Actinomadura bangladeshensis sp. nov. and *Actinomadura chokoriensis* sp. nov.

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The taxonomic position of two soil isolates, 3-46-b(3)^T and 3-45-a(11)^T, was clarified based on data from a polyphasic study. The organisms showed a combination of chemotaxonomic and morphological properties typical of the genus *Actinomadura*. They formed distinct phyletic lines in the *Actinomadura* 16S rRNA gene tree and were closely associated with the type strains of *Actinomadura meyeræ* (sequence similarity of 98.3–98.5%), *Actinomadura napierensis* (98.1–98.3%) and *Actinomadura latina* (96.4–96.8%). The level of 16S rRNA gene sequence similarity between the new isolates was 99.1%. The level of DNA–DNA hybridization between strains 3-46-b(3)^T and 3-45-a(11)^T was 43.6% and levels of relatedness between the two new isolates and the type strains of *A. meyeræ* and *A. napierensis* were 21.0–27.3%. On the basis of phenotypic and genotypic properties, the new isolates could be differentiated from each other and from their closest phylogenetic relatives. It is proposed that the organisms be classified as representing two novel species of the genus *Actinomadura*. The names proposed for these taxa are *Actinomadura bangladeshensis* sp. nov. [type strain 3-46-b(3)^T = JCM 13933^T = MTCC8057^T] and *Actinomadura chokoriensis* sp. nov. [type strain 3-45-a(11)^T = JCM 13932^T = MTCC8056^T].

The application of chemosystematic, numerical phenetic and molecular systematic methods has clarified the taxonomy of the genus *Actinomadura* and related taxa (Poscher *et al.*, 1985; Kroppenstedt *et al.*, 1990; Ochi *et al.*, 1991; Trujillo & Goodfellow, 1997, 2003; Quintana *et al.*, 2003a; Lee & Jeong, 2006; Le Roes & Meyers, 2007). The genus *Actinomadura* belongs to the family *Thermomonosporaceae* Stackebrandt *et al.* 1997 emend. Zhang *et al.* 2001, which also includes the genera *Actinocorallia* (Iinuma *et al.*, 1994; Zhang *et al.*, 2001; Lee, 2006), *Spirillospora* (Couch, 1963) and *Thermomonospora* (Henssen, 1957; Zhang *et al.*, 2001). Members of these genera form distinct phyletic lines in

trees based on 16S rRNA gene sequences (Zhang *et al.*, 1998, 2001) and can be distinguished from one another by using a combination of biochemical, chemical, morphological and physiological features (Zhang *et al.*, 1998, 2001; Trujillo & Goodfellow, 2003). At the time of writing, the genus *Actinomadura* encompasses 37 recognized species, which can be delineated based on a combination of genotypic and phenotypic data (Kroppenstedt & Goodfellow, 1991; Zhang *et al.*, 2001; Lu *et al.*, 2003; Lee & Jeong, 2006; Le Roes & Meyers, 2007).

As part of a screening programme for antibiotic-producing actinomycetes, strains 3-46-b(3)^T and 3-45-a(11)^T were isolated from sandy soil collected at Chokoria, Cox's Bazar, Bangladesh. The two strains showed morphological characteristics typical of the genera *Actinomadura* and *Nonomuraea*. Here we describe the characterization and classification of these strains and propose that they represent two novel species of the genus *Actinomadura*.

Bacterial strains were isolated by using the standard dilution plate method and were grown on humic acid-vitamin agar (Hayakawa & Nonomura, 1987) supplemented with cycloheximide (50 mg l⁻¹), nystatin (50 mg l⁻¹) and

Abbreviations: DAP, diaminopimelic acid; TBSA, tuberculostearic acid.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains 3-46-b(3)^T and 3-45-a(11)^T are AB331652 and AB331730, respectively.

A neighbour-joining tree based on nearly-complete 16S rRNA gene sequences showing the relationships between strains 3-46-b(3)^T, 3-45-a(11)^T and the type strains of all recognized species of the genus *Actinomadura* is available as supplementary material with the online version of this paper.

nalidixic acid (20 mg l^{-1}). After 21 days aerobic incubation at 30°C , the strains were transferred and purified on yeast extract-malt extract agar [medium 2 of the International *Streptomyces* Project (ISP); Shirling & 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 K_2HPO_4 , 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 15.0 g agar per litre distilled water (pH 7.2). For comparative purposes, *Actinomadura napierensis* JCM 13850^T and *Actinomadura meyeriae* JCM 13237^T were used.

Strains 3-46-b(3)^T and 3-45-a(11)^T were grown on ISP medium 3 agar and ISP medium 2 agar at 30°C for 21 days and then observed by light and scanning electron microscopy (model S-2400; Hitachi and model JSM-5600; JEOL). Samples for scanning electron microscopy were prepared as described by Itoh *et al.* (1989).

Phenotypic properties were examined by using the following standard methods. For cultural characterization, the isolates were grown for 21 days at 30°C on various agar media as described by Waksman (1950, 1961), Shirling & Gottlieb (1966) and Asano & Kawamoto (1986). Names and designations of colony colour were based on Jacobson *et al.* (1958). The temperature range and NaCl tolerance for growth were determined on ISP medium 3 (Shirling & Gottlieb, 1966). Utilization of carbohydrates (each at 1%) as sole carbon sources was tested by using ISP medium 9 according to a modification of the method of Stevenson (1967). Production of melanoid pigments was examined by using tyrosine agar (ISP medium 7).

Freeze-dried cells that were used for chemotaxonomic analyses were obtained from cultures grown in yeast-glucose (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 & Roberts (1974). Reducing sugars in whole-cell hydrolysates were analysed by the paper chromatography method of Becker *et al.* (1965). The *N*-acyl group of muramic acid in the peptidoglycan was determined according to the method of Uchida & Aida (1984). Phospholipids were extracted and identified by the method of Minnikin *et al.* (1984). Methyl esters of cellular fatty acids were analysed by TechnoSuruga Co., Ltd according to the instructions of the Microbial Identification System (MIDI) by using a gas chromatograph (model HP6890; Hewlett Packard) (Sasser, 1990). Isoprenoid quinones were extracted according to the method of Collins *et al.* (1977, 1984) and were analysed in an HPLC system (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 by using the HPLC method of Tamaoka & Komagata (1984). An equimolar mixture of nucleotides was digested by bacterial alkaline phosphatase and used as the qualitative standard.

Chromosomal DNA of strains 3-46-b(3)^T and 3-45-a(11)^T was isolated from biomass. Cells were freeze-dried and mechanically ground as described by Raeder & Broda (1985). Levels of DNA-DNA relatedness were measured fluorometrically by 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) and Ara & Kudo (2006, 2007a, b, c, d). The sequences were multiply 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 tree construction. A phylogenetic tree was constructed according to the neighbour-joining method (Saitou & Nei, 1987) contained in the CLUSTAL_X program, based on a comparison of 1241 nt present in all the strains as a result of elimination of gaps and ambiguous nucleotides from the sequences between positions 34 and 1491 (numbering according to the *Escherichia coli* 16S rRNA gene sequence). *Streptosporangium album* NBRC 13900^T was used as an outgroup. The confidence values of branches of the phylogenetic tree were determined by using bootstrap analyses based on 1000 resamplings (Felsenstein, 1985). Levels of 16S rRNA gene sequence similarity among

Fig. 1. Neighbour-joining tree based on nearly-complete 16S rRNA gene sequences showing the relationship between strains 3-46-b(3)^T and 3-45-a(11)^T and the type strains of closely related species of the genus *Actinomadura* in the family *Thermomonosporaceae*. *Streptosporangium album* DSM 43023^T was used as an outgroup. Numbers at nodes indicate levels of bootstrap support (%) based on a neighbour-joining analysis of 1000 resampled datasets; only values above 20% are shown. Bar, 0.01 substitutions per site. A more complete version of this tree is available as Supplementary Fig. S1.

Actinomadura strains were calculated manually after pairwise alignment with the CLUSTAL_X package.

Almost-complete 16S rRNA gene sequences for strains 3-46-b(3)^T (1497 nt) and 3-45-a(11)^T (1501 nt) were determined in this study and were compared with corresponding sequences of all recognized species within the genus *Actinomadura* and related taxa in the family *Thermomonosporaceae* Stackebrandt *et al.* 1997 emend. Zhang *et al.* 2001. A neighbour-joining tree (Fig. 1; see also Supplementary Fig. S1 in IJSEM Online) based on 16S rRNA gene sequences showed that the new isolates fell within the evolutionary radiation occupied by representatives of the genus *Actinomadura*. This generic assignment was supported by the results of our own and previous chemotaxonomic observations (Trujillo & Goodfellow, 2003; Lee & Jeong, 2006). Strains 3-46-b(3)^T and 3-45-a(11)^T contained meso-DAP, madurose, galactose, glucose, ribose and mannose in whole-organism hydrolysates (wall chemotype IIIB *sensu* Lechevalier & Lechevalier, 1970). Diagnostic phospholipids were phosphatidylglycerol and phosphatidylinositol (Table 1). Menaquinones were

MK-9(H₆) (59%), MK-9(H₈) (35%) and MK-9(H₄) (0.6%) for strain 3-46-b(3)^T and MK-9(H₆) (52%), MK-9(H₄) (22%), MK-9(H₈) (22%) and MK-9(H₂) (0.3%) for strain 3-45-a(11)^T (Table 1). Major fatty acids found in strain 3-46-b(3)^T were iso-C_{16:0} (23.1%), C_{17:0} (14.6%), C_{16:0} (9.5%), C_{15:0} (9.1%) and C_{17:1ω8c} (8.7%); minor amounts of 10-methyl C_{17:0} (5.8%), C_{18:1ω9c} (4.6%), C_{17:1ω9c} (4.3%), iso-C_{18:0} (3.2%), iso-C_{14:0} (3.0%), 10-methyl C_{18:0} [tuberculostearic acid (TBSA)] (2.4%) and summed feature 6 (2.3%) were also present. Major fatty acids for strain 3-45-a(11)^T were iso-C_{16:0} (24.0%), C_{17:0} (14.7%), C_{16:0} (12.0%), C_{17:1ω8c} (8.7%) and C_{15:0} (8.1%); minor amounts of 10-methyl C_{17:0} (5.0%), C_{18:1ω9c} (4.8%), iso-C_{18:0} (4.1%), iso-C_{14:0} (4.0%), C_{18:0} (2.3%), TBSA (2.2%) and summed feature 6 (2.3%) were also present (Table 1). These chemotaxonomic properties can be used to distinguish members of the genus *Actinomadura* from members of the genus *Nonomuraea* that form similar short chains of spores (Zhang *et al.*, 1998, 2001; Quintana *et al.*, 2003b; Ara *et al.*, 2007a, b).

Table 1. Differential characteristics between strains 3-46-b(3)^T and 3-45-a(11)^T and the type strains of *Actinomadura napierensis* and *Actinomadura meyeræ*

Data for reference strains were taken from Cook *et al.* (2005), Kroppenstedt *et al.* (1990), Kämpfer *et al.* (2005), Zhang *et al.* (1998), Poscher *et al.* (1985), Quintana *et al.* (2003a) and Trujillo & Goodfellow (2003). +, Positive; ±, moderate; –, negative; t, trace; ND, no data available.

Characteristic	3-46-b(3) ^T	3-45-a(11) ^T	<i>A. napierensis</i> B60 ^T	<i>A. meyeræ</i> A288 ^T
Aerial mycelium	Present	Present	Present	Absent/rare
Spore mass arrangement	Curved to hooked	Spiral	Spiral	Hooked
Spore surface ornamentation	Rough	Rough	Smooth	Warty
Number of spores	4–8	3–8	ND	ND
Diffusible pigment on ISP medium 7	–	–	+	–
Growth at 45 °C	±	+	ND	+
Polar lipids*	PG, PI	PG, PI	DPG, PI, PG	DPG, PI, PIMs
Menaquinone(s)	9(H ₆), 9(H ₈), 9(H ₄)	9(H ₆), 9(H ₄), 9(H ₈), 9(H ₂)	ND	9(H ₆)
Major fatty acids (%)	iso-C _{16:0} (23.1), C _{17:0} (14.6), C _{16:0} (9.5)	iso-C _{16:0} (24.0), C _{17:0} (14.7), C _{16:0} (12.0)	TBSA (36), C _{16:0} (28)	ND
Cell-wall sugars†	Mad (t), Gal, Glc, Man	Mad, Gal, Glc, Man, Rib	Gal, Glc, Mad, Man, Rha	Gal, Glc, Mad, Man, Rib
DNA G + C content (mol%)	70.27	72.43	70.0	ND
Utilization of:				
L-Arabinose	±	+	+	+
Cellobiose	+	+	ND	+
D-Fructose	±	±	+	+
Glycerol	±	+	+	ND
myo-Inositol	–	–	–	ND
Mannitol	±	±	+	ND
D-Mannose	–	–	ND	+
Raffinose	–	–	+	+
Sucrose	±	+	+	ND
D-Xylose	±	+	+	+

*DPG, Diphosphatidylglycerol; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIMs, PI mannosides.

†Gal, Galactose; Glc, glucose; Mad, madurose; Man, mannose; Rha, rhamnose; Rib, ribose.

The novel organisms were aerobic, Gram-positive, non-acid-alcohol-fast, non-motile actinomycetes that formed extensively branched substrate mycelia that carried aerial hyphae, which differentiated into chains of spores. Strains 3-46-b(3)^T and 3-45-a(11)^T produced well-developed branched substrate and aerial mycelium and did not fragment into bacillary or coccoid elements (Fig. 2). Curved to hooked, short spore chains of strain 3-46-b(3)^T consisted of about four to eight non-motile spores with a rough surface that were borne directly on aerial mycelium singly or in small clusters (Fig. 2a). Spiral (one turn) spore chains for strain 3-45-a(11)^T consisted of three to eight non-motile spores with a rough surface that were borne directly on the aerial mycelium (Fig. 2b). The two strains had different cultural characteristics (Tables 1 and 2) and these properties also served to distinguish the strains from each other and from phylogenetically closely related species that were included in the survey.

It is apparent from Fig. 1 that strains 3-46-b(3)^T and 3-45-a(11)^T belong to a distinct phylogenetic group. 16S rRNA gene sequence similarity between strains 3-46-b(3)^T and 3-45-a(11)^T was 99.1%. The new isolates were closely associated with the type strains of *Actinomadura meyeræ* (16S rRNA gene sequence similarity of 98.3–98.5%) and

Actinomadura napierensis (98.1–98.3%), although this relationship was not supported by a high bootstrap value (Fig. 1). Despite sharing relatively high 16S rRNA gene sequence similarities, the isolates could be distinguished from the type strains of their nearest phylogenetic neighbours, *Actinomadura meyeræ* and *Actinomadura napierensis*, on the basis of spore ornamentation, pigment production on ISP medium 7, carbon source utilization, growth at 45 °C and polar lipids (Table 1). Furthermore, the level of DNA–DNA relatedness between the two new isolates was $43.6 \pm 2.3\%$ (mean $\pm$ SD from four determinations) and levels between the new isolates and the type strains of *Actinomadura meyeræ* and *Actinomadura napierensis* were below 27%, well below the 70% cut-off point recommended for the delineation of genomic species (Wayne *et al.*, 1987).

It is clear from the genotypic and phenotypic data that strains 3-46-b(3)^T and 3-45-a(11)^T form a distinct clade within the genus *Actinomadura*. It is also evident from the phenetic and chemotaxonomic data that the two strains can be distinguished from each other and from the type strains of closely related species in the genus *Actinomadura*. In light of these data, it is proposed that strains 3-46-b(3)^T and 3-45-a(11)^T represent two novel species of the genus *Actinomadura*, for which the names *Actinomadura bangladeshensis* sp. nov. and *Actinomadura chokoriensis* sp. nov. are proposed.

Description of *Actinomadura bangladeshensis* sp. nov.

Actinomadura bangladeshensis (ban.gla.desh.en'sis. N.L. fem. adj. *bangladeshensis* pertaining to Bangladesh, where the type strain was isolated).

Forms a well-developed, branched substrate mycelium. Abundant aerial mycelium is present. Spore chains (four to eight spores) are curved to slightly hooked and spores are oval to cylindrical with a rough surface. Sporangia are not detected. Good growth occurs on ISP media 3, 4, 5 (glycerol-asparagine agar) and 7, glucose-yeast extract, Hickey–Tresner agar, Waksman agar and oatmeal-nitrate agar; moderate growth occurs on glucose-asparagine agar, yeast extract-malt extract agar and Bennett agar; and poor growth occurs on nutrient agar, water agar and Seino agar. Substrate mycelium colour is light wheat (2ea) to bamboo (2gc) on various agar media. Pale pink to white aerial mycelium and abundant sporulation are found on ISP media 3, 4 and 7, Hickey–Tresner agar, Waksman agar and oatmeal-nitrate agar after 21 days incubation at 28 °C. Soluble pigment is not produced. Aerobic. Gram-positive. Growth occurs at pH 5.0–9.0. The temperature range for growth is between 20 and 45 °C. Production of melanoid pigments is negative. Good growth occurs with 4% NaCl. The isomer of DAP is *meso*, and madurose, galactose, glucose and mannose occur in whole-cell hydrolysates. The *N*-acyl type of muramic acid is acetyl. Mycolic acids are absent. The polar lipids include

Fig. 2. Scanning electron micrographs of straight to curved spore chains on aerial mycelia of strains 3-46-b(3)^T (a) and short spiral spore chains of strain 3-45-a(11)^T (b) grown on oatmeal agar at 30 °C for 3 weeks. Bars, 2 µm (a), 5 µm (b).

Table 2. Differential cultural characteristics of strains 3-46-b(3)^T and 3-45-a(11)^T

Cultures were incubated at 30 °C for 3 weeks. Diffusible pigments were not formed on any of the agar media tested. Colour designations and codes in parentheses were taken from Jacobson *et al.* (1958). Growth and sporulation on aerial mycelium were scored as: ++, good; +, moderate; ±, poor; –, no growth and no spores formed.

Agar medium	3-46-b(3) ^T				3-45-a(11) ^T			
	Growth	Reverse colour	Aerial mycelium	Sporulation	Growth	Reverse colour	Aerial mycelium	Sporulation
ISP medium 2	+	Bamboo (2gc)	–	–	++	Tan (3ic)	–	–
ISP medium 3	++	Bamboo (2gc)	+	++	++	Light wheat (2ea)	++	++
ISP medium 4	++	Light beige (3ec)	+	++	++	Shell (3ca)	±	±
ISP medium 5	++	Light wheat (2ea)	–	–	++	Light wheat (2ea)	+	++
ISP medium 7	++	Red wood (6le)	+	++	++	Cinnamon brown (3lg)	–	–
Bennett	+	Bamboo (2gc)	–	–	++	Tan (3ic)	–	–
Glucose-asparagine	+	Light ivory (2ca)	±	+	+	Flesh pink (4ca)	–	–
Glucose-yeast extract	++	Honey gold (2ic)	–	–	++	Clove brown (3ni)	+	++
Hickey–Tresner*	++	Light tan (3gc)	+	++	++	Tan (3ic)	+	++
Nutrient	±	Bamboo (2gc)	–	–	+	Bamboo (2gc)	–	–
Oatmeal-nitrate	++	Shell pink (4ca)	+	++	++	Colourless	+	++
Seino (yeast extract-starch)	±	Light melon yellow (2ea)	–	–	+	Light ivory (2ca)	±	±
Water	±	Colourless	+	+	±	Colourless	±	±
Waksman	++	Colourless	+	++	++	Colourless	–	–

*Waksman (1961).

phosphatidylglycerol and phosphatidylinositol. The major menaquinone is MK-9(H₆); minor amounts of MK-9(H₈) and MK-9(H₄) are found. Major fatty acids (>8.0 % of the total) are iso-C_{16:0}, C_{17:0}, C_{16:0}, C_{15:0} and C_{17:1ω8c}. The G+C content of the DNA of the type strain is 70.27 mol%.

The type strain, 3-46-b(3)^T (=JCM 13933^T=MTCC8057^T), was isolated from sandy soil of Chokoria, Cox's Bazar, Bangladesh.

Description of *Actinomadura chokoriensis* sp. nov.

Actinomadura chokoriensis (cho.ko.ri.en'sis. N.L. fem. adj. *chokoriensis* pertaining to Chokoria, Bangladesh, the origin of the soil from which the type strain was isolated).

Forms a well-developed, branched substrate mycelium. Abundant aerial mycelium is present. Spore chains are spiral with three to eight spores. Sporangia are not detected. Spores are spherical to oval and the spore surface is rough. Good growth occurs on ISP media 2, 3, 4, 5 and 7, Bennett agar, glucose-yeast extract agar, Hickey–Tresner agar, Waksman agar and oatmeal-nitrate agar; moderate growth occurs on glucose-asparagine agar, nutrient agar and Seino agar; and poor growth occurs on water agar. Substrate mycelium colour is light wheat (cream) (2ea) to clove brown (3ni) on various agar media. White aerial

mycelium and sporulation are found on glycerol-asparagine, oatmeal agar, glucose-yeast extract, Hickey–Tresner agar, tap-water agar, oatmeal-nitrate and Seino agar media after 14 days incubation at 28 °C. Soluble pigment is not produced. Aerobic. Gram-positive. Growth occurs at pH 5.0–9.0. The temperature range for growth is between 20 and 45 °C. Production of melanoid pigments is negative. Good growth occurs with 4 % NaCl. The isomer of DAP is *meso*, and madurose, galactose, glucose, mannose and ribose occur in whole-cell hydrolysates. The *N*-acyl type of muramic acid is acetyl. Mycolic acids are absent. Polar lipids include phosphatidylglycerol and phosphatidylinositol. Phosphatidylcholine is absent. The major menaquinone is MK-9(H₆); minor amounts of MK-9(H₄) and MK-9(H₈) are found. The fatty acid profile is characterized by the presence of significant amounts (>8.0 % of the total) of iso-C_{16:0}, C_{17:0}, C_{16:0}, C_{17:1ω8c} and C_{15:0}. The G+C content of the DNA of the type strain is 72.43 mol%.

The type strain, 3-45-a(11)^T (=JCM 13932^T=MTCC8056^T), was isolated from sandy soil of Chokoria, Cox's Bazar, Bangladesh.

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