Streptomyces bangladeshensis sp. nov., isolated from soil, which produces bis-(2-ethylhexyl)phthalate

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The taxonomic position of an actinomycete strain isolated from soil from Natore, Bangladesh, was examined by using a polyphasic approach. The strain, designated AAB-4^T, was assigned to the genus *Streptomyces* on the basis of chemical and morphological criteria. It formed *Rectiflexibiles* aerial hyphae that carried long chains of rounded spores. The 16S rRNA gene of strain AAB-4^T was sequenced directly and then compared with those of previously studied streptomycetes following the generation of two phylogenetic trees by using maximum-likelihood and neighbour-joining algorithms. This confirmed the assignment of the novel strain to the genus *Streptomyces*. This strain showed a high level of 16S rRNA gene sequence similarity to *Streptomyces thermoviolaceus*, *Streptomyces thermodiastaticus* and *Streptomyces longisporus*, among others, but could be distinguished from them by phenotypic and physiological traits. This micro-organism produces bis-(2-ethylhexyl)phthalate, an antibacterial and antifungal agent. It is proposed that strain AAB-4^T be classified as a novel species within the genus *Streptomyces*, as *Streptomyces bangladeshensis* sp. nov. (type strain, AAB-4^T = LMG 22738^T = NRRL B-24326^T).

Introduction

The genus *Streptomyces* was proposed by Waksman & Henrici (1943) and classified in the family *Streptomycetaceae* on the basis of morphology and cell-wall chemotype. Streptomycetes are Gram-positive, aerobic micro-organisms with DNA G+C contents of 69–78 mol% (Korn-Wendish & Kutzner, 1992); they produce extensive branching substrate and aerial mycelia that develop into chains of spores by the formation of cross-walls in the multinucleate aerial filaments (Anderson & Wellington, 2001). The streptomycetes are widely used in industry because of their ability to produce numerous chemical compounds, including enzymes, antitumour agents and (in the main) antibiotics (Bérdy, 1995).

During routine screening for antibiotic-producing organisms from soil samples from different regions of Bangladesh, an actinomycete was isolated that produced the antibiotic bis-(2-ethylhexyl)phthalate. This strain, designated AAB-4^T, demonstrated a colonial morphology consistent with its assignment to the genus *Streptomyces*. The aim of the present investigation was to determine the taxonomic position of this organism, and the results suggest that it should be recognized as a novel species of the genus *Streptomyces*, for which the name *Streptomyces bangladeshensis* sp. nov. is proposed.

Strain AAB-4^T was isolated on yeast extract–glucose agar (Shirling & Gottlieb, 1966), using serial dilutions, and was selected by means of its antibacterial activity. The strain was maintained on Czapek–Dox (Shirling & Gottlieb, 1966) alkaline (pH 8.0) slants at 4 °C. Strain AAB-4^T was deposited in the Northern Regional Research Center Culture Collection (Peoria, IL, USA) as strain NRRL B-24326^T and in the BCCM/LMG Bacteria Collection (Ghent, Belgium) as strain LMG 22738^T.

Growth and sporulation of strain AAB-4^T were observed on standard media (Table 1); aerial spore-mass colour,

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Streptomyces bangladeshensis* strain AAB-4^T (=LMG 22738^T = NRRL B-24326^T) is AY750056.

A neighbour-joining dendrogram is available as a supplementary figure in IJSEM Online.

Table 1. Growth and characteristics of strain AAB-4^T on different media

Agar medium*	Growth†	Spore mass	Colony colour		Soluble pigments
			Aerial mycelia	Substrate mycelia	
YEME (ISP2)	+++	Abundant, yellow	Yellow-green	Beige	Olive green
Oatmeal (ISP3)	+++	Abundant, white	White	Yellowish white	None
Glycerol/asparagine	+++	Abundant, white	Pale white	Light yellow	None
Czapek-Dox	+++	Abundant, white	Grey	White	Yellow
Nutrient	+	Poor, white	Yellow	Yellow	None

*Media were as described by Shirling & Gottlieb (1966).

†+++ , Good growth; + , poor growth.

pigmentation of substrate mycelium and the production of diffusible pigments were recorded following incubation of the strain at 37 °C for 4–7 days. Peptone/yeast extract/iron agar and tyrosine agar (Shirling & Gottlieb, 1966) were used to score the production of melanin pigments.

Strain AAB-4^T was examined for various phenotypic properties, the results of which are listed in Table 2. Basal mineral salts agar (Hopwood, 1967) with 1 % (w/v) sole carbon sources was used to assess for carbon utilization.

Antibiotic resistance was determined at 37 °C on YEME medium (Shirling & Gottlieb, 1966), using the disc method (Al-Tai *et al.*, 1999). The ability of strain AAB-4^T to inhibit the growth of different bacteria was detected by using the plug technique (Beur *et al.*, 1966; Barry, 1980). Strain AAB-4^T spores were inoculated onto Czapek–Dox agar plates and agar discs (8 mm) of a 7-day-old culture grown at 37 °C were transferred to nutrient agar plates (Shirling & Gottlieb, 1966) that had previously been seeded with different test organisms (as listed in Table 3). The plates were kept

Table 2. Comparison of phenotypic properties of strain AAB-4^T, *S. thermoviolaceus* NRRL B-12374^T, *S. thermodiastaticus* NRRL B-5316^T and *S. longisporus* NRRL B-5336^T

Property	AAB-4 ^T	<i>S. thermoviolaceus</i>	<i>S. thermodiastaticus</i>	<i>S. longisporus</i>
Spore colour	Yellow	Grey	Grey	White
Pigmentation of substrate mycelium	White	Yellow/purple	Yellowish brown	White
Spore chain	<i>Rectiflexibiles</i>	Spiral*	Spiral*	<i>Rectiflexibiles</i>
Spore surface	Smooth	Tuberculate*	Tuberculate*	Smooth
Growth on sole carbon sources:†				
L-Arabinose	++	+	–	–
Fructose	++	++	+	–
Glucose	++	++	+	+
D-Xylose	+ / –	++	++	++
Mannitol	++	++	+ / –	++
Sucrose	++	++	+	–
Rhamnose	++	–	+ / –	++
Maltose	++	++	+	++
<i>m</i> -Inositol	++	+ / –	+ / –	++
Glycerol	++	++	++	+
Raffinose	++	++	+	+
Galactose	+	+	++	++
Melezitose	–	–	–	+
Mannose	++	++	++	++
Lactose	–	++	+	+
Trehalose	++	–	–	++

*Kim *et al.* (1999).

†–, No growth; +, poor growth; ++, moderate growth.

Table 3. Antimicrobial activities of strain AAB-4^T

Organism	Strain no.	Response*
<i>Bacillus subtilis</i>	QL-38	++
<i>Bacillus megaterium</i>	QL-40	+
<i>Staphylococcus aureus</i>	ATCC-259233	++
<i>Salmonella typhi</i>		++
<i>Shigella dysenteriae</i>	AL-35587	++
<i>Shigella flexneri</i>	AL-30372	++
<i>Escherichia coli</i>	FPFC-1407	++
<i>Pseudomonas aeruginosa</i>	CRL	+
<i>Candida albicans</i>	ATCC-10231	++
<i>Aspergillus niger</i>		++
<i>Aspergillus fumigatus</i>		+
<i>Penicillium digitatum</i>		+
<i>Epidermophyton floccosum</i>		+

* ++, Strongly positive; +, positive.

overnight at 4 °C and then incubated at 37 °C for 24 h. Tolerance of the novel strain to 0.2, 2, 4 and 7 % NaCl was determined on ISP9 medium (Shirling & Gottlieb, 1966).

Isolation of chromosomal DNA from strain AAB-4^T was carried out essentially as described by Hunter (1988). The 16S rRNA gene was amplified from purified DNA of the strain as described previously (Petrosyan *et al.*, 2003) using TripleMaster DNA polymerase (Eppendorf). The amplified fragment was purified from the gel by using a QIAquick Gel extraction kit (Qiagen) and then sequenced directly with an ABI Prism BigDye Terminator v2.0 Cycle Sequencing kit (Applied Biosystems). The sequences were obtained with a model 310 Genetic Analyser automated sequencer (Applied Biosystems).

For the identification of bis-(2-ethylhexyl)phthalate, mycelium from 7-day-old culture grown in Czapek–Dox broth (pH 8.0) (Shirling & Gottlieb, 1966) was separated by filtration and the culture filtrate was extracted twice with ethyl acetate. The organic fraction was evaporated under reduced pressure and the antibiotic was separated and purified by using chromatographic techniques. The pure compound was identified by using one-dimensional (¹H and ¹³C) and two-dimensional NMR data.

The colonial morphology of strain AAB-4^T was consistent with its assignment to the genus *Streptomyces* (Williams *et al.*, 1989). The strain formed a highly branched substrate mycelium and aerial hyphae that differentiated into long *Rectiflexibiles* spore-chains. On standard media, the colour of the substrate mycelium was beige and that of the aerial spore mass was yellow (Table 1). Strain AAB-4^T contained LL-diaminopimelic acid, as determined according to the methodology of Stanek & Roberts (1974).

To confirm that strain AAB-4^T was a streptomycete, we sequenced the almost-complete 16S rRNA gene from this

micro-organism and compared it with the 16S rRNA gene sequences of previously described streptomycetes. Strain AAB-4^T has a high percentage of nucleotide sequence similarity to *Streptomyces thermoviolaceus* NRRL B-12374^T (98 %), *Streptomyces thermodiastaticus* NRRL B-5316^T (98 %) and *Streptomyces longisporus* NRRL B-5336^T (97 %). These values correspond to 20–30 nt differences out of 1421 positions. Nucleotide similarity values within this range have been reported for several *Streptomyces* species with validly published names and which can be separated from each other on the basis of DNA relatedness data and phenotypic properties (Kim *et al.*, 1999, 2000).

The 16S rRNA gene sequence of strain AAB-4^T generated in this work (1421 nt; GenBank accession no. AY750056) was aligned with the 16S rRNA gene sequences of other streptomycetes obtained from the EMBL/GenBank dataset. The alignment of the sequences was carried out using CLUSTAL W software (Thompson *et al.*, 1994) and was adjusted manually. The alignment contained 1428 nt from 21 species. To determine which model of sequence evolution best fitted our dataset, a nested likelihood-ratio test was performed using the MODELTEST program, version 3.04 (Posada & Crandall, 1998). Phylogenetic relationships were inferred using the maximum-likelihood method (Felsenstein, 1981). Fifty random-taxon-addition heuristic searches with the tree bisection–reconnection branch-swapping option were conducted using PAUP* 4.0b10 software (Swofford, 2002). Genetic distances among species were estimated by using the Kimura method (Kimura, 1980). The relationships among taxa were also established by neighbour joining with the MEGA program (Kumar *et al.*, 2001). The robustness of the neighbour-joining and maximum-likelihood trees was evaluated using bootstrapping with 1000 and 10 000 replicates, respectively (Felsenstein, 1981).

Nucleotide frequencies for the 16S rRNA gene sequence dataset were 0.225 (A), 0.255 (C), 0.336 (G) and 0.182 (T). The heterogeneity of nucleotide frequencies across taxa was tested using the 'basefreq' option implemented in PAUP* ($\chi^2 = 1.230$, $P = 1.0$). The result indicates that rRNA gene nucleotide frequencies were not significantly heterogeneous across taxa, which is advantageous because the maximum-likelihood inference method performs optimally when nucleotide frequencies are homogeneous (Omilian & Taylor, 2001). The likelihood-ratio test indicated that the best model to fit the 16S rRNA gene dataset was Tamura–Nei (TrN) (Tamura & Nei, 1993), with an equal rate of substitution and a proportion of invariable sites of 0.9035. A maximum-likelihood analysis using this model yielded a single tree with a $-\ln$ score of 2646.11. The maximum-likelihood tree showed that strain AAB-4^T is a sister species of *S. longisporus* with a bootstrap value of 52 % (Fig. 1). The tree resulting from the neighbour-joining analysis yielded the same relationship between *S. bangladeshensis* and *Streptomyces longisporus*, with a low bootstrap value (34 %). The neighbour-joining dendrogram showed that the clade composed by both species was a sister clade to the species *S.*

Fig. 1. Maximum-likelihood tree based on 16S rRNA gene sequences. The tree was inferred from a dataset of 1428 nt from 21 strains of *Streptomyces*. The $-\ln$ likelihood is 2646.11. Branch lengths are proportional to the inferred number of nucleotide substitutions. Numbers below internal nodes show maximum-likelihood clade frequencies. GenBank accession numbers are in parentheses.

thermoviolaceus and *S. thermodiastaticus* (see Supplementary Figure, available in IJSEM Online).

The novel strain had a number of phenotypic characteristics that distinguish it from related micro-organisms. As can be seen in Table 2, strain AAB-4^T differs from *S. longisporus* in spore colour, soluble pigment formation, growth on L-arabinose, fructose, xylose and sucrose, and, less strikingly, on glucose, glycerol, raffinose, galactose, melezitose and lactose as sole carbon sources. The differences between AAB-4^T and *S. thermodiastaticus* and *S. thermoviolaceus* include such morphological traits as spore-chain shape and colour, pigmentation of substrate mycelium, and growth on rhamnose, *m*-inositol and lactose (for *S. thermoviolaceus*) and L-arabinose, D-xylose, mannitol, rhamnose, *m*-inositol and trehalose (for *S. thermodiastaticus*). The physiological properties of strain AAB-4^T are presented in the species description below.

When grown in Czapek–Dox broth (pH 8.0) medium, strain AAB-4^T produced the antimicrobial agent bis-(2-ethylhexyl)phthalate, previously isolated from *Streptomyces melanosporofaciens* (Kim *et al.*, 1991). The compound was

identified by ¹H and ¹³C NMR and showed significant activity against Gram-positive and -negative bacteria and some fungi (Table 3). However, these two micro-organisms are different with respect to their phenotypic characteristics (Kim *et al.*, 1990) and their 16S rRNA gene sequences (GenBank accession no. AJ391837).

The genotypic and phenotypic data suggest that strain AAB-4^T should be recognized as a novel species of the genus *Streptomyces*, for which we propose the name *Streptomyces bangladeshensis* sp. nov.

Description of *Streptomyces bangladeshensis* sp. nov.

Streptomyces bangladeshensis (ban.gla.desh.en'sis. N.L. masc. adj. *bangladeshensis* belonging to Bangladesh, the source of the soil from which the organism was isolated).

Aerobic, Gram-positive, moderately thermophilic actinomyces. Forms highly branched substrate mycelium and aerial hyphae that differentiate into long *Rectiflexibiles* chains of eight to ten spores. Aerial spore-mass colour is

yellow–green. Substrate mycelium is beige on standard media. Yellowish diffusible pigments are formed on Czapek–Dox agar. Melanin pigments are not produced on peptone/iron or tyrosine agars. Positive for H₂S production. Utilizes glucose, sucrose, *m*-inositol, mannitol, mannose, maltose, fructose, L-arabinose, rhamnose, glycerol, raffinose and trehalose as sole carbon sources. Growth occurs at 20–50 °C, at pH 6.0–11.0 and in the presence of 2% (w/v) NaCl, neomycin sulfate (50 µg ml⁻¹) and penicillin (10 IU ml⁻¹). Produces bis-(2-ethylhexyl)phthalate, an antimicrobial agent.

The type strain is AAB-4^T (=LMG 22738^T=NRRL B-24326^T). Isolated from soil from Natore, Bangladesh.

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