Antonie van Leeuwenhoek (2013) 103:293–298
DOI 10.1007/s10482-012-9809-9

ORIGINAL PAPER

Actinoplanes hulinensis sp. nov., a novel actinomycete isolated from soybean root (*Glycine max* (L.) Merr)

Yue Shen · Chongxi Liu · Xiangjing Wang · Junwei Zhao · Feiyu Jia · Yuqin Zhang · Liang Wang · Deguang Yang · Wensheng Xiang

Received: 21 June 2012 / Accepted: 6 September 2012 / Published online: 1 November 2012

Abstract A novel actinomycete, designated strain NEAU-M9^T, was isolated from soybean root (*Glycine max* (L.) Merr) and characterized using a polyphasic approach. 16S rRNA gene sequence similarity studies showed that strain NEAU-M9^T belonged to the genus *Actinoplanes*, being most closely related to *Actinoplanes campanulatus* DSM 43148^T (98.85 %), *Actinoplanes capillaceus* DSM 44859^T (98.70 %), *Actinoplanes lobatus* DSM 43150^T (98.30 %), *Actinoplanes auranticolor* DSM 43031^T (98.23 %) and *Actinoplanes sichuanensis* 03-723^T (98.06 %); similarity to other type strains of the genus *Actinoplanes* ranged from 95.87 to 97.56 %. The neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showed that the isolate formed a distinct phyletic line with *A. campanulatus* DSM 43148^T and *A. capillaceus* DSM 44859^T. This branching pattern was also supported by the tree constructed with the maximum-likelihood method. However, the low level of DNA–DNA relatedness allowed the isolate to be differentiated from the above-mentioned two *Actinoplanes* species. Moreover, strain NEAU-M9^T could also be distinguished from the most closely related species by morphological, physiological and characteristics. Therefore, it is proposed that strain NEAU-M9^T represents a novel *Actinoplanes* species, *Actinoplanes hulinensis* sp. nov. The type strain of *Actinoplanes hulinensis* is NEAU-M9^T (= CGMCC 4.7036^T = DSM 45728^T).

Keywords *Actinoplanes hulinensis* sp. nov. · Polyphasic taxonomy · 16S rRNA gene

Electronic supplementary material The online version of this article (doi:10.1007/s10482-012-9809-9) contains supplementary material, which is available to authorized users.

Y. Shen · D. Yang (✉)
College of Agriculture, Northeast Agricultural University, No. 59 Mucai Street, Xiangfang District, Harbin 150030, People's Republic of China
e-mail: deguangyang@sina.com

C. Liu · X. Wang · J. Zhao · F. Jia · L. Wang · W. Xiang (✉)
School of Life Science, Northeast Agricultural University, No. 59 Mucai Street, Xiangfang District, Harbin 150030, People's Republic of China
e-mail: xiangwensheng@yahoo.com.cn

Y. Zhang
Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, People's Republic of China

Introduction

The genus *Actinoplanes* was proposed by Couch (1950) for actinomycetes that produced motile spores with spherical, cylindrical, digitate, lobate, bottle or flask-shaped or very irregular sporangia with tufts of polar flagella. Subsequently, a detailed phenotypic analysis of the genus has been given by Goodfellow

et al. (1990), who determined the chemotaxonomic and phenotypic characteristics of species of *Actinoplanes* and reported that chemical and numerical taxonomic data supported the integrity of the genus. Furthermore, a comprehensive phylogenetic analysis of the genus has been given by Tamura and Hatano (2001). To date, 35 species of *Actinoplanes* with validly published names have been described. During a study on the ecological diversity of actinomycetes from soybean root, an aerobic actinomycete strain, NEAU-M9^T, 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-M9^T should be classified as representing a novel species of the genus *Actinoplanes*.

Materials and methods

Isolation and maintenance of the organism

Strain NEAU-M9^T was isolated from soybean root collected from Hulin, Heilongjiang province, north China (46°16′N, 133°36′E). In the open, the plant was tagged and stored in a clean plastic bag until used (~24 h). The root sample was air dried for 24 h at room temperature and then washed with an ultrasonic step (160W, 15 min) (KH-160TDV, Hechuang, China) to remove the surface soils and adherent epiphytes completely. After drying, the sample was cut into pieces of 5–10 mm in length and then subjected to a seven-step surface sterilization procedure: a 60-s wash in sterile tap water containing cycloheximide (100 mg l^{-1}) and nalidixic acid (20 mg l^{-1}), followed by a wash in sterile water, a 5-min wash in 5 % NaOCl, a 10-min wash in 2.5 % $Na_2S_2O_3$, a 5-min wash in 75 % ethanol, a wash in sterile water, and a final rinse in 10 % $NaHCO_3$ for 10 min. The rinsed root sample was transferred temporarily onto sterile filter paper to eliminate excess moisture and then placed on a plate of humic acid-vitamin agar (HV) (Hayakawa and Nonomura 1987) supplemented with cycloheximide (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 medium 3) and incubated at 28 °C for 2–3 weeks.

Morphological, cultural and physiological characteristics

Morphology of the sporangia and surface ornamentation of the sporangiospores were observed by scanning electron microscopy (Hitachi S-3400N) using cultures grown on ISP3 agar for 21 days. Spore motility was assessed by light microscopic (Nikon ECLIPSE E200) observation of cells suspended in phosphate buffer (pH 7.0, 1 mM). Cultural characteristics were determined after 3 weeks at 28 °C by methods used in the international *Streptomyces* project (ISP) (Shirling and Gottlieb 1966). Color determination was done with color chips from the ISCC-NBS Color Charts standard samples no 2106 (Kelly 1964). Growth over a range of temperatures, pH values and NaCl concentrations were tested using modified YEME medium (yeast extract 0.3 %, sucrose 10.3 %, tryptone 0.5 %, malt extract 0.3 %, glucose 1.0 % w/v, pH 7.2). Decomposition of cellulose, hydrolysis of starch, reduction of nitrate, liquefaction of gelatin and production of H_2S were examined as described previously (Gordon et al. 1974). Carbon source utilization was tested using carbon source utilization (ISP9) medium (Shirling and Gottlieb 1966) supplemented with a final concentration of 1 % of the tested carbon sources. Nitrogen source utilization was examined using the basal medium recommended by Williams et al. (1983) supplemented with a final concentration of 0.1 % of the nitrogen sources tested.

Chemotaxonomic characterization

Biomass for chemical studies was prepared by growing the strain in GY medium (glucose 1.0 %, yeast extract 1.0 %, $K_2HPQ_4{\cdot}3H_2O$ 0.05 %, $MgSO_4{\cdot}7H_2O$ 0.05 % w/v, pH 7.2–7.4) 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 (DAP) in the whole cell hydrolysates were analysed by HPLC method using Agilent TC-C_{18} Column (250 × 4.6 mm i.d. 5 μm) with a mobile phase consisting of acetonitrile: 0.05 mol l^{-1} phosphate buffer pH 7.2 = 15:85 at a flow rate of 0.5 ml min^{-1}. The peak detection used an Agilent G1321A fluorescence detector with a 365 nm excitation and 455 nm longpass emission filters (McKerrow et al. 2000). The *N*-acyl group of muramic acid in peptidoglycan was determined by the method of

Uchida et al. (1999). The whole-organism sugars were analyzed according to the procedures developed by Lechevalier and 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 analyzed by HPLC–UV method using Agilent Extend-C_{18} Column (150 × 4.6 mm, i.d. 5 μm), typically at 270 nm. The mobile phase was acetonitrile-propyl alcohol (60:40, v/v) and the flow rate was set to 1.0 ml min^{-1} and the run time was 60 min (Wu et al. 1989). Cellular fatty acids were analysed by GC–MS using the method of Xiang et al. (2011).

DNA base composition and DNA–DNA hybridization

The G+C content of the genomic DNA was determined by the thermal denaturation method as described by Mandel and Marmur (1968), and *Escherichia coli* JM109 was used as the reference strain. DNA–DNA relatedness test was performed in triplicate using the optical renaturation method as described by De Ley et al. (1970).

DNA preparation, amplification and determination of 16S rRNA sequence

Genomic DNA was extracted as described previously by Lee et al. (2003) and PCR amplification of 16S rRNA gene was carried out according to the procedures described by Loqman et al. (2009). The 16S rRNA gene sequence of strain NEAU-M9^T was aligned with multiple sequences obtained from the GenBank/EMBL/DDBJ databases using Clustal X 1.83 software. The alignment was manually verified and adjusted prior to the construction of phylogenetic trees. Phylogenetic trees were generated with the neighbour-joining (Saitou and Nei 1987) and maximum-likelihood (Felsenstein 1981) algorithms using molecular evolutionary genetics analysis (MEGA) software version 5.05 (Tamura et al. 2011). The stability of the clades in the trees was appraised using a bootstrap value with 1,000 repeats (Felsenstein 1985). A distance matrix was generated using Kimura's two-parameter model (Kimura 1980). All positions containing gaps and missing data were eliminated from the dataset (complete deletion option).

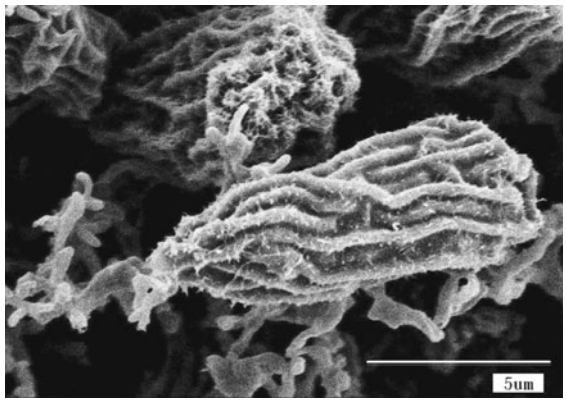

Fig. 1 Scanning electron micrograph of strain NEAU-M9^T grown on ISP3 medium for 21 days at 28 °C. *Bar* 5 μm

Results and discussion

Morphological observation of a 21-day-old culture of strain NEAU-M9^T grown on ISP3 agar revealed it had the typical characteristics of genus *Actinoplanes*. Strain NEAU-M9^T was observed to produce branched, non-fragmenting substrate hyphae which bore characteristic sporangia. The mature spore vesicles (4.56 × 10.72 μm) were found to be bell-shaped, and the surface of which were adorned by short hairs (Fig. 1). Sporangiospores were found to be motile. Strain NEAU-M9^T was determined to grow well on ISP3 and ISP4 agar; moderate growth was observed on ISP2, ISP6 and ISP7 agar and poor growth on ISP5 agar, Aerial mycelia were not detected on any of the tested ISP media. The substrate mycelium of strain NEAU-M9^T was observed to be pale yellow to deep reddish orange on all media tested. No diffusible pigment was detected on any of the tested media (Supplementary Table S1).

Growth of strain NEAU-M9^T was found to occur in the pH range 7–10 and 0–1 % NaCl (w/v), with optimum growth at pH 8.0 and 0 % NaCl (w/v). The temperature range for growth was determined to be 18–32 °C, with the optimum temperature being 28 °C. Strain NEAU-M9^T contained *meso*-diaminopimelic acid as diamino acid. Whole-cell hydrolysates were found to contain galactose, mannose and ribose (Supplementary Fig. S1). The acyl type of the cell wall polysaccharides was determined to be glycolyl. The phospholipid profile was found to consist of phosphatidylethanolamine and phosphatidylinositol (phospholipid type PII; Lechevalier et al. 1977) (Supplementary Fig. S2). The menaquinones detected

were MK-9(H_4) (59.8 %), MK-9(H_6) (40.3 %). The cellular fatty acid profile was determined to be composed of $C_{14:0}$ (4.7 %), $C_{15:0}$ (1.1 %), $C_{16:0}$ (50.0 %), $C_{16:1}\omega7c$ (1.5 %), $C_{17:0}$ cyclo (1.2 %), $C_{18:1}\omega9c$ (5.0 %) and $C_{18:0}$ (35.2 %) (fatty acids representing <1.0 % of the total are not reported) (Supplementary Table S2).

The almost-complete 16S rRNA gene sequence (1,511 nt) of strain NEAU-M9^T was determined and deposited as JQ073723 in the GenBank/EMBL/DDBJ databases. Blast sequence analysis of the 16S rRNA gene sequence showed that the strain was affiliated to the genus *Actinoplanes*. The closest phylogenetic relatives were *Actinoplanes campanulatus* DSM 43148^T (98.85 %), *Actinoplanes capillaceus* DSM 44859^T (98.70 %), *Actinoplanes lobatus* DSM 43150^T (98.30 %), *Actinoplanes auranticolor* DSM 43031^T (98.23 %) and *Actinoplanes sichuanensis* 03-723^T (98.06 %); lower sequence similarities (<97.56 %) were found with the type strains of all other members of the genus *Actinoplanes* with validly published names. The phylogenetic tree (Fig. 2) based on 16S rRNA gene sequences showed that strain NEAU-M9^T formed a distinct phyletic line with *A. campanulatus* DSM 43148^T and *A. capillaceus* DSM 44859^T, an association that was supported by maximum-likelihood algorithm employed (Supplementary Fig. S3) and by a 86 % bootstrap value in the neighbour-joining analysis. To establish the precise taxonomic position of strain NEAU-M9^T, DNA–DNA hybridizations performed between the novel isolate and *A. campanulatus* DSM 43148^T, *A. capillaceus* DSM 44859^T and *A. lobatus* DSM 43150^T; the levels of DNA–DNA relatedness between them were 52.2 ± 0.7, 50.8 ± 0.7 and 15.2 ± 1.4 %, respectively. These values were below the threshold value of 70 % recommended by Wayne et al. (1987) for assignment of strains to the same species. Strain NEAU-M9^T was noted to share many morphological and chemotaxonomic characteristics with other species of the genus *Actinoplanes*, including the development of motile sporangiospores; the scanty presence of aerial hyphae; MK-9(H_4) as the predominant menaquinone, the presence of *meso*-diaminopimelic acid in the whole-cell peptidoglycan; and phosphatidylethanolamine as the diagnostic phospholipid (Couch 1950; Goodfellow and Cross 1984; Kothe 1987; Vobis 1989; Kämpfer et al. 2007). However, the whole-cell sugar patterns (absence of arabinose and xylose) and major cellular fatty acid compositions ($C_{16:0}$ and $C_{18:0}$) of strain NEAU-M9^T could differentiate it from its most related type strains of the genus *Actinoplanes*, which contain arabinose and xylose as characteristic sugars and iso-$C_{15:0,}$ iso-$C_{16:0}$ and $C_{17:1}\omega9c$ as predominant fatty acids (Supplementary Table S2). In addition, the isolate could also be clearly distinguished from the most closely related type strains of the genus

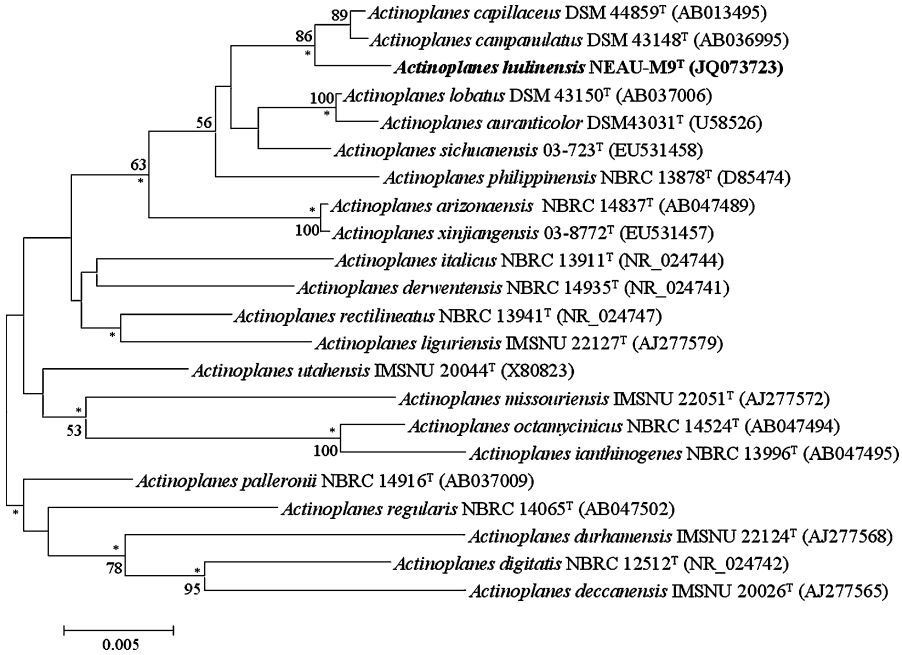

Fig. 2 Neighbour-joining tree showing the phylogenetic position of strain NEAU-M9^T and related taxa based on 16S rRNA gene sequences. *Asterisks* indicate branches that were also found using maximum-likelihood methods. *Numbers at branch points* indicate bootstrap percentages (based on 1,000 replicates); only values >50 % are indicated. *Bar* 0.005 substitutions per nucleotide position

Table 1 Differential physiological characteristics of strain NEAU-M9^T and the type strains of related *Actinoplanes* species

Characteristic	1	2	3	4	5	6
Utilization of						
Tyrosine	+	−	+	+	+	+
Galactose	+	−	+	−	−	+
Glucose	+	+	+	+	−	+
Lactose	+	−	−	+	+	−
Mannose	+	+	+	−	+	−
Raffinose	+	−	−	+	−	−
Nitrate reduction	−	−	+	+	−	+
Gelatinase	+	−	−	−	−	+

Strains 1, NEAU-M9^T; 2, *A. campanulatus* DSM 43148^T; 3, *A. capillaceus* DSM 44859^T; 4, *A. lobatus* DSM 43150^T; 5, *A. auranticolor* DSM 43031^T; 6, *A. sichuanensis* 03-723^T. Data for reference strains are taken from previous study (Sun et al. 2009). +, positive; –, negative

Actinoplanes based on physiological and biochemical characteristics as summarized in Table 1. Data from the present polyphasic taxonomic study, together with the DNA–DNA hybridization data, indicated that strain NEAU-M9^T represents a novel species of the genus *Actinoplanes*, for which we propose the name *Actinoplanes hulinensis* sp. nov.

Description of *Actinoplanes hulinensis* sp. nov.

Actinoplanes hulinensis (hu.lin.en′sis. N.L. masc. adj. *hulinensis*, pertaining to Hulin, China, from where the sample was collected).

Aerobic actinomycete which produces branched, non-fragmenting substrate hyphae which carry bell-shaped spore vesicles (4.56 × 10.72 μm). Sporangiospores are motile and the surface is covered by short hairs. Substrate mycelium is pale yellow to deep reddish orange on ISP2, ISP3, ISP4, ISP5, ISP6 and ISP7 media. Aerial mycelium is absent. Soluble pigment and melanin are not formed. Negative for decomposition of cellulose, reduction of nitrate and production of H_2S and positive for hydrolysis of starch and liquefaction of gelatin. Arabinose, fructose, galactose, glucose, inositol, lactose, maltose, mannose, mannitol, raffinose, rhamnose, sorbitol and sucrose are utilized as sole carbon sources but ribose and xylose are not utilized. Alanine, arginine, asparagine, creatine, glycine, glutamine, threonine and tyrosine are utilized as sole nitrogen sources but arginine are not. Tolerates up to 1 % NaCl and grows at temperatures between 18 and 32 °C, with an optimum temperature of 28 °C. Growth occurs at initial pH values between 7 and 10, the optimum being pH 8.0. Whole-cell hydrolysates contains *meso*-DAP. Whole-cell hydrolysates comprise galactose, mannose and ribose. The acyl type of the cell wall polysaccharides is glycolyl. The major menaquinone are MK-9(H_4) and MK-9(H_6). The phospholipid profile comprises phosphatidylethanolamine and phosphatidylinositol (phospholipid type PII). Major fatty acids are $C_{16:0}$ and $C_{18:0}$. The G+C content of the DNA is 67.90 mol%.

The type strain is NEAU-M9^T (= CGMCC 4.7036^T = DSM 45728^T), isolated from soybean root collected from Hulin, Heilongjiang province, China. The GenBank/EMBL/DDBJ database accession number of the 16S rRNA sequence of strain NEAU-M9^T is JQ073723.

Acknowledgments This work was supported in part by grants from NKPBR (No. 2010CB126102) and NNSF (No. 30971937 and 30771427) of China, the Special Fundation for Scientific and Technological Innovation Research of Harbin (No. 2011 RFXXN038), NSF of Heilongjiang Province (No. C201029), and NIH grant GM086184.

References

Collins MD (1985) Isoprenoid quinone analyses in bacterial classification and identification. In: Goodfellow M, Minnikin DE (eds) Chemical methods in bacterial systematics. Academic, London, pp 267–284

Couch JN (1950) *Actinoplanes*, a new genus of the *Actinomycetales*. J Elisha Mitchell Sci Soc 66:87–92

De Ley J, Cattoir H, Reynaerts A (1970) The quantitative measurement of DNA hybridization from renaturation rates. Eur J Biochem 12:133–142

Felsenstein J (1981) Evolutionary trees from DNA sequences: a maximum likelihood approach. J Mol Evol 17:368–376

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791

Goodfellow M, Cross T (1984) Classification. In: Goodfellow M, Mordarski M, Williams ST (eds) The Biology of the Actinomycetes. Academic, London, pp 7–164

Goodfellow M, Stanton LJ, Simpson KE, Minnikin DE (1990) Numerical and chemical classification of *Actinoplanes* and some related actinomycetes. J Gen Microbiol 136:19–36

Gordon RE, Barnett DA, Handerhan JE, Pang CHN (1974) *Nocardia coeliaca*, *Nocardia autotrophica*, and the nocardin strain. Int J Syst Bacteriol 24:54–63

Hayakawa M, Nonomura H (1987) Humic acid-vitamin agar, a new medium for the selective isolation of soil actinomycetes. J Ferment Technol 65:501–509

Kämpfer P, Huber B, Thummes K, Grün-Wollny I, Busse HJ (2007) *Actinoplanes couchii* sp. nov. Int J Syst Evol Microbiol 57:721–724

Kelly KL (1964) Inter-society color council-national bureau of standards color-name charts illustrated with centroid colors published in US

Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120

Kothe HW (1987) Die Gattungen *Actinoplanes* und ihre Stellung innerhalb der *Actinomycetales*. PhD thesis, University of Marburg

Lechevalier MP, Lechevalier HA (1980) The chemotaxonomy of actinomycetes. In: Actinomycete taxonomy, special publication 6, Society for Industrial Microbiology, Arlington, pp 277–284

Lechevalier MP, De Bièvre C, Lechevalier HA (1977) Chemotaxonomy of aerobic actinomycetes: phospholipid composition. Biochem Syst Ecol 5:249–260

Lee YK, Kim HW, Liu CL, Lee HK (2003) A simple method for DNA extraction from marine bacteria that produce extracellular materials. J Microbiol Methods 52:245–250

Loqman S, Barka EA, Clément C, Ouhdouch Y (2009) Antagonistic actinomycetes from Moroccan soil to control the grapevine gray mold. World J Microbiol Biotechnol 25: 81–91

Mandel M, Marmur J (1968) Use of ultraviolet absorbance temperature profile for determining the guanine plus cytosine content of DNA. Methods Enzymol 12B:195–206

McKerrow J, Vagg S, McKinney T, Seviour EM, Maszenan AM, Brooks P, Seviour RJ (2000) A simple HPLC method for analysing diaminopimelic acid diastereomers in cell walls of gram-positive bacteria. Lett Appl Microbiol 30: 178–182

Minnikin DE, O'Donnell AG, Goodfellow M, Alderson G, Athalye M, Schaal K, Parlett JH (1984) An integrated procedure for the extraction of bacterial isoprenoid quinones and polar lipids. J Microbiol Methods 2:233–241

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Shirling EB, Gottlieb D (1966) Methods for characterization of *Streptomyces* species. Int J Syst Bacteriol 16:313–340

Sun W, Dong GX, Zhang YQ, Wei YZ, Li QP, Yu LY, Klenk HP, Zhang YQ (2009) *Actinoplanes sichuanensis* sp. nov. and *Actinoplanes xinjiangensis* sp. nov. Int J Syst Evol Microbiol 59:2763–2768

Tamura T, Hatano K (2001) Phylogenetic analysis of the genus *Actinoplanes* and transfer of *Actinoplanes minutisporangius* Ruan et al. 1986 and '*Actinoplanes aurantiacus*' to *Cryptosporangium minutisporangium* comb. nov. and *Cryptosporangium aurantiacum* sp. nov. Int J Syst Evol Microbiol 51:2119–2125

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739

Uchida K, Kudo T, Suzuki K, Nakase T (1999) A new rapid method of glycolate test by diethyl ether extraction, which is applicable to a small amount of bacterial cells of less than one milligram. J Gen Appl Microbiol 45:49–56

Vobis G (1989) The *Actinoplanetes*. In: Williams ST, Sharpe ME, Holt JG (eds) Bergey's manual of systematic bacteriology, 4th edn. Williams and Wilkins, Baltimore, pp 2418–2428

Wayne LG, Brenner DJ, Colwell RR, Grimont PAD, Kandler O, Krichevsky MI, Moore LH, Moore WEC, Murray RGE et al (1987) International committee on systematic bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. Int J Syst Bacteriol 37:463–464

Williams ST, Goodfellow M, Alderson G, Wellington EMH, Sneath PHA, Sackin MJ (1983) Numerical classification of *Streptomyces* and related genera. J Gen Microbiol 129: 1743–1813

Wu C, Lu X, Qin M, Wang Y, Ruan J (1989) Analysis of menaquinone compound in microbial cells by HPLC. Microbiology [English translation of Microbiology (Beijing)] 16:176–178

Xiang WS, Liu CX, Wang XJ, Du J, Xi LJ, Huang Y (2011) *Actinoalloteichus nanshanensis* sp. nov., isolated from the rhizosphere of a fig tree (*Ficus religiosa*). Int J Syst Evol Microbiol 61:1165–1169