Actinoalloteichus nanshanensis sp. nov., isolated from the rhizosphere of a fig tree (Ficus religiosa)

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A Gram-positive, aerobic actinomycete, designated strain NEAU 119T, was isolated from the rhizosphere of a fig tree and was characterized using a polyphasic approach. The isolate formed branching, non-fragmenting vegetative hyphae and produced black pigment on yeast extract/malt extract (ISP medium 2). The G+C content of the DNA was 76.6 mol%. The organism had chemotaxonomic characteristics typical of the genus Actinoalloteichus and was closely related to the type strains of Actinoalloteichus cyanogriseus, Actinoalloteichus spitiensis and Actinoalloteichus hymeniacidonis, currently the only three recognized species of the genus Actinoalloteichus, sharing 16S rRNA gene similarities of 96.4, 96.6 and 98.1 %, respectively. However, the results of DNA–DNA hybridization studies demonstrated that the novel strain showed only 46.8 % relatedness with the type strain of A. hymeniacidonis. In addition, a set of phenotypic characteristics also readily distinguished strain NEAU 119T from the type strains of recognized species of the genus Actinoalloteichus. According to the above data, it is proposed that strain NEAU 119T represents a novel species, Actinoalloteichus nanshanensis sp. nov. The type strain of Actinoalloteichus nanshanensis is NEAU 119T (=CGMCC 4.5714T=NBRC 106685').

The genus Actinoalloteichus proposed by Tamura et al. (2000) originated from the taxonomic studies on its phenotypic and genotypic properties by Liu et al. (1984), Itoh et al. (1987) and Tamura & Hatano (1998). At present, there are three recognized species of the genus Actinoalloteichus, namely Actinoalloteichus cyanogriseus (Tamura et al., 2000), Actinoalloteichus spitiensis (Singla et al., 2005) and Actinoalloteichus hymeniacidonis (Zhang et al., 2006). As part of a programme to discover actinomycetes with novel antibiotic production properties, an aerobic actinomycete strain, NEAU 119T, was isolated. In this study, the taxonomic status of this strain is reported based on phylogenetic, chemotaxonomic and phenotypic evidence. It is proposed that strain NEAU 119T should be classified as representing a novel species of the genus Actinoalloteichus.

Strain NEAU 119T was isolated from mud of the rhizosphere of a fig tree (Ficus religiosa) collected from Nanshan Temple, Guangxi Province, south China (23° 6’ N 109° 36’ E). The collection site was located in the subtropical rainforest region, where annual average rainfall is 1250–1750 mm, the annual average temperature is ~16–23 °C and the soil pH is 6.5. The strain was isolated using the standard dilution plate method and grown on humic acid-vitamin agar (HV) (Hayakawa & Nonomura, 1987) supplemented with nystatin (50 mg l\(^{-1}\)) and nalidixic acid (20 mg l\(^{-1}\)). After 21 days of aerobic incubation at 28 °C, colonies were transferred and purified on oatmeal agar (ISP 3 medium) and maintained as glycerol suspensions (20%, v/v) at −80 °C.

Cultures grown on oatmeal agar for 2–3 weeks at 28 °C were observed by light and scanning electron microscopy. Cultural characteristics were observed on a number of standard media (Table 1) after 2 weeks at 28 °C. The utilization of sole carbon source, decomposition of urea and cellulose, hydrolysis of asesculin and hippurate, utilization of calcium malate, sodium oxalate and sodium succinate, reduction of nitrate, growth in Sabouraud’s dextrose broth and MacConkey agar, gelatin liquefaction and H₂S production were examined as described previously (Gordon et al., 1974; Yokota et al., 1993). Growth over a range of temperatures, pH values and NaCl concentrations...
was tested using modified Bennett’s agar (Williams et al., 1983). Resistance to antibiotics was examined according to Goodfellow & Orchard (1974). Biomass for chemical studies was prepared by growing the strain in tryptic soy broth (TSB) in Erlenmeyer flasks for 7 days at 28 °C. 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 & 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). Extracts were analysed by the HPLC-UV method using a Prominence LC-20A C18 column (150 × 4.6 mm, i.d. 5 μm), typically at 270 nm. The mobile phase was acetonitrile/propyl alcohol (60:40, v/v), the flow rate was set to 1.0 ml min⁻¹ and the run time was 60 min. The injection volume was 20 μl and the chromatographic column was controlled at 40 °C (Wu et al., 1989). Mycolic acids were checked by the acid methanolysis method as described previously (Minnikin et al., 1980). Biomass for fatty acid analysis was prepared by scraping growth from oatmeal agar. Fatty acids were analysed by GC-MS performed on a GC instrument (6890N; Agilent) and an autosampler injector (7683-B; Agilent). A capillary column HP-5MS (5 % phenyl methylsiloxane) with dimensions of 30 m × 0.25 mm × i.d. 0.25 μm film thickness (Agilent Technologies) was used for the separation of fatty acid methyl esters. The initial temperature of 150 °C was maintained for 2 min, raised to 230 °C at the rate of 4 °C min⁻¹, and then kept at 230 °C for 5 min. The split ratio was 1 : 50 and helium was used as a carrier gas with a flow rate of 0.8 ml min⁻¹. The injector and detector temperatures were 240 and 260 °C, respectively. The MS was operated in the electron impact (EI) mode at 70 eV in the scan range of 50–550 m/z (Miller, 1982; Wang et al., 2005).

Strain NEAU 119T showed a range of properties that were consistent with its classification in the genus Actinoalloteichus. The organism formed light blue aerial mycelium with straight spore chains (see Supplementary Fig. S1 available in IJSEM Online) and the branched vegetative hyphae tended to produce septa and fragment after 3 weeks of incubation on oatmeal agar. Growth of the novel strain occurred over the pH range 6–10 and at 0–4 % NaCl (w/v), with optimum growth at pH 8.5 and 0 % NaCl (w/v). The temperature range for growth was 20–37 °C, with the optimum temperature being 28 °C. Detailed physiological characteristics are presented in the species description. The strain shared higher physiological similarity with the phylogenetically closest species, A. hymeniacidonis, as shown in Table 2. However, it could be readily differentiated from A. hymeniacidonis by the inability to hydrolyse starch or utilise sorbitol, sodium citrate and sodium succinate. Strain NEAU 119T contained meso-A2pm as the diagnostic diamino acid of the cell wall and galactose, glucose, mannose, ribose and rhamnose as the diagnostic sugars in whole-organism hydrolysates. Mycolic acids were absent. The major menaquinone was MK-9(H₄) (78.91 %) and small amounts of MK-10(H₄) (16.76 %), MK-9(H₃) (1.96 %), MK-8(H₄) (1.40 %) and MK-9(H₂) (0.96 %) were also present. The phospholipid profile consisted of phosphatidylethanolamine, phosphatidylglycerol, phosphatidylglycerol and phosphatidylglycerol mannoside. The cellular fatty acid profile was composed of C₁₅:0 iso (6.31 %), C₁₅:0 anteiso (19.48 %), C₁₅:0 2H (2.05 %), C₁₆:0 3H (6.43 %), C₁₆:0 iso (21.45 %), C₁₇:0 cyclo (1.52 %), C₁₇:0 iso (1.02 %), C₁₇:0 anteiso (14.86 %), C₁₇:1iso (5.61 %), C₁₇:0 (5.30 %) and C₁₈:0 16 (14.69 %) fatty acids representing <1.00 % of the total were not reported. Strain NEAU 119T shared many chemotaxonomic characteristics with other species of the genus Actinoalloteichus: the cell-wall chemotype was III, the major menaquinone was MK-9(H₄), the major fatty acids were iso- and anteiso-branched fatty acids, and mycolic acids were absent. However, the amounts of MK-10(H₄) and C₁₈:0 saturated fatty acid in this strain were higher than found for those of the other species of this genus. The fatty acid C₁₇:0 cyclopropane acid was only present in strain NEAU 119T.

Genomic DNA of strain NEAU 119T was extracted as described previously by Lee et al. (2003) and PCR amplification of the 16S rRNA gene was carried out according to the procedures outlined by Logman et al. (2009). DNA–DNA relatedness tests were performed by using the optical renaturation method as described by De

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### Table 1. Growth and cultural characteristics of strain NEAU 119T

<table>
<thead>
<tr>
<th>Agar medium</th>
<th>Growth</th>
<th>Aerial mycelium</th>
<th>Substrate mycelium</th>
<th>Diffusible pigment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast extract/malt extract</td>
<td>Poor</td>
<td>Bluish grey; poor</td>
<td>Brownish black</td>
<td>Black</td>
</tr>
<tr>
<td>(ISP 2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oatmeal (ISP 3)</td>
<td>Abundant</td>
<td>Light blue; abundant</td>
<td>Greysih black</td>
<td>Yellowish grey</td>
</tr>
<tr>
<td>Inorganic salts/starch (ISP 4)</td>
<td>Poor</td>
<td>Colourless; poor</td>
<td>Colourless</td>
<td>None</td>
</tr>
<tr>
<td>Glycerol/asparagine (ISP 5)</td>
<td>Poor</td>
<td>Blue; poor</td>
<td>Colourless</td>
<td>None</td>
</tr>
<tr>
<td>Peptone/yeast extract/iron</td>
<td>Poor</td>
<td>Light bluish grey; poor</td>
<td>Blush black</td>
<td>Black</td>
</tr>
<tr>
<td>(ISP 6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tyrosine (ISP 7)</td>
<td>Poor</td>
<td>Brownish grey; moderate</td>
<td>Reddish brown</td>
<td>Yellowish grey</td>
</tr>
<tr>
<td>Modified Bennett’s</td>
<td>Moderate</td>
<td></td>
<td>Brownish black</td>
<td>Black</td>
</tr>
</tbody>
</table>
Ley et al. (1970) and the renaturation took place at 85 °C. The DNA G+C content of the genomic DNA was determined by the thermal denaturation method as described by Mandel & Marmur (1968).

The 16S rRNA gene sequence of strain NEAU 119\textsuperscript{T} was compared with those of the other strains obtained from the GenBank database. CLUSTAL_X 1.83 software was used to determine the matching alignments of multiple sequences and MEGA 4.0 was employed to calculate the evolutionary distances. The matrix distances were calculated from the sequence data using the 'Kimura two-parameter' model (Kimura, 1980). The phylogenetic tree was then constructed with the neighbour-joining method available in the MEGA 4.0 software package (Saitou & Nei, 1987). The stability of the topology of the phylogenetic tree was assessed by using the bootstrap method with 1000 repetitions (Felsenstein, 1985).

BLAST sequence analysis of the 16S rRNA gene sequence also indicated that strain NEAU 119\textsuperscript{T} was affiliated to the genus *Actinoalloteichus*. Based on EzTaxon analysis, the similarity between strain NEAU 119\textsuperscript{T} and *A. cyanogriseus*, *A. spitiensis* and *A. hymeniacidonis* was 96.4, 96.6 and 98.1%, respectively. However, sequence similarities with all other recognized species of related genera were lower than 95%. DNA–DNA hybridization experiments revealed 46.8% DNA–DNA relatedness between strain NEAU 119\textsuperscript{T} and the type strain of *A. hymeniacidonis*, which was well below the 70% value that is considered to be the threshold for the delineation of genomic species (Wayne et al., 1987). Phylogenetic analysis including members of the genus *Actinoalloteichus* and related genera showed that the novel strain formed a distinct lineage within the monophyletic group of the genus *Actinoalloteichus* and was supported with high bootstrap values in all tree-making methods (data not presented). A reduced maximum-parsimony tree is shown in Fig. 1.

In conclusion, it is evident from the genotypic, chemotaxonomic and phenotypic data that strain NEAU 119\textsuperscript{T} represents a novel species of the genus *Actinoalloteichus*, for which the name *Actinoalloteichus nanshanensis* sp. nov. is proposed.

**Description of Actinoalloteichus nanshanensis** sp. nov.

*Actinoalloteichus nanshanensis* (nan.sha.nen’sis. N.L. masc. adj. nanshanensis pertaining to the Nanshan Temple in Guangxi Province in south China, from where the sample was collected).

Gram-positive, non-acid-fast, strictly aerobic actinomycete with branching hyphae. Non-fragmenting substrate mycelia are present within 3 weeks of cultivation. Aerial mycelia with straight spore (0.6 × 0.8 μm) chains aggregate. Grows

Table 2. Differential physiological characteristics of strain NEAU 119\textsuperscript{T} and *A. hymeniacidonis* HPA177\textsuperscript{T}

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>NEAU 119\textsuperscript{T}</th>
<th><em>A. hymeniacidonis</em> HPA177\textsuperscript{T}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Utilization of:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sorbitol</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Hydrolysis of starch</td>
<td>–</td>
<td>+</td>
</tr>
</tbody>
</table>

![Fig. 1. Neighbour-joining tree showing the phylogenetic position of strain NEAU 119\textsuperscript{T} and related taxa based on 16S rRNA gene sequences. *Streptomyces ambofaciens* ATCC 23877\textsuperscript{T} was used as the root organism. The numbers on the branches are confidence limits (expressed as percentages) estimated from a bootstrap analysis with 1000 replicates. Bar, 0.01 substitutions per nucleotide position.](image-url)
well on oatmeal agar at 20–37 °C. A black soluble pigment is produced on yeast extract/malt extract agar and peptone/yeast extract/iron agar. Negative in tests for the decomposition of urea and cellulose, growth in Sabouraud’s dextrose broth and MacConkey agar, hydrolysis of aesculin and hippurate, utilization of calcium malate, sodium oxalate, sodium succinate and reduction of nitrate and positive for gelatin liquefaction and H₂S production. Resistant to ampicillin (100 mg L⁻¹), carbenicillin (100 mg L⁻¹), gentamicin sulfate (10 mg L⁻¹), nalidixic acid (20 mg L⁻¹), nystatin (50 mg L⁻¹), rifamycin (50 mg L⁻¹), streptomycin sulfate (100 mg L⁻¹) and thiostrapton (30 mg L⁻¹), but sensitive to apramycin (30 mg L⁻¹), chloramphenicol (30 mg L⁻¹) and kanamycin (10 mg L⁻¹). Fructose, glucose, maltose, mannose, mannotol, rhamnose, sucrose and xylose are utilized as sole carbon sources, but arabinose, raffinose and sorbitol are not utilized. Tolerates up to 4 % (w/v) NaCl and grows at temperatures between 20 and 37°C, with an optimum temperature of 28 °C; cannot grow at 15 or 45 °C. Growth occurs at initial pH values between 6 and 10, the optimum being pH 8.5. The cell-wall chemotype is III. The major menaquinone is MK-9(H₄); small amounts of MK-10(H₄), MK-9(H₆), MK-8(H₄) and MK-9(H₂) are also present. The phospholipid profile comprises mainly phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol and phosphatidylglycerol mannoside. Major fatty acids are C₁₆:0 iso, C₁₅:0 anteiso and C₁₇:0 anteiso and C₁₈:1. The type strain, NEAU 119T (=CGMCC 4.5714T=NBRC 106685T), was isolated from mud of the rhizosphere of a fig tree. The G+C content of the DNA of the type strain is 76.6 mol%.

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**References**


