Actinoallomurus acanthiterrae sp. nov., an actinomycete isolated from rhizosphere soil of the mangrove plant *Acanthus ilicifolius*

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A novel actinobacterium strain, 2614A723^T, was isolated from rhizosphere soil of mangrove plant *Acanthus ilicifolius* collected at Touyuan, Wenchang, Hainan province, China. A phylogenetic analysis based on 16S rRNA gene sequences indicated that strain 2614A723^T formed a distinct phyletic line in the genus *Actinoallomurus*, the 16S rRNA gene tree sharing similarities of 98.35 %, 98.07 % and 97.86 % with *Actinoallomurus spadix* NBRC 14099^T, *Actinoallomurus purpureus* TTN02-30^T and *Actinoallomurus luridus* TT02-15^T, respectively. Strain 2614A723^T contained lysine and *meso*-diaminopimelic acid in the cell wall peptidoglycan and madurose, galactose and xylose in the whole-cell sugars. The predominant menaquinones were MK-9(H_4) and MK-9(H_6). The major polar phospholipids were phosphatidylglycerol and diphosphatidylglycerol. The predominant fatty acids were iso-$C_{16:0}$ and anteiso-$C_{17:0}$. These chemotaxonomic data confirmed the affiliation of strain 2614A723^T to the genus *Actinoallomurus*. It is apparent from the combined phenotypic data, biochemical tests and DNA–DNA hybridization values that strain 2614A723^T should be classified in the genus *Actinoallomurus* as a representative of a novel species. The name *Actinoallomurus acanthiterrae* sp. nov. is proposed with strain 2614A723^T (=CCTCC AA 2012001^T=DSM 45727^T) as the type strain.

The genus *Actinoallomurus* was first established by Tamura *et al.*(2009) as a member of the family *Thermomonosporaceae*. Members of this genus have been found in different environments, including soils, plants and dung (Pozzi *et al.*, 2011; Tamura *et al.*, 2009; Indananda *et al.*, 2011) and have the potential to produce metabolites arising from different biosynthetic pathways (Pozzi *et al.*, 2011). In the course of studying actinomycetes from *Acanthus ilicifolius* rhizosphere soil in different mangroves of China, strain 2614A723^T were isolated on oatmeal agar (Shirling & Gottlieb, 1966) supplemented with novobiocin (25 μg ml^{-1}), nystatin (30 μg ml^{-1}), nalidixic acid (10 μg ml^{-1}) and $K_2Cr_2O_7$ (20 μg ml^{-1}) and incubated at 28 °C for 30 days. The isolate was purified and maintained on glucose–yeast extract–malt agar medium (ISP medium 2; Shirling & Gottlieb, 1966) and preserved as a suspension of mycelia fragments in glycerol (20 % v/v) at −20 °C for study and at −80 °C for long-term preservation. Its taxonomic position was investigated using a polyphasic approach.

The morphological and physiological characteristics of strain 2614A723^T, *Actinoallomurus spadix* NBRC 14099^T, *Actinoallomurus luridus* TT02-15^T and *Actinoallomurus purpureus* TTN02-30^T were tested simultaneously. Growth and cultural characteristics were examined by using various agar media (ISP 2–7) (Shirling & Gottlieb, 1966) and yeast extract–starch agar medium [YS: yeast extract 2 g l^{-1}, starch 10 g l^{-1} and agar15 g l^{-1}, NITE Biological Resource Center (NBRC) medium No.1053] used for *Actinoallomurus purpureus* TTN02-30^T. All the test media had a final pH of 5.8–6.0. Cultures were incubated at 28 °C and observations were recorded after 7, 14, 21 and 28 days. Cell morphology was observed by scanning electron microscopy (TM3000, Hitachi) after incubation on ISP medium 3 for 30 days at

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of 2614A723^T is JQ085862.

A supplementary figure and two supplementary tables are available with the online version of this paper.

28 °C. Specimens for electron microscopy were fixed in 2.5 % glutaraldehyde, dehydrated, critical-point dried and coated with gold as described by Lee & Jeong (2006), before observation. Strain 2614A723^T grew well on ISP 2, ISP 3, ISP 6, ISP 7 and YS medium. Moderate growth was observed on ISP 4 and ISP 5. Poor aerial mycelium was present on ISP medium 2 and YS medium, good aerial mycelium was only present on ISP medium 3. Soluble pigment was produced on ISP 7 agar (Table S1 available in IJSEM online). Strain 2614A723^T formed short spiral chains of oval and smooth-surfaced spores (Fig. 1), similar to other species of this genus that form oval to rod-shaped and irregular-sized spores at maturity.

The ability of the strain to grow at a range of temperatures (4, 18, 28, 34, 37, 42 and 50 °C) was tested on modified Bennett's agar (Jones, 1949). pH range (pH 4.0–9.0 at intervals of half a pH unit) were determined by using double-concentration YS liquid medium supplemented with an equal pH and amount of phosphate buffer solution. Tolerance of NaCl [0–6 %(w/v), at intervals of 0.5 %] for growth were estimated by using YS liquid medium shaken at 200 r.p.m., 28 °C for 7–14 days. Utilization of sole carbon and nitrogen sources was examined on ISP medium 9 (Shirling & Gottlieb, 1966) including each ether-sterilized compound at a final concentration of 1 % (w/v) for sugars or 0.5 % (w/v) for amino acids. Gelatin liquefaction, starch hydrolysis and reaction on milk were tested as described by Gordon *et al.* (1974) and Goodfellow (1971). Catalase activity was detected by the production of bubbles after the addition of a drop of 3 % (v/v) H_2O_2. Oxidase activity was determined by the oxidation of tetramethyl-*p*-phenylenediamine. Nitrate reductase and hydrolysis of aesculin were examined as described by MacFaddin (1980). Hydrolysis of carboxymethyl-cellulose was examined on modified Bennett's agar (Jones, 1949) as described by Teather & Wood (1982): after an appropriate incubation period (one or two weeks) at 28 °C, the Bennett's agar medium was flooded with an aqueous solution of Congo red (1 mg ml^{-1} for media containing carboxymethyl cellulose or carboxymethyl pachyman; 0.2 mg ml^{-1} for media containing oat β-D-glucan) for 15 min. The Congo red solution was then poured off, and plates were further treated by flooding with 1 M NaCl for 15 min. The visualized zones of hydrolysis could be stabilized for at least 2 weeks by flooding the agar with 1 M HCl, which changes the dye colour to blue and inhibits further enzyme activity. Other physiological and biochemical tests were performed according to the established methods of Williams *et al.* (1983) and Kämpfer *et al.* (1991). The physiological properties of the novel strain are summarized and compared with those of related species of the genus *Actinoallomurus* in detail in Table 1.

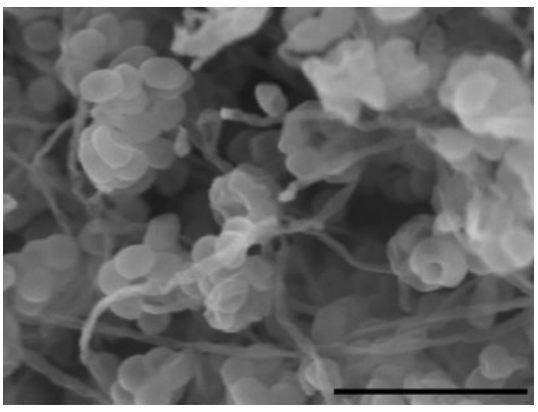

Fig. 1. Scanning electron micrograph of cells of strain 2614A723^T grown on oatmeal agar (ISP 3) medium for 3 weeks at 28 °C. Bar, 5 μm.

Biomass for chemical and molecular systematic studies was obtained from culture in liquid YS medium on a rotary shaker at 220 r.p.m., 34 °C for 5–7 days at exponential growth phase. Amino acids and sugars in whole-cell hydrolysates were analysed according to the procedure of Lechevalier & Lechevalier (1980) by TLC. The acyl type of the cell wall was analysed according to the method of Uchida & Aida (1984). Phospholipids were extracted, examined by two-dimensional TLC and identified using previously described procedures (Minnikin *et al.*, 1984; Collins & Jones, 1980). Menaquinones were extracted according to the protocol of Collins *et al.* (1977) and determined by HPLC (Tamaoka *et al.*, 1983). Fatty acids were extracted by the method of Sasser (1990) and the composition was determined by GC–MS performed on a GC instrument (7890A; Agilent) coupled with a mass selective detector (MS-5975C; Agilent) and an autosampler injector (7693-A; Agilent). A capillary column HP-5MS (5 % phenylmethylsiloxane) with dimensions of 30 m × 250 μm × 0.25 μm film thickness (Agilent 19091N-133) was used for the separation of fatty acid methyl esters. The initial temperature of 100 °C was maintained for 5 min, raised to 240 °C at the rate of 10 °C min^{-1}. Injected volume of sample was 1 μl and helium was used as a carrier gas at a flow rate of 0.8 ml min without split. The injector and detector temperatures were 230 °C and 250 °C, respectively. The MS was operated in the electron multiplier voltage (EMV) mode at 988 V in the scan range of 50–400 *m/z*.

The whole-cell hydrolysates of strain 2614A723^T contained *meso*-diaminopimelic acid, alanine, lysine, glycine and glutamic acid. Madurose, xylose and galactose were detected as the whole-cell sugars. The phospholipids comprised phosphatidylglycerol, diphosphatidylglycerol and phosphatidylinositol (Fig. S1.). The acyl type of the muramic acid is *N*-acetyl. Strain 2614A723^T contained menaquinones MK-9 (H_4) (67.41 %), MK-9 (H_6) (27.66 %) as major menaquinones and a small amount of MK-9 (H_2) (4.91 %) (Table 1). The major fatty acids present in strain 2614A723^T were iso-$C_{16:0}$ and anteiso-$C_{17:0}$ (Table S2).

Preparation of genomic DNA and PCR amplification of the 16S rRNA gene were carried out following the procedure of Goodfellow *et al.* (2007). The resultant nearly complete 16S rRNA gene sequence (1483 nt) was compared with

Table 1. Differential properties of strain 2614A723^T and members of the most closely related species of the genus *Actinoallomurus*

Strains: 1, strain 2614A723^T; 2, *A. spadix* NBRC 14099^T; 3, *A. luridus* NBRC 103683^T; 4, *A. purpureus* NBRC 103687^T. *meso*-DAP, *meso*-Diaminopimelic acid; Gal, galactose; Mad, madurose; Xyl, xylose; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; PL, unknown phospholipid. +, Positive; W, weakly positive; −, negative.

Characteristic	1	2	3	4
Growth at/in:				
Temperature (°C)	18–37	18–42	18–42	18–34
NaCl (%, w/v)	0–3.5	0–2.5	0–2.5	0–4.5
pH	5.5–7.0	6–6.5	5.5–6.5	5.5–6.5
Nitrogen utilization				
L-Asparagine	+	W	+	−
L-Glutamic acid	+	+	+	W
L-Glycine	+	+	W	+
L-Methionine	+	W	W	W
L-Ornithine	+	+	+	W
L-Proline	+	+	+	W
L-Phenylalanine	+	+	+	W
L-Tryptophan	+	W	W	W
L-Valine	+	+	+	W
Carbon utilization				
(+)-D-cellobiose	+	W	−	−
Dextrin	+	+	+	+
(+)-D-Galactose	+	+	−	W
Glycerol	+	+	W	+
Inositol	+	W	−	−
Lactose	+	+	−	+
Mannitol	+	+	−	−
D-Mannose	+	+	−	−
(+)-D-Melezitose hydrate	+	+	−	−
(+)-D-Melibiose	+	+	−	−
D-Sorbitol	+	+	−	−
L-Sorbose	W	−	−	−
Starch (soluble)	W	W	−	−
Sucrose	+	+	−	−
(+)-D-Trehalose	+	+	W	+
(+)-D-Xylose	+	+	−	−
Degradation tests				
Tween 80	W	+	+	+
Gelatin liquefaction	W	+	+	−
Nitrate reductase	−	+	−	−
Amino acid*	Ala, Glu, Gly, Lys, *meso*-DAP	Ala, Glu, Lys, *meso*-DAP	Ala, Glu, Lys, *meso*-DAP	Ala, Glu, Lys, *meso*-DAP
Sugar*	Gal, Mad, Xyl	Mad, Gal	Mad, Gal	Mad, Gal
DNA G+C content (mol%)*	71.3	74	72	70
Menaquinones*	MK-9(H_4), MK-9(H_6), MK-9(H_2)	MK-9(H_6), MK-9(H_8)	MK-9(H_6), MK-9(H_8), MK-9(H_4)	MK-9(H_6), MK-9(H_8)
Phospholipids*	PG, DPG, PI	PG, DPG	PG, DPG	PG, DPG

*Data for reference strains *A. spadix* NBRC 14099^T, *A. luridus* NBRC 103683^T and *A. purpureus* NBRC 103687^T from Tamura *et al.* (2009).

available 16S rRNA gene sequences of type strains of species of the genus *Actinoallomurus* from GenBank using the BLAST program and the EzTaxon server (http://www.eztaxon.org, Chun *et al.*, 2007) to determine an approximate phylogenetic affiliation. The gene sequences were aligned with CLUSTAL W Multiple alignment (Thompson, J.D. *et al.*, 1994), which is an accessory application of BioEdit software. The two end gap positions were cut manually with corresponding sequences of the related type species. Phylogenetic trees were inferred using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) tree-making algorithms in MEGA version 4.0 (Tamura *et al.*, 2007). Evolutionary distance matrices were generated via the neighbour-joining methods, as described by Jukes

and Cantor (1969). The resultant unrooted tree topologies were evaluated by bootstrap analyses (Felsenstein 1985) based on 1000 resamplings of the neighbour-joining dataset. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option).

Strain 2614A723^T shared its highest 16S rRNA gene sequence similarity with the type strain of *A. spadix* NBRC 14099^T, 98.35 %, a value which corresponded to 21 nucleotide differences and three nucleotide gaps out of 1451 locations. The corresponding 16S rRNA similarities with the type strains of *A. purpureus* TTN02-30^T and *A. luridus* TT02-15^T were 98.07 % and 97.86 %, respectively, values equivalent to 27 nucleotide differences and one nucleotide gap and 29 nucleotide differences and two nucleotide gaps, respectively. It was evident from the dendrogram shown in Fig. 2 that the new isolate formed a distinct branch with *A. spadix* NBRC 14099^T, *A. purpureus* TTN02-30^T and *A. luridus* TT02-15^T. This was also supported in the maximum-parsimony tree.

Chromosomal DNA was extracted as described by Pospiech & Neumann (1995). The G+C content of the genomic DNA was determined by using the HPLC method (Mesbah *et al.*, 1989). DNA–DNA hybridizations between strain 2614A723^T and *A. spadix* NBRC 14099^T, *A. purpureus* TTN02-30^T and *A. luridus* TT02-15^T were performed to determine whether the isolate represented a novel species. DNA–DNA relatedness values were determined using a modified nylon membrane hybridization method described previously by Sui *et al.* (2011).

The DNA–DNA relatedness values between strain 2614A723^T and the type strains of *A. spadix* NBRC 14099^T, *A. purpureus* TTN02-30^T and *A. luridus* TT02-15^T averaged out at 17.15±0.36 %, 12.75±0.71 % and 42.92±0.28 %, respectively, values well below the 70 % cut-off point recommended by Wayne *et al.* (1987) for the delineation of genomic species. In addition, the DNA G+C content of strain 2614A723^T was 71.3 mol%. This value is within the range for the genus *Actinoallomurus* (Tamura *et al.*, 2009).

The isolate could be distinguished from other species of the genus *Actinoallomurus* with validly published names using a combination of phenotypic properties. Physiological and chemotaxonomic analyses indicated that strain 2614A723^T belonged to the genus *Actinoallomurus*. The differential properties of strain 2614A723^T from the most closely related species of the genus *Actinoallomurus* (Table 1), 16S rRNA gene sequence and DNA–DNA hybridization analyses indicated that it represented a novel species. It is evident from the genotypic and phenotypic data that strain 2614A723^T can be distinguished from other known species of the genus *Actinoallomurus*. It is therefore proposed that the strain be classified in the genus *Actinoallomurus* as *Actinoallomurus acanthiterrae* sp. nov.

Description of *Actinoallomurus acanthiterrae* sp. nov.

Actinoallomurus acanthiterrae [a.can.thi.ter′ra.e. L. n. *acanthus* name of a plant and also a botanical generic name (*Acanthus*); L. n. *terra* soil; N.L. gen. n. *acanthiterrae* of soil of holly-leaved acanthus, denoting the source of the type strain].

Cells are aerobic and Gram-positive. Good growth occurs on ISP 2, ISP 3, ISP 6, ISP 7 and yeast extract-starch medium, moderate on ISP 4 and ISP 5. Poor white aerial mycelium was present on ISP medium 2 and YS medium, well-developed white aerial mycelium was present on ISP medium 3 and differentiated to short spiral chains of oval to smooth-surfaced spores. Substrate mycelium is well-developed and light yellowish to dark brown. Pink, soluble pigment was formed on ISP 7 agar. Growth occurs at 18–37 °C (optimum, 28–37 °C), pH 5.5–7 (optimum, pH 6–7) and in the presence of up to 3.5 % (w/v) NaCl (optimum, 0 % NaCl). Positive in tests for catalase production, starch hydrolysis, aesculin degradation and degradation of Tween 20 and 40; weakly positive for Tween 80 degradation, milk coagulation, gelatin liquefaction and peptonization, negative for carboxymethylcellulose degradation, urease production and nitrate reduction. L-alanine, L-asparagine, L-glutamic

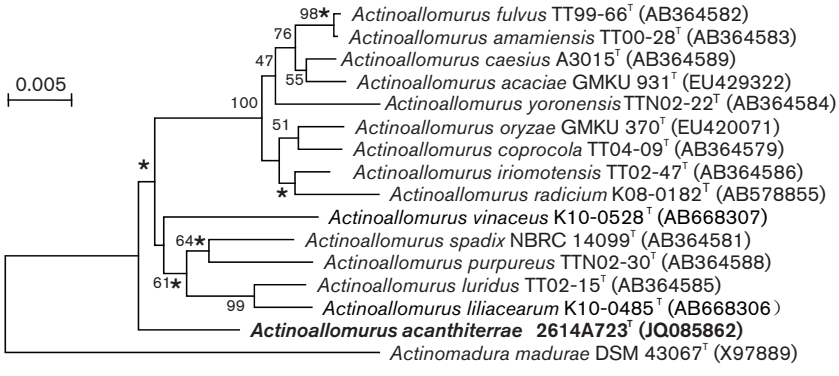

Fig. 2. Neighbour-joining phylogenetic tree, based on nearly complete 16S rRNA gene sequences (1451 nt), showing the relationships between strain 2614A723^T and related representative species of the genus *Actinoallomurus*. Numbers at branching points indicate bootstrap percentages (based on 1000 replications). Only bootstrap values of more than 40 % are shown. Asterisks indicate phyletic lines that were recovered using maximum-parsimony and neighbour-joining tree-making algorithms. The sequence of *Actinomadura madurae* DSM 43067^T was used as an outgroup. Bar, 0.005 substitutions per nucleotide position.

acid, L-glycine, L-hydroxyproline, L-methionine, L-ornithine, L-phenylalanine, L-proline, L-serine, L-tryptophan and L-valine were degraded as sole nitrogen source; L-arginine, L-cysteine, L-histidine and L-lysine cannot be used; (+)-D-cellobiose, dextrin, (+)-D-galactose, glycerol, inositol, lactose, mannitol, D-mannose, (+)-D-melezitose hydrate, (+)-D-melibiose, D-raffinose pentahydrate, D-sorbitol, L-sorbose (weakly), soluble starch (weakly), sucrose, (+)-D-trehalose, trisodium citrate and (+)-D-xylose were utilized as sole carbon and energy sources, but not (−)-D-arabinose, D-fucose, D-glucose, inulin, maltose, D-ribose, L-(+)-rhamnose, D-salicin, sodium acetate and succinic acid (Table 1). The peptidoglycan is rich in *meso*-diaminopimelic acid and contains *N*-acetyl muramic acid. Madurose, xylose and galactose are the major sugars in whole-organism hydrolysates. Tetrahydrogenated menaquinones with nine isoprene units are the predominant isoprenologue. The phospholipids are phosphatidylglycerol, diphosphatidylglycerol and phosphatidylinositol. The fatty acids profile obtained from yeast extract–starch medium culture contains: iso-$C_{14:0}$, iso-$C_{15:0}$, anteiso-$C_{15:0}$, $C_{15:0}$, iso-$C_{16:0}$, $C_{16:1}\omega9c$, $C_{16:1}\omega11c$, $C_{16:0}$, 10-methyl-$C_{16:0}$, iso-$C_{17:0}$, anteiso-$C_{17:0}$, $C_{17:1}\omega9c$, $C_{17:0}$, $C_{17:1}\omega11c$, iso-$C_{18:0}$, anteiso-$C_{18:0}$; $C_{18:1}\omega9c$,and $C_{18:0}$, 10-methyl-$C_{18:0}$.

The type strain, 2614A723^T (=CCTCC AA 2012001^T=DSM 45727^T), was isolated from rhizosphere soil of mangrove plant *Acanthus ilicifolius* collected at Touyuan, Wenchang, Hainan province, China. The G+C content of the genomic DNA of the type strain is 71.3 mol%

Acknowledgements

This research was supported by the National Natural Science Foundation of China (grant number 31170467).

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