

Streptomyces staurosporininus sp. nov., a staurosporine-producing actinomycete

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The taxonomic position of a staurosporine-producing actinomycete isolated from a hay meadow soil was determined using a polyphasic approach. The organism had chemical and morphological characteristics consistent with its classification in the genus *Streptomyces* and formed a distinct branch between the *Streptomyces lydicus* and *Streptomyces noursei* clades in the 16S rRNA *Streptomyces* gene tree. DNA–DNA relatedness values between the isolate and its nearest phylogenetic neighbours, namely *Streptomyces lydicus* NBRC 13058^T and *Streptomyces chattanoogensis* NBRC 12754^T, were 53 % and 40 %, respectively. The isolate was also readily distinguished from the type strains of these species using a combination of morphological and other phenotypic properties. On the basis of these results, it is proposed that isolate BK179^T (=KACC 20912^T =NRRL B-24850^T) be classified as the type strain of *Streptomyces staurosporininus* sp. nov.

Members of the genus *Streptomyces* remain a unique source of natural products, including clinically significant antibiotics, antimetabolites and antitumour agents (Watve *et al.*, 2001; Fiedler *et al.*, 2005; Olano *et al.*, 2009), hence the continued interest in screening streptomycetes for new bioactive compounds (Bérdy, 2005; Goodfellow & Fiedler, 2010). Another remarkable feature of the genus is the large number of described species it contains, nearly 600 at the time of writing (Euzéby, 2011). The subgeneric classification of the genus, although complex, is being clarified by the application of judiciously chosen genotypic and phenotypic procedures (Kim *et al.*, 1996; Kämpfer *et al.*, 2008; Kumar & Goodfellow, 2010; Rong & Huang, 2010). These procedures are also being used to detect novel and rare streptomycetes for screening programmes (Hohmann *et al.*, 2009a, b) as it is becoming counterproductive to screen common members of the genus as this practice leads to the costly rediscovery of known antibiotics (Busti *et al.*, 2006; Lam, 2007).

In a continuation of our screening programme, a presumptively novel *Streptomyces* strain, isolate BK179^T, was isolated from a hay meadow soil. A polyphasic study based on genotypic and phenotypic procedures showed that the

isolate belonged to a novel species of the genus *Streptomyces*. The novel species, *Streptomyces staurosporininus*, was most closely related to, but readily differentiated from, the type strain of *Streptomyces lydicus*.

Isolate BK179^T was recovered from a plate of starch-casein agar (Küster & Williams, 1964), supplemented with cycloheximide and nystatin (each 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 Cackle Park Experimental Farm, Northumberland, UK (national grid reference NZ 200913). The organism was maintained on oatmeal agar slopes [International *Streptomyces* Project (ISP) medium 3; Shirling & Gottlieb, 1966] at 4 °C and as a mixture of mycelial fragments and spores in 20 % (v/v) glycerol at –20 °C. Biomass for chemotaxonomic and molecular systematic studies was grown in shake flasks of tryptone-yeast extract broth (Shirling & Gottlieb, 1966) for 7 days at 28 °C, harvested by centrifugation and washed twice in distilled water; cells for chemical analysis were freeze-dried.

The 16S rRNA gene of the isolate was analysed as described previously (Kim *et al.*, 1996). The resultant, almost complete sequence (1457 nt) was aligned manually against corresponding sequences of representatives of the genus *Streptomyces* using MEGA 4 software (Tamura *et al.*, 2007).

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain BK179^T is FR692111.

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 PHYLML (Guindon & Gascuel, 2003) software packages. The Jukes & Cantor (1969) model was used to generate an evolutionary distance matrix for the neighbour-joining algorithm. The topology of the neighbour-joining tree was evaluated by a bootstrap analysis (Felsenstein, 1985) based on 1000 resampled datasets. *Streptomyces macrosporus* DSM 41499^T (GenBank accession no. Z68099) and *Streptomyces megasporus* DSM 41476^T (Z68100) were used as outgroups.

Isolate BK179^T formed a distinct phyletic line between the *Streptomyces lydicus* and *Streptomyces noursei* clades, the taxonomic status of which was supported by high bootstrap values and by all of the tree-making algorithms (Fig. 1). The organism was most closely related to the type strains of *S. lydicus* and *Streptomyces chattanoogensis*, sharing sequence similarities of 99.2 and 99.1 %, respectively, values which corresponded to 11 and 13 nt differences across 1435 and 1430 locations. The corresponding similarities with members of the *Streptomyces noursei* clade fell within the range 98.4–98.9 %, values equivalent to 16 and 23 nt differences.

DNA–DNA relatedness values between isolate BK179^T and *Streptomyces chattanoogensis* NBRC 12754^T and *Streptomyces lydicus* NBRC 13058^T were determined using the nitrocellulose filter hybridization method as described by Seldin & Dubnau (1985). Probe DNA samples were labelled using the non-radioactive digoxigenin (DIG) High Prime System (Roche), hybridized DNA visualized using DIG luminescent detection kits (Roche) and DNA–DNA relatedness quantified using a densitometer (Bio-Rad). It was evident from the resultant data that isolate BK179^T belongs to a distinct genomic species as it shared DNA–DNA relatedness values

of 40.0 (± 5.2) % and 53.0 (± 3.5) % with *Streptomyces chattanoogensis* and *Streptomyces lydicus*, respectively; values well below the 70 % cut-off point recommended for the assignment of strains to the same genomic species (Wayne *et al.*, 1987).

Isolate BK179^T was examined for chemical markers considered to be characteristic of members of the genus *Streptomyces*. Standard procedures were used to determine the isomer of diaminopimelic acid (A₂pm; 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 the type strain of *Streptomyces lydicus* as control. Fatty acids were extracted from the isolate, methylated and analysed by GC using the standard Sherlock Microbial Identification (MIDI) system, version 5 (MIDI, 1999; Sasser, 1990), and the DNA base composition of the isolate was determined using the thermal denaturation method of Marmur & Doty (1962) with *Escherichia coli* K-12 as a control.

The isolate contained major amounts of LL-diaminopimelic acid but lacked diagnostic sugars in whole-organism hydrolysates (wall chemotype I *sensu* Lechevalier & Lechevalier, 1970), contained *N*-acetyl muramic acid moieties and hexa- and octahydrogenated menaquinones with nine isoprene units [MK-9 (H₆, H₈)] as predominant isoprenoid quinones. The cellular fatty acid profile consisted of major amounts of iso-C_{16:0} (19.5 %), C_{16:0} (17.0 %) and anteiso-C_{15:0} (16.8 %) components, lower amounts (<10 %) of iso-C_{15:0} (7.8 %), C_{17:0} cyclo (7.6 %), anteiso-C_{17:0} (6.1 %) and iso-C_{14:0} (5.7 %) and trace amounts of iso-C_{16:1} ω 7c/C_{15:0} 2-OH, iso-C_{17:0}, iso-C_{16:1} H, iso-C_{17:1} ω 9c and anteiso-C_{17:1} ω 9c. The DNA G+C content of the isolate was 71.2 %. All of these properties are consistent with the classification of the strain in the genus *Streptomyces* (Williams *et al.*, 1983; Manfio *et al.*, 1995; Anderson & Wellington, 2001; Kämpfer, 2012).

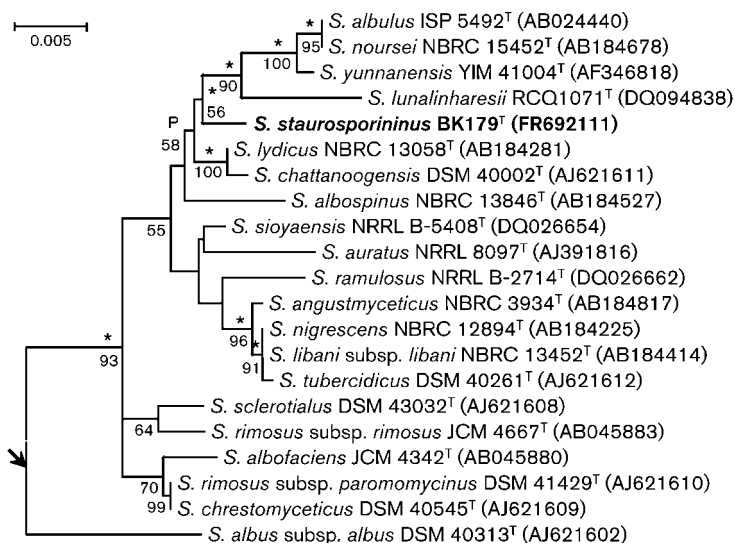


Fig. 1. Neighbour-joining tree based on a nearly complete 16S rRNA gene sequence (1457 nt) showing relationships between isolate BK179^T and closely related species of the genus *Streptomyces*. Asterisks indicate branches of the tree that were also recovered with the maximum-likelihood and maximum-parsimony tree-making algorithms; P indicates branches which were also recovered by the maximum-parsimony tree-making algorithm. Numbers at nodes are percentage bootstrap values based on 1000 resampled datasets; only values above 50 % are given. The arrow indicates the inferred root position using *Streptomyces macrosporus* DSM 41499^T (GenBank accession no. Z68099) and *Streptomyces megasporus* DSM 41476^T (Z68100) as outgroup. Bar, 0.005 substitutions per nucleotide position.

The isolate was also examined for cultural and morphological features after growth on several standard nutrient media at 28 °C for 3 weeks. Cultural properties were sought using glucose-yeast extract-malt extract (GYM; DSMZ medium 65) and modified Bennett's (Jones, 1949) agars and on tryptone-yeast extract, yeast extract-malt extract, oatmeal, inorganic salts-starch, glycerol-asparagine and tyrosine agars, that is, on ISP media 1–5 and 7 (Shirling & Gottlieb, 1966). Spore arrangement and spore surface ornamentation were observed by examining a gold-coated, dehydrated preparation prepared from the ISP3 plate, using a scanning electron microscope (Cambridge Steroscan 240 instrument) and the procedure described by O'Donnell *et al.* (1993). The isolate formed an extensively branched white substrate mycelium and aerial hyphae which differentiated into spiral chains of ridged ornamented spores on oatmeal agar. It produced a pale yellow substrate mycelium on glucose-yeast extract-malt extract, modified Bennett's, inorganic salts-starch, glycerol-asparagine and tyrosine agars, but did not grow on the remaining media.

Strain BK179^T was investigated by HPLC-diode array analysis for the production of secondary metabolites. The strain was cultivated in a 500 ml Erlenmeyer flask on a rotary shaker at 120 r.p.m. and 27 °C in a complex medium that consisted of 10 g soluble starch, 10 g glucose, 10 g glycerol, 2.5 g corn steep powder, 5 g Bacto peptone, 2 g

yeast extract, 1 g NaCl, 3 g CaCO₃ and 1 litre tap water; the pH was adjusted to 7.3 prior to sterilization. Samples were taken between 48 and 144 h of cultivation, and extracts were prepared from the culture filtrate using ethyl acetate and from the mycelium using methanol/acetone (1:1). Aliquots of the extracts were analysed by reversed-phase HPLC and diode array monitoring using a standard protocol (Fiedler, 1993). The dominant peak in the HPLC chromatogram, which reached a maximal intensity at 144 h of cultivation, was identified as staurosporine by its characteristic UV-visible spectrum and retention time of 7.4 min; these results were identical to those of the reference compound staurosporine in the HPLC-UV-Vis database which contained data on 950 natural products, mainly antibiotics (Fiedler, 1993). Staurosporine was originally discovered from a culture broth of *Saccharothrix aerocolonigenes* subsp. *staurosporeus* strain AM-2282 during a screening programme for microbial alkaloids using a TLC detection method (Ömura *et al.*, 1977; Takahashi *et al.*, 1995).

Strain BK179^T and the type strains of *Streptomyces chattanoogensis* and *Streptomyces lydicus* were examined for a restricted range of phenotypic properties known to be of value in streptomycete systematics (Table 1). These tests and some additional ones carried out on the isolate were performed using the media and methods described by Williams *et al.* (1983). The isolate, unlike the type strain of *Streptomyces lydicus*, used rhamnose but not D-fructose as

Table 1. Characteristics that separate strain BK179^T from the type strains of phylogenetically closely related species of the genus *Streptomyces*

Strains: 1, BK179^T; 2, *Streptomyces chattanoogensis* ISP 5002^T; 3, *Streptomyces lydicus* ISP 5461^T. Data acquired in this study.

Characteristic	1	2	3
Appearance on inorganic-salt starch agar			
Aerial spore-mass colour	White	Yellow	Yellow
Reverse colour	White	White	Grey-yellow
Soluble pigment	–	Yellow	–
Spore surface	Rugose	Spiny	Smooth
Biochemical tests			
Nitrate reduction	+	+	–
Hydrogen sulphide production	+	+	–
Degradation of:			
Adenine	–	+	+
Casein	+	+	–
Hypoxanthine	+	–	+
Xylan	+	–	–
Growth on sole carbon sources (1%, w/v)			
L-Arabinose	+	–	+
Dextran	+	–	+
D-Fructose	–	+	+
Rhamnose	+	–	–
D-Xylose	+	–	+
Growth at pH 4.0	+	–	–
Resistance to Gentamicin sulphate (8 µg ml ⁻¹)	+	–	–

sole carbon source, degraded casein and xylan but not adenine, reduced nitrate, produced hydrogen sulphide and grew at pH 4.0. Similarly, unlike the type strain of *Streptomyces chattanoogensis*, it used L-arabinose, dextran, rhamnose and D-xylose, but not D-fructose, as sole sources of carbon, degraded hypoxanthine and xylan but not adenine and grew at pH 4.0. All three strains formed spiral spore chains but can be distinguished on the basis of spore ornamentation; the novel isolate formed ridged ornamented spores, and the type strains of *Streptomyces chattanoogensis* and *Streptomyces lydicus* formed smooth and smooth or spiny spores, respectively (Williams *et al.*, 1983).

Strain BK179^T can be distinguished from the type strains of its nearest neighbours, namely *Streptomyces chattanoogensis* and *Streptomyces lydicus*, based on 16S rRNA gene sequence, DNA–DNA relatedness, and phenotypic and morphological data. It is, therefore, proposed that the organism be recognized as a novel species, *Streptomyces staurosporininus* sp. nov.

Description of *Streptomyces staurosporininus*

Streptomyces staurosporininus (stau.ro.spo'ri.ni.nus. N.L. neut. n. *staurosporinum* staurosporine; L. masc. suff. *-inus* adjectival suffix used with the sense of belonging to or related to; N.L. masc. adj. *staurosporininus* related to staurosporine, producing the antibiotic staurosporine).

Aerobic, Gram-positive, non-acid–alcohol-fast actinomycete which forms an extensively branched substrate mycelium which carries aerial hyphae that differentiate into long spiral chains of ridged ornamented spores on oatmeal agar. Grows from 10 to 30 °C, from pH 4.0 to 10.0 and in the presence of NaCl (5.0 %). Aesculin, allantoin and arbutin are hydrolysed. Degrades elastin and uric acid, but not cellulose, chitin, guanine or pectin. D-Arabitol, cellobiose, maltose, D-mannitol, melibiose, raffinose, myo-inositol and starch are used as sole carbon sources, but not adonitol, D-sorbitol or L-sorbose (1 %, w/v). Susceptible to ($\mu\text{g ml}^{-1}$): novobiocin (8), rifampicin (16) and vancomycin hydrochloride (2), but not to ampicillin (4), cephaloridine hydrochloride (2), kanamycin sulphate (8), penicillin G (2), streptomycin sulphate (4) or tetracycline hydrochloride (8). Resistant to lysozyme (0.05 %, w/v). Additional properties are cited in the text and in Table 1. Contains LL-diaminopimelic acid, N-acetyl muramic acid moieties, and hexa- and octahydrogenated menaquinones with nine isoprene units [MK-9 (H₆, H₈)] as predominant menaquinones. The major cellular fatty acids are iso-C_{16:0}, C_{16:0} and anteiso-C_{15:0}. Produces staurosporine. The DNA G + C content is 71.2 %.

The type and only strain, BK179^T (=KACC 20912^T =NRRL B-24850^T), was isolated from hay meadow plot 6 at Cackle Park Experimental Farm, Northumberland, UK. The species description is based on a single strain and hence serves as a description of the type strain.

Acknowledgements

B.-Y.K. gratefully acknowledges receipt of an Overseas Research Scholarship and a Newcastle University Postgraduate Scholarship. T.D.Z. is grateful to the Conselho Nacional de Desenvolvimento Científico e Tecnológico for a fellowship to study in the UK (grant 201066/2009-2).

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