Streptomyces brevispora sp. nov. and *Streptomyces laculatispora* sp. nov., actinomycetes isolated from soil

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The taxonomic positions of two actinomycetes isolated from a hay meadow soil sample were determined using a polyphasic approach. The isolates had chemical and morphological properties typical of streptomycetes and formed a distinct 16S rRNA gene subclade together with the type strain *Streptomyces drozdowiczii* NRRL B-24297^T. DNA–DNA relatedness studies showed that the three strains belonged to different genomic species. The organisms were also distinguished using a combination of phenotypic properties. On the basis of these data it is proposed that the isolates be assigned to the genus *Streptomyces* as *Streptomyces brevispora* sp. nov. and *Streptomyces laculatispora* sp. nov., with BK160^T (=KACC 21093^T =NCIMB 14702^T) and BK166^T (=KACC 20907^T =NCIMB 14703^T) as the respective type strains.

The genus *Streptomyces* encompasses nearly 600 described species (Euzéby, 2011), members of which are the source of a broad range of secondary metabolites, notably antibiotics (Bérdy, 2005; Goodfellow & Fiedler, 2010). The subgeneric classification of the genus, while complex, is being improved by the application of genotypic and phenotypic procedures (Lanoot *et al.*, 2005; Goodfellow *et al.*, 2007; Rong & Huang, 2010). These procedures are also effective in the reassignment of misclassified species of the genus *Streptomyces* (Tamura *et al.*, 2008; Kumar & Goodfellow, 2010) and in the recognition of new members of the taxon isolated from diverse natural habitats (Semêdo *et al.*, 2004; Xu *et al.*, 2006; Xiao *et al.*, 2009; Zhao *et al.*, 2010).

The present investigation was designed to establish the taxonomic positions of two *Streptomyces* strains isolated from a hay meadow soil sample. A polyphasic taxonomic study based on a combination of genotypic and phenotypic procedures showed that isolates BK160^T and BK166^T should be recognized as two novel species of the genus *Streptomyces*, *Streptomyces brevispora* sp. nov. and *Streptomyces laculatispora* sp. nov., respectively.

Strains BK160^T and BK166^T were isolated on starch-casein agar (Küster & Williams, 1964), supplemented with

cycloheximide and nystatin (at 25 µg ml⁻¹), which had been inoculated with a pre-heated (55 °C for 20 min) soil suspension and incubated at 28 °C for 21 days. The soil sample was collected from Palace Leas meadow hay plot 6 (Atalan *et al.*, 2000) at Cockle Park Experimental Farm, Northumberland, UK (National Grid reference NZ 200913). The organisms were maintained on oatmeal agar slopes (International *Streptomyces* Project - ISP medium 3; Shirling & Gottlieb, 1966) at 4 °C and as mixtures of mycelial fragments and spores in 20% (v/v) glycerol at –80 °C. Biomass for the chemotaxonomic and molecular systematic studies was grown in shake flasks of tryptone-yeast extract broth (ISP medium 1; Shirling & Gottlieb, 1966) for 7 days at 28 °C, harvested by centrifugation and washed twice in distilled water; cells for the chemotaxonomic studies were freeze-dried.

Genomic DNA was extracted from the isolates and PCR amplification and 16S rRNA gene sequencing achieved using procedures described previously (Kim *et al.*, 1996). The resultant, almost complete 16S rRNA gene sequences (1439 to 1457 nt) were aligned manually against corresponding sequences of representatives of the genus *Streptomyces* using MEGA 4 software (Tamura *et al.*, 2007). Phylogenetic trees were inferred by using the maximum-likelihood (Felsenstein, 1981), maximum-parsimony (Fitch, 1971) and neighbour-joining (Saitou & Nei, 1987) tree-making algorithms drawn from the MEGA 4 (Tamura *et al.*, 2007) and PHYL (Guindon

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains BK160^T and BK166^T are FR692104 and FR692106, respectively.

& Gascuel, 2003) packages. The Jukes & Cantor (1969) model was used to generate evolutionary distance matrices for the neighbour-joining data. Topologies of the resultant trees were evaluated by bootstrap analysis (Felsenstein, 1985) of the neighbour-joining method based upon 1000 replicates using MEGA 4 software. *Kitasatospora griseola* JCM 3339^T (GenBank accession no. U93320) was used as the outgroup.

Isolates BK160^T and BK166^T formed a distinct subclade in the *Streptomyces* 16S rRNA gene tree together with the type strain of *Streptomyces drozdowiczii*, a taxon which was supported by all tree-making algorithms and by a bootstrap value of 74 % (Fig. 1). The isolates shared a 16S rRNA gene sequence similarity of 99.9 %, a value which corresponded to 2 nt differences over 1434 locations. Isolate BK166^T is the strain that is most closely related to *S. drozdowiczii* NRRL B-24297^T; the two organisms share a 16S rRNA gene similarity of 99.4 %, a value equivalent to 7 nt differences over 1434 locations. Similarly, isolate BK160^T shares a 16S rRNA similarity of 99.4 % with the type strain of *S. drozdowiczii*, a value equivalent to 9 nt differences over 1439 sites. The *S. drozdowiczii* subclade is loosely associated with the *Streptomyces niveus* subclade, which also contains the type strains of *Streptomyces laceyi* and *Streptomyces spheroides*; the latter two species have been considered to be synonyms of *S. niveus* (Tamura *et al.*, 2008).

DNA–DNA relatedness values between the isolates and between them and the type strain of *S. drozdowiczii* were determined using the nitrocellulose membrane filter hybridization procedure described by Seldin & Dubnau (1985). DNA probes were labelled using the non-radioactive digoxigenin (DIG) High Prime System (Roche), hybridized DNA visualized using a DIG luminescent detection kit (Roche), and DNA–DNA relatedness quantified using a densitometer (Bio-Rad). The resultant data showed that all of the strains belong to different genomic species as they shared DNA–DNA relatedness values

between 25.6 and 54.5 % (Table 1); values well below the 70 % cut-off point recommended for the assignment of strains to the same genomic species (Wayne *et al.*, 1987). The two most closely related strains, isolates BK160^T and BK166^T, shared a DNA–DNA relatedness value of 54.5 %.

Isolates BK160^T and BK166^T were examined for chemical markers considered to be characteristic of strains of the genus *Streptomyces*. Standard procedures were used to determine the isomers of diaminopimelic acid (Hasegawa *et al.*, 1983), predominant menaquinones (Collins, 1985), muramic acid type (Uchida *et al.*, 1999) and diagnostic whole-cell sugars (Hasegawa *et al.*, 1983), using appropriate controls. Fatty acids from the isolates and from *S. drozdowiczii* NRRL B-24297^T were extracted, methylated and analysed by GC using the standard Sherlock Microbial Identification (MIDI) system, version 5 (MIDI, 1999; Sasser, 1990). The DNA base composition of the isolates was determined using the procedure described by Gonzalez & Saiz-Jimenez (2002).

Isolates BK160^T and BK166^T contained major amounts of LL-diaminopimelic acid but lacked diagnostic sugars in whole-organism hydrolysates (wall chemotype I *sensu* Lechevalier & Lechevalier, 1970), contained N-acetylated muramic acid, and contained hexa- and octahydrogenated menaquinones with nine isoprene units [MK-9 (H₆, H₈)] as predominant isoprenologues in a ratio of 5:1 and 4:2, respectively. The cellular fatty acid profiles consisted mainly of saturated straight-chain and iso- and anteiso-branched-chain components (Table 2), hence all three strains belong to fatty acid type 2c *sensu* Kroppenstedt (1985); the fatty acid composition of the *S. drozdowiczii* strain was found to be similar to that recorded by Semêdo *et al.* (2004). The DNA G + C content of each of the isolates was 69.2 %. All of these properties support the classification of the strains in the genus *Streptomyces* (Williams *et al.*, 1983; Manfio *et al.*, 1995; Anderson & Wellington, 2001).

Fig. 1. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences showing relationships between isolates BK160^T and BK166^T and between them and strains of phylogenetically close species of the genus *Streptomyces*. Asterisks indicate branches of the tree that were also recovered with the maximum-likelihood and maximum-parsimony tree-making algorithms; P indicates branches which were also recovered with the maximum-parsimony tree-making algorithm. Numbers at nodes are percentage bootstrap values based on 1000 resampled datasets; only values above 50 % are given. Arrow indicates the inferred root position using *Kitasatospora griseola* JCM 3339^T (GenBank accession no. U93320) as outgroup. Bar, 0.005 substitutions per nucleotide position.

Table 1. 16S rRNA gene sequence similarities and DNA–DNA relatedness values between isolates BK160^T, BK166^T and *S. drozdowiczii* NRRL B-24297^T

Values above the diagonal are DNA–DNA relatedness values (%) whereas those below the diagonal are nucleotide difference/16S rRNA gene sequence similarities (%).

Strain	BK160 ^T	BK166 ^T	<i>S. drozdowiczii</i> NRRL B-24297 ^T
BK160 ^T	–	54.5	30.2
BK166 ^T	2/99.9	–	25.6
<i>S. drozdowiczii</i> NRRL B-24297 ^T	9/99.4	7/99.5	–

The isolates were examined for cultural and morphological features following growth on several standard media for three weeks at 28 °C. Cultural properties were sought using glucose-yeast extract-malt extract (GYM; DSMZ

medium 65) and modified Bennett's (Jones, 1949) agars, and on oatmeal, inorganic salts-starch, glycerol-asparagine and tyrosine agars (ISP media 3, 4, 5 and 7; Shirling & Gottlieb, 1966). Spore arrangement and spore surface

Table 2. Diagnostic characteristics of strains BK160^T and BK166^T and their closest phylogenetic relatives in the genus *Streptomyces*

Strains: 1, isolate BK160^T; 2, isolate BK166^T; 3, *S. drozdowiczii* NRRL B-24297^T. +, Positive; –, negative.

Characteristic	1	2	3
Appearance on inorganic salt-starch agar			
Spore-mass colour	None	White	Grey
Substrate mycelium colour	White	Pale yellow	Yellow–brown
Production of soluble pigment	–	–	+
Melanin production on tyrosine agar	–	+	+
Degradation tests			
Arbutin	+	+	–
Cellulose	–	–	+
Elastin	+	+	–
Xanthine	+	+	–
Xylan	+	+	–
Enzyme activity			
Allantoin hydrolysis	–	+	–
Pectin hydrolysis	–	–	+
Growth on sole carbon sources			
L-Arabinose	–	+	+
D-Mannose	+	–	+
D-Xylose	+	+	–
L-Rhamnose	–	+	–
D-Mannitol	+	+	–
myo-Inositol	+	+	–
Growth at 37 °C	+	–	+
Tolerance of (µg ml ^{–1}):			
Streptomycin sulphate (16)	–	+	–
Rifampicin (5)	–	–	+
Kanamycin sulphate (30)	–	+	–
Vancomycin hydrochloride (0.25)	–	–	–
Major fatty acids (expressed as %)			
iso-C _{14:0}	4.9	5.5	6.0
anteiso-C _{15:0}	36.4	29.5	18.0
iso-C _{15:0}	5.9	10.1	19.0
C _{16:0}	8.5	9.0	6.0
iso-C _{16:0}	19.9	18.5	22.0
anteiso-C _{17:0}	12.6	8.9	7.0
iso-C _{17:0}	3.2	4.7	10.0

ornamentation were observed by examining gold-coated, dehydrated preparations from the ISP 3 cultures, using a scanning electron microscope (Cambridge Stereoscan 240 instrument) and the procedure described by O'Donnell *et al.* (1993).

Isolate BK160^T formed a branched white substrate mycelium and sparse white aerial hyphae which differentiated into straight to flexuous (*Rectiflexibilis*) chains of smooth-surfaced spores on oatmeal agar (Fig. 2a). The organism grew well on ISP 7, did not grow on GYM agar and grew poorly on all of the other media. Similarly, isolate BK166^T produced an extensive branched light-brown substrate mycelium and abundant greyish aerial hyphae which differentiated into straight to flexuous chains of square-like smooth-surfaced spores on oatmeal agar (Fig. 2b). This organism grew well on all remaining media producing light-brown soluble pigments on ISP 3, ISP 5, ISP 7 and modified Bennett's agars.

The isolates and the type strain of *S. drozdowiczii* were examined for a range of phenotypic properties using media and methods described by Williams *et al.* (1983). It can be seen from Table 2 that strains BK160^T and BK166^T can be readily distinguished from one another and from *S. drozdowiczii* NRRL B-24297^T using a combination of

phenotypic properties and by quantitative differences in fatty acid composition (Table 2). Thus, isolate BK166^T, unlike BK160^T, hydrolysed allantoin, grew on L-arabinose and L-rhamnose as sole carbon sources, and was tolerant of kanamycin sulphate (30 µg ml⁻¹) and streptomycin sulphate (16 µg ml⁻¹). Unlike the two isolates, the type strain of *S. drozdowiczii* hydrolysed pectin and degraded cellulose, but not xanthine or xylan. Additional phenotypic properties are cited in the species descriptions.

In conclusion, the genotypic and phenotypic data show that isolates BK160^T and BK166^T represent two novel species of the genus *Streptomyces*. It is, therefore, proposed that these organisms be recognized as *Streptomyces brevispora* sp. nov. and *Streptomyces laculatispora* sp. nov., respectively.

Description of *Streptomyces brevispora* sp. nov.

Streptomyces brevispora (bre.vi.spo'ra. L. adj. *brevis* small; N.L. n. *spora* a seed and in biology a spore; N.L. fem. n. *brevispora* the small spore, referring to the small size of the spores).

Aerobic, Gram-positive, non-acid-alcohol-fast actinomycete which forms a branched substrate mycelium that bears aerial hyphae which differentiate into short, straight chains of smooth-surfaced spores (0.7–0.8 × 0.7–0.9 µm) on oatmeal agar. Grows from 4 to 37 °C and from pH 5.0 to 9.0, but not in the presence of 7.0% (w/v) NaCl. Hydrolyses chitin, starch and uric acid, but not guanine or tributyrin. L-Arabitol, cellobiose, D-galactose, D-glucose, maltose, melibiose, D-salicin and D-sorbitol are used as sole carbon sources for energy and growth, but not D-sorbose (at 1%, w/v) or oxalic acid (at 0.2%, w/v). Susceptible (µg ml⁻¹ unless indicated) to: cephaloridine hydrochloride (2), gentamicin sulphate (8), novobiocin (8) and tetracycline hydrochloride (8), but not to ampicillin (4), ciprofloxacin (2), lincomycin hydrochloride (8) or penicillin G (2 IU ml⁻¹). Lysozyme (0.05%, w/v) sensitive. Additional properties are cited in the text and in Table 2. Chemotaxonomic properties are typical of the genus *Streptomyces*. The DNA G + C content is 69.2 mol%.

The type strain, BK160^T (=KACC 21093^T =NCIMB 14702^T), was isolated from soil. The species description is based on a single strain and hence serves as the description of the type strain.

Description of *Streptomyces laculatispora* sp. nov.

Streptomyces laculatispora (la.cu.la.ti.spo'ra. L. adj. *laculatus* four-cornered; N.L. n. *spora* a seed and in biology a spore; N.L. fem. n. *laculatispora* the four-cornered spore, referring to the square-like spores).

Aerobic, Gram-positive, non-acid-alcohol-fast actinomycete which forms a branched substrate mycelium that carries aerial hyphae which differentiate into short, straight

Fig. 2. Electron micrograph of (a) strain BK160^T and (b) strain BK166^T grown on an oatmeal agar for 3 weeks at 28 °C. Bars, 5 µm.

chains of smooth-surfaced, square-like spores ($0.7\text{--}0.8 \times 0.7\text{--}0.8\ \mu\text{m}$) on oatmeal agar. Grows from 4 to 30 °C, from pH 5.0 to 9.0, and in the presence of 7.0 % (w/v) NaCl. Hydrolyses casein, starch and uric acid, but not chitin, guanine or tributyrin. L-Arabitol, cellobiose, D-galactose, D-glucose, maltose, melibiose, D-salicin and D-sorbitol are used as sole carbon sources for energy and growth, but not D-sorbose (at 1.0 %, w/v) or oxalic acid (at 0.2 %, w/v). Susceptible ($\mu\text{g ml}^{-1}$ unless indicated) to: cephaloridine hydrochloride (2), gentamicin sulphate (8), novobiocin (8) and tetracycline hydrochloride (8), but not to ampicillin (2), ciprofloxacin (2), clindamycin hydrochloride (10), lincomycin hydrochloride (8) or penicillin G ($2\ \text{IU ml}^{-1}$). Lysozyme (0.05 %, w/v) resistant. Additional properties are cited in the text and in Table 2. Chemotaxonomic properties are typical of the genus *Streptomyces*. The DNA G+C content is 69.2 mol%.

The type strain, BK166^T (=KACC 20907^T =NCIMB 14703^T), was isolated from soil. The species description is based on a single strain and hence serves as the description of the type strain.

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