

# *Streptomyces cocklensis* sp. nov., a dioxamycin-producing actinomycete

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The taxonomic position of a streptomycete isolated from soil collected from Cockle Park Experimental Farm, Northumberland, UK, was determined by using a polyphasic approach. The organism had chemical and morphological features consistent with its classification in the genus *Streptomyces*. 16S rRNA gene sequence analysis supported classification of the strain in the genus *Streptomyces* and showed that it formed a distinct phyletic line loosely associated with members of the *Streptomyces yeochonensis* clade. It was related most closely to *Streptomyces paucisporeus* 1413<sup>T</sup> (98.6% 16S rRNA gene sequence similarity), but could be distinguished from the latter based on the low level of DNA–DNA relatedness (40%). It was readily distinguished from the type strains of all species assigned to the *S. yeochonensis* clade based on a combination of phenotypic properties. Strain BK168<sup>T</sup> (=KACC 20908<sup>T</sup>=NCIMB 14704<sup>T</sup>) should therefore be classified as the type strain of a novel species of the genus *Streptomyces*, for which the name *Streptomyces cocklensis* sp. nov. is proposed. The organism produces the antibiotic dioxamycin.

Members of the genus *Streptomyces* remain a rich source of novel bioactive compounds, notably antibiotics (Bérdy, 2005; Goodfellow & Fiedler, 2010). Another unique feature of the genus is the large number of species it contains, almost 600 at the time of writing (<http://www.bacterio.cict.fr/s/streptomycesa.html>). Relationships within this taxonomically complex genus have been clarified by the application of genotypic and phenotypic procedures (Manfio *et al.*, 1995; Anderson & Wellington, 2001; Lanoot *et al.*, 2004, 2005a) so that it is now apparent that many type strains can be assigned to distinct multi-membered 16S rRNA gene clades (Lanoot *et al.*, 2005b; Liu *et al.*, 2005; Xu *et al.*, 2006; Goodfellow *et al.*, 2007; Quintana *et al.*, 2008; Kumar & Goodfellow, 2010; Rong & Huang, 2010), including the *Streptomyces yeochonensis* clade (Kim *et al.*, 2004; Xu *et al.*, 2006). Members of this taxon are able to grow from pH 4.5 to 7.5 with optimal growth between pH 5.0 and 5.5, i.e. they are neutrotolerant acidophilic streptomycetes.

The present study was designed to establish the taxonomic status of a *Streptomyces* strain that was isolated from an

acid soil and found to grow from pH 4.5 to 10.0. It is apparent from the results of the present study that this strain, designated BK168<sup>T</sup>, merits recognition as a representative of a novel species of the genus *Streptomyces*. The novel species is loosely associated with the *S. yeochonensis* 16S rRNA gene clade.

Strain BK168<sup>T</sup> was isolated from a plate of starch-casein agar (Küster & Williams, 1964), supplemented with cycloheximide and nystatin (each at 25 µg ml<sup>-1</sup>), which had been inoculated with a pre-heated soil suspension (55 °C for 20 min) and incubated at 28 °C for 21 days. The soil sample had been 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 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 studies were freeze-dried.

Genomic DNA was extracted from the isolate and PCR amplification and 16S rRNA gene sequencing were achieved as described previously (Kim *et al.*, 1996). The

Abbreviation: ISP, International *Streptomyces* Project.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain BK168<sup>T</sup> is FR692107.

Two supplementary figures are available with the online version of this paper.

resultant, almost-complete 16S rRNA gene sequence of strain BK168<sup>T</sup> was aligned manually against corresponding sequences of representatives of the genus *Streptomyces* by using the software MEGA4 (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 MEGA4 (Tamura *et al.*, 2007) and PHYL (Guindon & Gascuel, 2003) packages. The Jukes & Cantor (1969) model was used to generate evolutionary distance matrices for the neighbour-joining algorithm. Topologies of the resultant trees were evaluated by bootstrap analysis (Felsenstein, 1985) of the maximum-likelihood method based upon 1000 replicates by using MEGA4. *Streptomyces macrosporus* DSM 41449<sup>T</sup> (GenBank accession no. Z68099), *Streptomyces megasporus* DSM 41476<sup>T</sup> (Z68100) and *Streptomyces scabrisporus* KM-4927<sup>T</sup> (AB030585) were used as the outgroup.

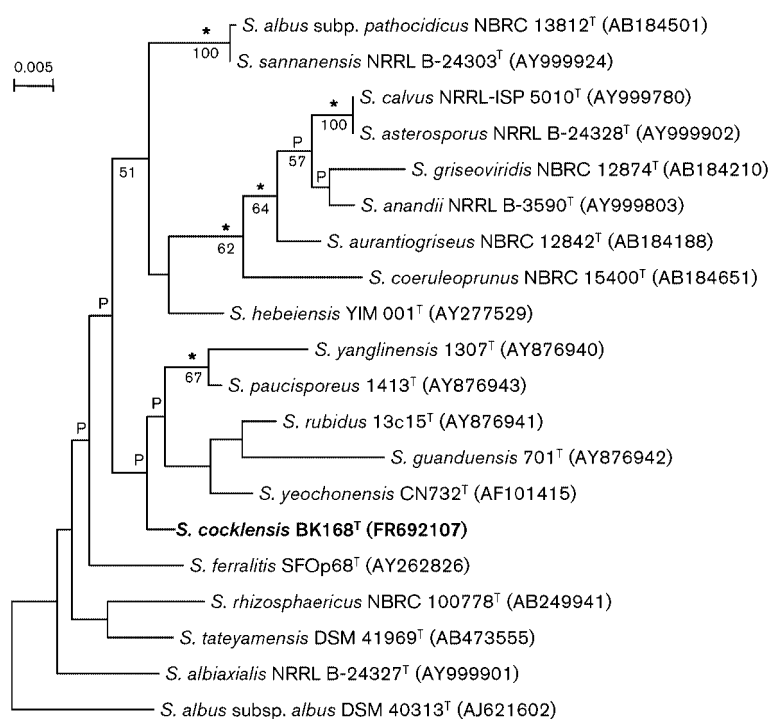
It can be seen from Fig. 1 that strain BK168<sup>T</sup> forms a distinct phyletic line that is loosely associated with the *S. yeochonensis* 16S rRNA gene clade in the maximum-likelihood and maximum-parsimony analyses. However, this relationship was not supported by high bootstrap values or by neighbour-joining analysis, in which the organism was recovered in the *S. yeochonensis* 16S rRNA gene clade. The isolate was most closely related to the type strain of *Streptomyces paucisporeus*; the two strains shared 98.6% 16S rRNA gene sequence similarity, a value which corresponded to 20 nt differences at 1401 locations. Levels of 16S rRNA gene sequence similarity between strain BK168<sup>T</sup> and the type strains of other members of the clade

were 98.4% with *Streptomyces yanglinensis* and *S. yeochonensis*, 98.3% with *Streptomyces rubidus* and 97.6% with *Streptomyces guanduensis*.

The level of DNA–DNA relatedness between the novel isolate and the type strain of *S. paucisporeus*, its nearest phylogenetic neighbour, was determined by using the nitrocellulose membrane filter hybridization procedure described by Seldin & Dubnau (1985). DNA probes were labelled using the non-radioactive digoxigenin High Prime System (Roche), hybridized DNA was visualized by using a digoxigenin luminescent detection kit (Roche) and the level of DNA–DNA relatedness was quantified by using a densitometer (Bio-Rad). The two strains shared 40% DNA–DNA relatedness, a value well below the 70% cut-off recommended for the assignment of strains to the same genomic species (Wayne *et al.*, 1987).

Isolate BK168<sup>T</sup> was examined for chemical markers that are characteristic of *Streptomyces* strains. Standard procedures were used to determine the diaminopimelic acid isomers (Hasegawa *et al.*, 1983), predominant menaquinones (Collins, 1985), muramic acid type (Uchida *et al.*, 1999) and diagnostic whole-cell sugars (Hasegawa *et al.*, 1983), by using appropriate controls. Fatty acids were extracted, methylated and analysed by GC with the standard Sherlock Microbial Identification system (MIDI, 1999). The DNA G + C content of the isolate was determined by using the procedures described by Gonzalez & Saiz-Jimenez (2002).

Strain BK168<sup>T</sup> contained major amounts of LL-diaminopimelic acid and N-acetylated muramic acid in the cell-wall peptidoglycan, lacked diagnostic sugars in whole-organism



**Fig. 1.** Maximum-likelihood tree based on nearly complete 16S rRNA gene sequences showing the relationships between strain BK168<sup>T</sup> and the type strains of phylogenetically closely related *Streptomyces* species. Asterisks indicate branches of the tree that were also recovered with the neighbour-joining and maximum-parsimony tree-making algorithms; 'P' indicates branches that were also recovered with just the maximum-parsimony tree-making algorithm. Numbers at nodes are percentage bootstrap values based on 1000 resampled datasets; only values above 50% are given. *Streptomyces macrosporus* DSM 41449<sup>T</sup> (GenBank accession no. Z68099), *Streptomyces megasporus* DSM 41476<sup>T</sup> (Z68100) and *Streptomyces scabrisporus* KM-4927<sup>T</sup> (AB030585) were used as the outgroup. Bar, 0.005 substitutions per nucleotide position.

hydrolysates (wall chemotype I *sensu* Lechevalier & Lechevalier, 1970), and contained hexa-, octa- and tetra-hydrogenated menaquinones with nine isoprene units [MK-9(H<sub>6</sub>, H<sub>8</sub>, H<sub>4</sub>)] as predominant isoprenologues in a ratio of 3:3:2, respectively, and minor amounts of MK-9(H<sub>2</sub>). The cellular fatty acid profile consisted of major amounts of anteiso-C<sub>15:0</sub> (27.8 %), C<sub>16:0</sub> (23.8 %) and iso-C<sub>16:0</sub> (19.4 %), lesser amounts (<10 %) of anteiso-C<sub>17:0</sub> (9.5 %), iso-C<sub>15:0</sub> (7.4 %) and iso-C<sub>14:0</sub> (5.8 %), and trace amounts of iso-C<sub>17:0</sub> and C<sub>14:0</sub>. The DNA G+C content of strain BK168<sup>T</sup> was 73.2 mol%. All of these properties are in line with the classification of the isolate in the genus *Streptomyces* (Williams *et al.*, 1989; Manfio *et al.*, 1995; Anderson & Wellington, 2001).

The isolate was examined for cultural and morphological features after growth on several standard media at 28 °C for 3 weeks. Cultural properties were investigated by using glucose-yeast extract-malt extract (DSMZ medium 65) and modified Bennett's (Jones, 1949) agars and on tryptone-yeast extract (ISP medium 1), yeast extract-malt extract (ISP medium 2), oatmeal (ISP medium 3), inorganic salts-starch (ISP medium 4), glycerol-asparagine (ISP medium 5) and

tyrosine (ISP medium 7) agars (Shirling & Gottlieb, 1966). Spore arrangement and spore surface ornamentation were observed by examining a gold-coated, dehydrated preparation from the oatmeal agar plate with a scanning electron microscope (Cambridge Stereoscan 240) and the procedure described by O'Donnell *et al.* (1993). The organism formed an extensively branched brownish substrate mycelium and greyish aerial hyphae, which differentiated into straight to flexuous chains of smooth-surfaced spores on oatmeal agar (Supplementary Fig. S1 in IJSEM Online). Reddish-brown diffusible pigments were formed on glycerol-asparagine and oatmeal agars (Supplementary Fig. S2 in IJSEM Online). The isolate grew well on all of the tested media except tryptone-yeast extract agar.

Strain BK168<sup>T</sup> was included in our HPLC-diode array screening programme to determine whether it produced novel secondary metabolites. It was cultivated in shake flasks of oatmeal broth (20 g oatmeal, 5 ml trace element solution, 1 l tap water; pH 7.3) with samples taken between 48 and 144 h of cultivation; extracts were prepared from the culture filtrates by using ethyl acetate and from mycelia by using methanol/acetone (1:1).

**Table 1.** Differential characteristics of strain BK168<sup>T</sup> and the type strains of phylogenetically closely related *Streptomyces* species

Strains: 1, BK168<sup>T</sup>; 2, *S. guanduensis* JCM 13274<sup>T</sup>; 3, *S. paucisporeus* JCM 13276<sup>T</sup>; 4, *S. rubidus* JCM 13277<sup>T</sup>; 5, *S. yanglinensis* JCM 13275<sup>T</sup>; 6, *S. yeochonensis* NRRL B-24245<sup>T</sup>.

Characteristic	1	2	3	4	5	6
Appearance on oatmeal agar						
Aerial hyphae	Sparse	Sparse	Sparse	Abundant	Abundant	Abundant
Aerial spore-mass colour	Grey	White to grey	Grey	Mahogany	Grey	Grey
Melanin production on tyrosine agar	—	—	+	—	—	—
Degradation of (% w/v):						
Aesculin (0.1)	+	+	+	+	+	+
Casein (1.0)	+	—	+	—	—	+
Hypoxanthine (0.4)	—	+	—	—	+	—
Xylan (0.4)	+	+	—	+	+	—
Growth on sole carbon sources (at 1 % w/v)						
L-Arabinose	—	+	+	+	+	—
D-Fructose	—	+	—	+	+	—
Glycerol	—	+	—	+	+	—
D-Mannose	—	+	—	+	+	—
Raffinose	—	+	—	+	+	—
L-Rhamnose	—	+	+	—	+	—
L-Sorbose	—	+	+	+	+	—
Sucrose	—	+	—	+	+	—
Trehalose	—	+	+	—	+	—
D-Xylose	—	+	—	+	+	—
Growth at:						
pH 4.5	+	+	+	+	+	+
pH 7.5	+	—	+	—	—	+
pH 10.0	+	—	—	—	—	—
10 °C	+	—	+	—	—	—
37 °C	—	+	—	+	+	+

Aliquots of the extracts were analysed by reversed-phase HPLC and diode array monitoring according to a standard protocol (Fiedler, 1993). The dominant peak in the HPLC chromatogram of the extracts was identified by HPLC–diode array detection–electrospray ionization–MS analysis as dioxamycin based on its characteristic UV-VIS profile and molecular mass of 736.3 Da. Dioxamycin, a benz[*a*]anthraquinone antibiotic, was first isolated from *Streptomyces* sp. MH406-SF1, which is closely related to *Streptomyces xantholiticus* (Sawa *et al.*, 1991).

Strain BK168<sup>T</sup> and the type strains of *S. guanduensis*, *S. paucisporeus*, *S. rubidus*, *S. yanglinensis* and *S. yeochonensis* were examined for a range of phenotypic properties by using the media and methods described by Williams *et al.* (1983). Strain BK168<sup>T</sup> could be distinguished from the above taxa, including the type strain of its nearest neighbour, *S. paucisporeus*, based on a combination of phenotypic properties, notably by its ability to degrade xylan and failure to grow on L-arabinose, L-rhamnose, L-sorbose or trehalose as sole carbon sources for energy and growth (Table 1). Additional phenotypic properties are given in the species description below.

It is evident from the genotypic and phenotypic data presented that strain BK168<sup>T</sup> can be distinguished from the type strains of species classified in the *S. yeochonensis* 16S rRNA gene clade. We therefore propose that strain BK168<sup>T</sup> represents a novel species of the genus *Streptomyces*, for which the name *Streptomyces cocklensis* sp. nov. is proposed.

### Description of *Streptomyces cocklensis* sp. nov.

*Streptomyces cocklensis* (cock.len'sis. N.L. masc. adj. *cocklensis* of or belonging to Cockle, referring to Cockle Park Experimental Farm, the source of the type strain).

Aerobic, Gram-positive, non-acid–alcohol-fast actinomycete that forms an extensively branched substrate mycelium which carries aerial hyphae that differentiate into short, straight chains of smooth-surfaced spores (0.9–1.0 × 1.5–1.7 µm) on oatmeal agar. Grows at 10–30 °C and at pH 4.0–10.0, but not in the presence of 3.0% (w/v) NaCl. Hydrolyses elastin and uric acid, but not arbutin, chitin, pectin or starch. Cellobiose, D-glucose and maltose are used as sole carbon sources for energy and growth, but not D-galactose, D-mannose, L-arabitol, melibiose, D-salicin, D-sorbitol or D-xylitol (at 1%, w/v). Susceptible to (µg ml<sup>-1</sup>) cephaloridine hydrochloride (2), streptomycin sulphate (4) and vancomycin hydrochloride (2), but not to ampicillin (4), ciprofloxacin (2), kanamycin sulphate (8), penicillin G (2 IU ml<sup>-1</sup>), rifampicin (15) or tetracycline hydrochloride (8). Additional properties are cited in the text or in Table 1. Chemotaxonomic properties are typical of the genus *Streptomyces*.

The type and only strain, BK168<sup>T</sup> (=KACC 20908<sup>T</sup> =NCIMB 14704<sup>T</sup>), was isolated from hay meadow plot 6 soil at Cockle Park Experimental Farm, Northumberland, UK. The species description is based on a single strain and

hence serves as a description of the type strain. The DNA G + C content of the type strain is 73.2 mol%.

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