—	9	—
MK-9(H ₄)	64	75	82
MK-9(H ₆)	23	2	2
MK-9(H ₈)	12	—	—
MK-10(H ₄)	—	3	9

Fig. 1. Scanning electron micrograph of strain HPA177^T grown on yeast extract/malt extract agar for 14 days at 28 °C. Bar, 5 μm.

Fig. 2. Neighbour-joining tree showing the phylogenetic position of strain HPA177^T and related taxa based on 16S rRNA gene sequences. *Streptomyces ambofaciens* ATCC 23877^T was used as the root organism. Numbers at nodes represent levels of bootstrap support, based on neighbour-joining analyses of 1000 resampled datasets. Solid circles represent clades that were also recovered in the maximum-parsimony tree. Bar, 0.01 substitutions per nucleotide position.

For 16S rRNA gene sequencing, genomic DNA of HPA177^T was extracted as described previously by Lee *et al.* (2003) and PCR amplification and sequencing of the 16S rRNA gene were performed as described by Webster & Hill (2001). The resultant sequence (1470 bp) was compared with those

Table 3. Differential physiological characteristics of strain HPA177^T, *A. cyanogriseus* IFO 14455^T and *A. spitiensis* DSM 44848^T

Strains: 1, HPA177^T; 2, *A. cyanogriseus* IFO 14455^T; 3, *A. spitiensis* DSM 44848^T. ++, Good; +, moderate; +/-, poor; -, none.

Characteristic	1	2	3
Utilization of:			
Arabinose	—	+/-	—
Fructose	+	—	—
Glucose	++	++	—
Maltose	++	++	—
Mannitol	++	+	+
Mannose	++	+	—
Raffinose	—	—	+
Xylose	+	+	—
Rhamnose	+	++	—
Sucrose	+	+/-	+
Sorbitol	+	+	—
Sodium citrate	+	—	+
Sodium succinate	—	—	+
Decomposition of casein	+	—	+
Hydrolysis of starch	+	+/-	+
Resistance to methyl violet	+	—	—
Pigmentation in:			
ISP 1	+	—	—
ISP 6	+	+	—

deposited in public databases. Phylogenetic analysis was performed using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1972) methods in the PHYLIP package version 3.6 (Felsenstein, 1985), after multiple alignment of data by CLUSTAL X version 1.8 (Thompson *et al.*, 1997). Evolutionary distances were calculated according to the two-parameter model of Kimura (1980). Tree topologies were evaluated by bootstrap analyses (Felsenstein, 1985) based on 1000 replications. It is clear from Fig. 2 that strain HPA177^T forms a distinct lineage within the evolutionary radiation encompassed by the genus *Actinoalloteichus*, supported by both tree-making algorithms and by a 99% bootstrap value recorded using the neighbour-joining method. Sequence similarity calculations after neighbour-joining analysis indicated that the isolate shared relatively low similarities with its closest relatives, *A. spitiensis* DSM 44848^T (97.1%) and *A. cyanogriseus* IFO 14455^T (96.7%). Sequence similarities to all other species of related genera with validly published names were lower than 95%. Strain HPA177^T could also be distinguished from members of the genus *Actinoalloteichus* using a combination of phenotypic properties (Table 3).

It is evident from the genotypic and phenotypic data that strain HPA177^T should be classified as a representative of a novel *Actinoalloteichus* species; the name *Actinoalloteichus hymeniacidonis* sp. nov. is proposed.

Description of *Actinoalloteichus hymeniacidonis* sp. nov.

Actinoalloteichus hymeniacidonis (hy.me.ni.a.ci'do.nis. N.L. gen. n. *hymeniacidonis* of *Hymeniacidon*, the generic name of the marine sponge *Hymeniacidon perleve*, the source of the type strain).

Gram-positive, strictly aerobic actinomycete with branching hyphae. Non-fragmenting substrate mycelia are present within 3 weeks of cultivation. Aerial mycelia with straight spore ($0.6 \times 0.8 \mu\text{m}$) chains aggregate. Grows well on yeast extract/malt extract agar and oatmeal agar at $20\text{--}37^\circ\text{C}$. A black soluble pigment is produced on yeast extract/malt extract agar and peptone/yeast extract/iron agar. Decomposition of urea, growth in Sabouraud's dextrose broth and MacConkey agar, hydrolysis of aesculin and hippurate, utilization of calcium malate, sodium oxalate and sodium succinate and reduction of nitrate are all negative. Fructose, glucose, maltose, mannose, mannitol, rhamnose, sucrose, sorbitol and xylose are utilized as sole carbon sources, but arabinose, inositol and raffinose are not. Grows weakly at 15°C and does not grow at 45°C . The cell wall chemotype is III. The major menaquinone is MK-9(H_4); small amounts of MK-9(H_6) and MK-9(H_8) are also present. The phospholipid profile comprises mainly phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannoside. Major fatty acids are 15:0 anteiso (20%), 17:1 ω 8c (19%), 16:0 iso (16%) and 17:0 (11%).

The type strain is HPA177^T (=CGMCC 4.2500^T=JCM 13436^T), isolated from the marine sponge *Hymeniacidon perleve* in Dalian, China.

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