Full Paper

***Actinomadura maheshkhaliensis* sp. nov., a novel actinomycete isolated from mangrove rhizosphere soil of Maheshkhali, Bangladesh**

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The taxonomic position of one soil isolate 13-12(50)^T was clarified by a polyphasic study. The organism showed a combination of chemotaxonomic and morphological properties typical of the genus *Actinomadura*. It formed a distinct phyletic line in the *Actinomadura* 16S rRNA gene tree and was closely associated with *Actinomadura mexicana* (sequence similarity 99.5%), *Actinomadura glauciflava*, *Actinomadura citrea* (sequence similarity 99.4%) and *Actinomadura madurae* (sequence similarity 99.2%). The result of DNA-DNA hybridizations between 13-12(50)^T and *Actinomadura mexicana* was 49.9%. On the basis of the phenotypic, chemotaxonomic and genotypic properties, the isolate was differentiated from its closest phylogenetic relatives. It is proposed that the organism be classified as a novel species of the genus *Actinomadura*. The name proposed for the new taxa are *Actinomadura maheshkhaliensis* sp. nov. [(13-12(50)^T=JCM 13934^T=MTCC 8055^T].

Key Words—*Actinomadura maheshkhaliensis* sp. nov.; polyphasic taxonomy; 16S rDNA sequencing

Introduction

The genus *Actinomadura* belongs to the family *Thermomonosporaceae* Stackebrandt et al., 1997 emend. Zhang et al. 2001 (Stackebrandt et al., 1997; Zhang et al., 2001) which includes the genera *Actinocorallia* (Iinuma et al., 1994; Lee, 2006; Zhang et al., 2001), *Spirillospora* (Couch, 1963) and *Thermomonospora*

(Henssen, 1957; Zhang et al., 2001). Members of these taxa form distinct phyletic lines in the 16S rRNA gene tree (Zhang et al., 1998, 2001) and can be distinguished from one another using a combination of biochemical, chemical, morphological and physiological features (Trujillo and Goodfellow, 2003; Zhang et al., 1998, 2001). The application of chemo-systematic, numerical phenetic and molecular systematic methods has clarified the taxonomy of the genus *Actinomadura* and related taxa (Ara et al., 2008; Kroppenstedt et al., 1990; Lee and Jeong, 2006; Le Roes and Meyers, 2007; Ochi et al., 1991; Poschner et al., 1985; Quintana et al., 2003a; Trujillo and Goodfellow, 1997, 2003). The genus *Actinomadura* currently encompasses 37 recognized species. As part of a screening program for antibiotic-producing actinomycetes, 13-12(50)^T

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The DDBJ accession number for the 16S rRNA gene sequences of strain 13-12(50)^T is AB331731.

was isolated from muddy soil collected at Maheshkhali, Cox's Bazar, Bangladesh. The strain 13-12(50)^T showed typical morphological characteristics of the genera *Actinomadura* and *Nonomuraea*. In this paper we describe the characterization and classification of this strain and propose *Actinomadura maheshkhaliensis* sp. nov.

Materials and Methods

The strain was isolated using the standard dilution plate method and grown on humic acid-vitamin agar (HV) (Hayakawa and Nonomura, 1987) supplemented with cycloheximide (50 mg L⁻¹), nystatin (50 mg L⁻¹) and nalidixic acid (20 mg L⁻¹) (Ara and Kudo, 2007a,b). After 21 days of aerobic incubation at 30°C, the strain was transferred and purified on yeast extract-malt extract agar [(medium 2 of the International *Streptomyces* Project (ISP medium 2)] (Shirling and Gottlieb, 1966) and maintained as working cultures on Seino (yeast extract-starch) agar containing 15.0 g soluble starch, 4.0 g yeast extract, 0.5 g K₂HPO₄, 0.5 g MgSO₄ · 7H₂O and 15.0 g agar in 1 L of distilled water (pH 7.2). For comparative purposes *Actinomadura mexicana* JCM 13236^T, *Actinomadura glauciflava* JCM 6161^T, *Actinomadura citrea* JCM 3295^T and *Actinomadura madurae* JCM 7436^T were used in this study.

Strain 13-12(50)^T was grown on ISP medium 3 agar and ISP medium 2 agar media at 30°C for 21 days and then observed by light and scanning electron microscopy (model S-2400, Hitachi and model JSM-5600, JEOL). The sample for scanning electron microscopy was prepared as described by Itoh et al. (1989) and Ara et al., (2007a,b).

The phenotypic properties were examined using the following standard methods. For cultural characterization, the isolate was grown for 21 days at 30°C on various agar media as described by Waksman (1950, 1961), Shirling and Gottlieb (1966) and Asano and Kawamoto (1986). The *Color Harmony Manual* (Jacobson et al., 1958) was used to determine the names and designations of colony colors. Utilization of carbohydrates (conc. 1%) as sole carbon source was tested by using ISP medium 9 according to the modified method of Stevenson (1967). Production of melanoid pigments was examined using tyrosine agar (ISP medium 7).

Freeze-dried cells that were used for chemotaxonomic analyses were obtained from cultures grown in

yeast-dextrose (YD) or trypticase soy broth (TSB) on a rotary shaker for 2 weeks at 28°C. The isomer of diaminopimelic acid (DAP) was determined by using TLC as described by Stanek and Roberts (1974). Reducing sugars in whole-cell hydrolysates were analyzed by the paper chromatography method of Becker et al. (1965). The *N*-acyl group of muramic acid in peptidoglycan was determined by the method of Uchida and Aida (1984). Phospholipids were extracted and identified by the method of Minnikin et al. (1984). Methyl esters of cellular fatty acids were analyzed by TechnoSuruga Co., Ltd. (Japan) according to the instructions of the Microbial Identification System (MIDI) using a gas chromatograph (model HP6890, Hewlett-Packard) (Sasser, 1990). Isoprenoid quinones were extracted by the method of Collins et al. (1977, 1984) and were analyzed by a HPLC (model 802-SC, Jasco) equipped with a CAPCELL PAK C18 column (Shiseido) (Tamaoka et al., 1983). Preparation and detection of methyl esters of mycolic acids were carried out as described by Tomiyasu (1982). The G+C content of the DNA was determined using the HPLC method of Tamaoka and Komagata (1984). An equimolar mixture of nucleotides was digested by bacterial alkaline phosphatase and used as the qualitative standard.

Chromosomal DNA of strain 13-12(50)^T was isolated from biomass. The cells were freeze-dried and mechanically ground as described by Raeder and Broda (1985). DNA-DNA relatedness was measured fluorometrically using the microplate hybridization method (Ezaki et al., 1989). Hybridization was carried out overnight at 54°C. Genomic DNA extraction, PCR-mediated amplification of the 16S rRNA gene and sequencing of the PCR products were carried out as described by Nakajima et al. (1999). The sequences were aligned with selected sequences obtained from the GenBank/EMBL/DDBJ databases by using the Clustal X program (Thompson et al., 1997). The alignment was manually verified and adjusted prior to construction of a phylogenetic tree. A phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987) contained in the Clustal X program, based on the comparison of 1,241 nucleotides present in all the strains as a result of elimination of gaps and ambiguous nucleotides from the sequences between positions 34 and 1491 (*Escherichia coli* position number). *Streptosporangium album* NBRC 13900^T was used as an out-group. The confidence values of branches of the phylogenetic tree were determined using boot-

strap analyses based on 1,000 resamplings (Felsenstein, 1985). The values for 16S rRNA gene sequence similarity (%) among *Actinomadura* strains were manually calculated after pair wise alignment using the Clustal X package.

Results and Discussion

An almost-complete 16S rRNA gene sequences for strain 13-12(50)^T (1,493 nucleotides) was determined in this study and compared with the corresponding sequence of all species with validly described names of the genus *Actinomadura* in the family *Thermomonosporaceae*. A neighbor-joining tree (Fig. 2) based on 16S rRNA gene sequences showed that the organism falls within the evolutionary radiation occupied by representatives of the genus *Actinomadura*. The generic assignment was supported by the results of our own

and the earlier chemotaxonomic observations (Lee and Jeong, 2006; Trujillo and Goodfellow, 2003). Strain 13-12(50)^T contained *meso*-diaminopimelic acid, madurose, galactose, glucose and mannose in whole-organism hydrolysates (wall chemotype IIIB *sensu*; Lechevalier and Lechevalier, 1970). Diagnostic phospholipids were phosphatidylglycerol and phosphatidylinositol (Table 1). The major menaquinones were observed as MK-9(H₆) (59%) and MK-9(H₈) (34%) (Table 1). Fatty acids with major amounts for strain 13-12(50)^T were C_{16:0} (45.9%), 10-methyloctadecanoic acid (tuberculostearic acid) (10MeC_{18:0}) (18.0%), C_{18:1} ω9c (14.6%) including small amounts of C_{18:0} (6.8%), sum in feature 3 (4.1%), C_{14:0} (3.0%), C_{17:0} (1.5%) and iso-C_{16:0} (1.2%) (Table 1). These chemotaxonomic properties serve to distinguish members of the genus *Actinomadura* from the members of the genus *Nonomuraea* that form similar short chains of spores (Ara et

Table 1. Physiological, morphological, chemotaxonomic properties and G+C mol% of the *Actinomadura maheshkhaliensis* 13-12(50)^T.

Characteristic	13-12(50) ^T
Spore-chain morphology	
Spore chain	Hooks to spiral
Spore ornamentation	Irregular to warty
Number of spores	3–10
Growth on ISP 3 medium	
Aerial mycelium	Yellowish-white
Substrate mycelium	Yellow
Soluble pigment	Faint yellow
Polar lipids	Phosphatidylglycerol (PG) and phosphatidylinositol (PI)
Sugars	Madurose (trace), galactose, glucose, mannose
Mycolic acid	Not present
<i>N</i> -acetylated muramic acid type	Acetyl
Major menaquinones	MK-9(H ₆) (59%), MK-9(H ₈) (34%)
Fatty acids (%)	Major fatty acid: C _{16:0} (45.9%), 10MeC _{18:0} (18.0%) and C _{18:1} ω9c (14.6%) Minor fatty acid: C _{18:0} (6.8%), sum in feature 3 (4.1%), C _{14:0} (3.0%), C _{17:0} (1.5%) and iso-C _{16:0} (1.2%)
DNA G+C content (mol%)	72.7
Utilization of:	
L-Arabinose	+
Sucrose	±
D-Xylose	+
<i>myo</i> -Inositol	±
D-Mannose	±
D-Fructose	–
L-Rhamnose	+
D-Raffinose	–
Cellobiose	+
Glycerol	±
Mannitol	±

Fig. 1. Scanning electron micrographs of *Actinomadura maheshkhaliensis* 13-12(50)^T showing hooked and spiral short chains of warty spores on aerial mycelium grown on oatmeal agar after 3 weeks at 28°C. Bar 1 µm.

al., 2007a,b; Quintana et al., 2003b; Zhang et al., 1998, 2001).

The organism was an aerobic, Gram-positive, non-acid-alcohol-fast, non-motile actinomycete that formed extensively branched substrate mycelia that carried aerial hyphae, which differentiated into short chains of spores. Strain 13-12(50)^T produced well-developed branched substrate and aerial mycelium and did not fragment into bacillary or coccoid elements (Table 3 and Fig. 1). Curvy to hooked and spiral short spore chains (one turn) of strain 13-12(50)^T were composed of about 3 to 10 non-motile spores with a warty surface that were borne directly on aerial mycelium singly or in small clusters (Fig. 1).

It is apparent from Fig. 2 that strain 13-12(50)^T belong to a distinct phylogenetic group. The 16S rRNA gene sequence showed similarity between 13-12(50)^T and the closely associated *Actinomadura mexicana* (sequence similarity 99.5%), *Actinomadura glauciflava*,

Actinomadura citrea (sequence similarity 99.4%) and *Actinomadura madurae* (sequence similarity 99.2%) (Fig. 2). Despite sharing relatively high 16S rRNA gene sequence similarities, the isolate can be differentiated from the type strains of the phylogenetically closest neighbors, *A. mexicana* and *A. glauciflava*, on the basis of one or more cultural characteristics (growth intensity, abundance of aerial mycelium and color, substrate mycelium color and diffusible pigmentation on various agar media) and chemotaxonomic characteristics (whole cell sugar, phospholipids, menaquinones and fatty acid components) (Table 2). Moreover, DNA-DNA relatedness studies are now commonly used to resolve the finer taxonomic relationships between representative of such closely related species. In the present investigation, it is evident that isolate 13-12(50)^T belongs to a distinct genomic species, as it shares a mean DNA-DNA reassociation value of 49.9% with the phylogenetically closely related type strain *A. mexicana*. This value is well below the 70% cut-off point recommended for the delineation of genomic species (Wayne et al., 1987).

It is clear from the genotypic data that strain 13-12(50)^T forms a distinct clade within the genus *Actinomadura*. It is also evident from the phenetic and chemotaxonomic data that the strain can be distinguished from closely related type strains in the genus *Actinomadura*. In light of these data, it is proposed that strain 13-12(50)^T be classified in the genus *Actinomadura* as *Actinomadura maheshkhaliensis*.

Description of *Actinomadura maheshkhaliensis* sp. nov.

Actinomadura maheshkhaliensis (*ma.hesh.kha.li.en'sis*. N.L. fem. adj. maheshkhaliensis, pertaining to Maheshkhali, Bangladesh).

Forms a well-developed, branched substrate mycelium. Abundant aerial mycelium is present. Spore chains (3–10 spores) are curvy, slightly hooked to spiral and spores are oval to spherical with a rough, nodular surface. Sporangia are not detected. Good growth occurs on ISP medium 2, ISP medium 4, ISP medium 7, ISP medium 3, nutrient agar, Waksman no. 1 agar and Seino agar and poor growth on water agar. Substrate mycelium color is light wheat (2ca), yellow to lemon yellow (1ca) on various agar media. Pale yellowish to white aerial mycelium and abundant sporulation occurs on ISP medium 3, ISP medium 4 and Seino agar media after 21 days of incubation at 28°C. A faint

Fig. 2. Neighbor-joining tree based on nearly complete 16S rRNA gene sequences showing relationships between *Actinomadura maheshkhaliensis* 13-12(50)^T and all the valid species of the genus *Actinomadura*.

Streptosporangium album was used as an outgroup. Numbers at nodes indicate the levels of bootstrap support (%) based on a neighbor-joining analysis of 1,000 resampled datasets; only values above 20% are shown. Bar, 0.01 substitutions per site.

yellow soluble pigment is produced on ISP medium 3. Aerobic. Gram-positive. The isomer of DAP is *meso* and madurose, galactose, glucose and mannose occur in the whole-cell hydrolysates. The *N*-acyl type of muramic acid is acetyl. Mycolic acids are absent. The polar lipids include phosphatidylglycerol and phosphatidylinositol. The major menaquinone is MK-9(H₈) with minor amounts of MK-9(H₆). Fatty acids with major amounts of C_{16:0} (45.9%), 10MeC_{18:0} (18.0%), C_{18:1} ω9c (14.6%) including small amounts of C_{18:0} (6.8%), sum in feature 3 (4.1%), C_{14:0} (3.0%), C_{17:0} (1.5%) and

iso-C_{16:0} (1.2%). The G+C content of the DNA is 72.7 mol%. Isolated from muddy rhizosphere soil of Maheshkhali mangrove forest, Cox's Bazar, Bangladesh. The type strain is 13-12(50)^T (=JCM 13934^T =MTCC 8055^T).

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Table 2. Differential physiological, morphological, chemotaxonomic and G+C mol% properties of the *Actinomadura maheshkhaliensis* 13-12(50)^T, *A. glauciflava* and *A. mexicana*.

Characteristic	13-12(50) ^T	<i>A. glauciflava</i> ^a	<i>A. mexicana</i> ^b
Spore-chain morphology			
Spore chain	Hooks to spirals (1 turn)	Hooks to spirals (1 turn)	Hooks
Spore ornamentation	Irregular to warty	Warty	Warty
Number of spores	3–10	6–11	4–7
Spore diameter (μm)	0.5	1.0	1.0
Growth on ISP 2 medium			
Aerial mycelium	Moderate, yellowish-white	Abundant, bluish-green	None
Substrate mycelium	Light wheat	Yellow-brown	Cream yellow
Soluble pigment	Faint yellow	Yellow	Yellow
Polar lipids	Phosphatidylglycerol (PG) and phosphatidylinositol (PI)	PG, PI	PG, PI, PIMs
Sugars	Madurose (t), galactose, glucose, mannose	Madurose, galactose, glucose, mannose, ribose	Madurose, galactose, glucose, mannose, ribose
Major menaquinone(s)	MK-9(H ₆) (59%), MK-9(H ₈) (34%)	MK-9(H ₆) (80.0%), MK-9(H ₄) (12.0%), MK-9(H ₈) (10.0%)	MK-9(H ₆), MK-9(H ₄)
Major fatty acid (%)	C _{16:0} (45.9%), 10MeC _{18:0} (18.0%) and C _{18:1 ω9c} (14.6%)	Hexadecanoic (~40.0%), 14-methylpentadecanoic (~20.0%), 10-methyloctadecanoic (~20.0%)	ND
DNA G+C content (mol%)	72.7	72.0	ND

Data were taken from the present study and from Lu et al. (2003)^a and Quintana et al. (2003a)^b.

Table 3. Cultural characteristics of *Actinomadura maheshkhaliensis* 13-12(50)^T.

Agar medium	13-12(50) ^T
	Growth/reverse color/aerial mycelium/sporulation
Salts-starch (ISP-4)	++/pale orange (5ca)/++/++
Tyrosine (ISP-7)	++/lemon yellow (1la)/++/++
Nutrient	++/light wheat (2ca)/–/–
Yeast-malt extract (ISP-2)	++/light wheat (2ca)/++/++
Oatmeal (ISP-3)	++/yellow (1ca)/++/++
Water	±/pale yellow (1ca)/±/±
Waksman no. 1	++/light ivory (2ca)/±/±
Seino (yeast-starch) ^a	++/light melon yellow (2ca)/++/++

Cultures were incubated at 30°C for 3 weeks. Color designations and codes in parentheses were taken from the *Color Harmony Manual* (Jacobson et al., 1958). Growth and sporulation on aerial mycelium are scored as: (++) , good; (+) , moderate; (±) , poor; – , no growth and no spore formed.

^aSeino agar medium: (N-Z amine, 0.3%; yeast extract, 0.1%; meat extract, 0.1%; CaCO₃, 0.3% and agar, 1.2%).

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