Streptomyces herbaceus sp. nov., *Streptomyces incanus* sp. nov. and *Streptomyces pratens* sp. nov., isolated from the soil of a hay meadow

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The taxonomic positions of three streptomycetes isolated from a soil sample from a hay meadow were determined using a polyphasic approach. The isolates had chemical and morphological properties typical of the genus *Streptomyces* and, in phylogenetic analyses based on 16S rRNA gene sequences, formed a distinct subclade that was most closely related to the *Streptomyces prasinus* subclade. DNA–DNA relatedness studies showed that the novel strains belonged to three different genomic species. The novel strains could be distinguished from one another and from the type strains of the species classified in the *S. prasinus* subclade using a combination of genotypic and phenotypic properties. On the basis of these data, it is proposed that the novel strains be assigned to the genus *Streptomyces* as *Streptomyces herbaceus* sp. nov., *Streptomyces incanus* sp. nov. and *Streptomyces pratens* sp. nov., with BK119^T (=KACC 21001^T =CGMCC 4.5797^T), BK128^T (=KACC 21002^T =CGMCC 4.5799^T) and BK138^T (=KACC 20904^T =CGMCC 4.5800^T) as the respective type strains.

The discovery that the genomes of streptomycetes contain a large number of gene clusters that encode secondary metabolites (Bentley *et al.*, 2002; Ikeda *et al.*, 2003; Ohnishi *et al.*, 2008) underlines the importance of these organisms as sources of novel and clinically significant bioactive compounds, notably antibiotics (Goodfellow & Fiedler, 2010). Another remarkable feature of the genus *Streptomyces* is the large number of described species it contains: nearly 600 at the time of writing (Euzéby, 2011). The subgeneric classification of the genus, while complex, has been clarified by the application of genotypic and phenotypic procedures (Goodfellow *et al.*, 2007; Rong & Huang, 2010) that have also been used to circumscribe novel species isolated from clinical (Quintana *et al.*, 2008) and environmental sources (Nagai *et al.*, 2011; Zucchi *et al.*, 2012). In the present polyphasic study, strains BK119^T, BK128^T and BK138^T were isolated from the soil of a hay meadow and shown to represent three novel *Streptomyces* species.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Streptomyces herbaceus* BK119^T, *Streptomyces incanus* BK128^T and *Streptomyces pratens* BK138^T are FR692091, FR692095 and FR692098, respectively.

A supplementary table is available with the online version of this paper.

Strains BK119^T, BK128^T and BK138^T were isolated on starch-casein agar (Küster & Williams, 1964) supplemented with cycloheximide and nystatin (each at 25 μg ml^{-1}), after incubation at 28 °C for 21 days. They came from a soil sample collected from plot 6 of the Palace Leas hay meadow (Atalan *et al.*, 2000) at Cockle Park Experimental Farm, Ulgham, Morpeth, Northumberland, UK. The organisms were maintained on oatmeal agar slopes [International *Streptomyces* Project (ISP) medium 3; Shirling & Gottlieb, 1966] at 4 °C and also 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 glucose-yeast extract-malt extract broth (ISP medium 2; Shirling & Gottlieb, 1966) at 28 °C for 7 days, harvested by centrifugation and then washed twice in distilled water; biomass for the chemotaxonomic studies was freeze-dried.

The phylogenetic positions of the three novel strains were determined by 16S rRNA gene sequence analysis. Genomic DNA was extracted from the biomass preparations, and PCR amplification and 16S rRNA gene sequencing were achieved, using the procedures described by Kim *et al.* (2010). The resultant, almost complete 16S rRNA gene sequences (1458–1460 nt) were aligned manually against

corresponding sequences of representatives of the genus *Streptomyces*, using the MEGA 3.0 software package (Kumar *et al.*, 2004). Phylogenetic trees were inferred by using the maximum-parsimony (Fitch, 1971), minimum-evolution (Rzhetsky & Nei, 1992) and neighbour-joining (Saitou & Nei, 1987) algorithms. The Jukes & Cantor (1969) model was used to generate evolutionary distance matrices for the neighbour-joining data. The resultant tree topologies were evaluated by bootstrap analysis (Felsenstein, 1985) based on 1000 resampled datasets, again using MEGA 3.0. The 16S rRNA gene sequence of *Streptacidiphilus albus* JL83^T was used as the outgroup for each of the trees (Fig. 1).

The three novel strains formed a distinct subclade in each of the constructed trees, in a clustering that was supported by moderate bootstrap values (Fig. 1). The two most closely related strains, BK119^T and BK128^T, showed a 16S rRNA gene sequence similarity of 99.4 %, a value that corresponded to eight nucleotide differences over 1427 locations. Strain BK138^T showed a 16S rRNA gene sequence similarity of 99.0 % with both strain BK119^T and strain BK128^T, a value equivalent to a difference of either 14 or 15 nt. The subclade formed by the three novel strains was most closely related to the *Streptomyces prasinus* subclade (the status of which was supported by all three treeing algorithms and by very high bootstrap values; Fig. 1). These two subclades united to form a distinct branch, the taxonomic integrity of which was also underpinned by all of the algorithms and by a high bootstrap value. The novel strains appeared most closely related to *Streptomyces hirsutus* NBRC 12786^T, with 16S rRNA gene sequence similarities (98.4–98.6 %) that corresponded to differences of 20–25 nt. Of the three novel isolates, strain BK119^T was found to be the one most closely related to *S. hirsutus* NBRC 12786^T.

The level of DNA–DNA relatedness between the novel strains was investigated, in duplicate, by using the fluorometric microplate method of Ezaki *et al.* (1989) with the modifications described by He *et al.* (2005). The thermal denaturation method described by Gonzalez & Saiz-Jimenez (2002) was used to estimate genomic DNA G+C contents. The highest level of DNA–DNA relatedness, 45.6 % ± 0.8 %, was found between strains BK119^T and BK138^T. The corresponding values between these two strains and strain BK128^T were 20.8 ± 2.2 % and 24.5 ± 0.1 %, respectively. As the level of DNA–DNA relatedness observed between any two of the novel strains fell well below the 70 % cut-off point recommended for the delineation of

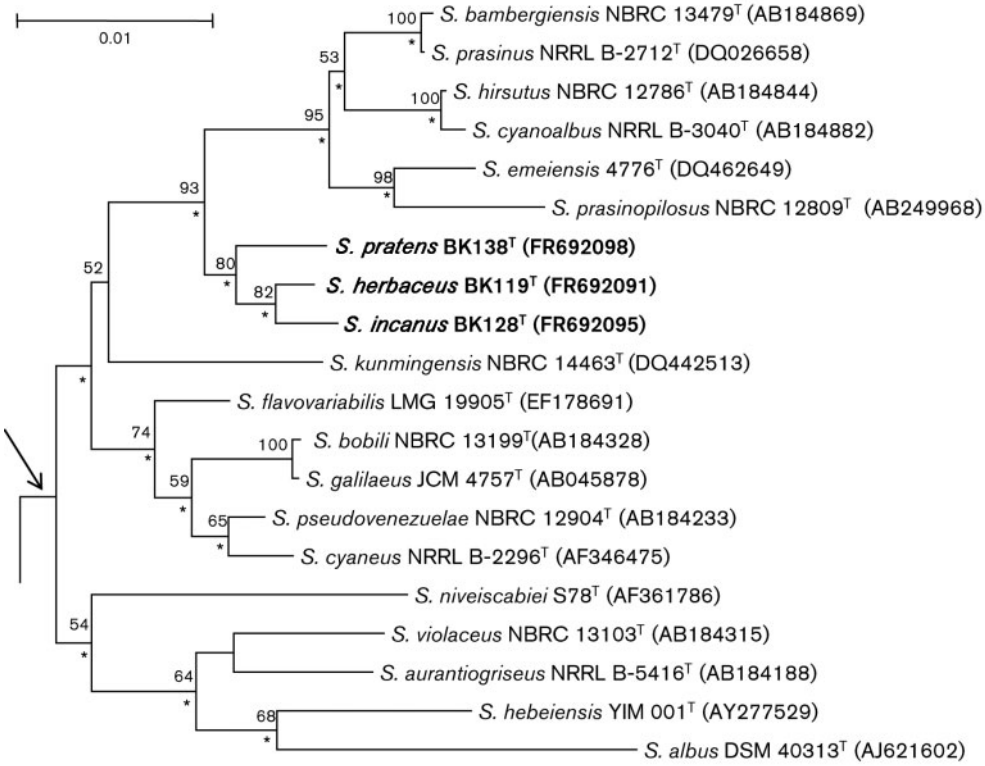

Fig. 1. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences (1418–1434 bp), showing the relationships between strains BK119^T, BK128^T and BK138^T and between them and the type strains of related *Streptomyces* species. Asterisks indicate branches of the tree that were also recovered with the maximum-parsimony and minimum-evolution tree-making algorithms. Only bootstrap values >50 %, expressed as percentages of 1000 replications, are shown at branch points. The arrow indicates the inferred root position. Bar, 0.01 substitution per nucleotide position.

genomic species (Wayne *et al.*, 1987), the novel strains apparently represented three different genomic species. The genomic DNA G+C contents of strains BK119^T, BK128^T and BK 138^T were 70.2, 69.2 and 73.2 mol%, respectively.

The novel strains were examined for the chemical markers considered to be typical of the genus *Streptomyces*. Standard procedures were used to determine the predominant menaquinones (Collins *et al.*, 1985), muramic acid type (Uchida *et al.*, 1999), mycolic acids (Minnikin *et al.*, 1980), diagnostic whole-cell sugars (Hasegawa *et al.*, 1983) and isomers of diaminopimelic acid (Hasegawa *et al.*, 1983), using appropriate controls. Fatty acids were extracted from the strains, methylated, analysed by gas chromatography (6890; Hewlett Packard) and identified using version 5 of the Sherlock Microbial Identification System (MIDI) and the ACTINO database (Sasser, 1990).

When cultivated in ISP2 broth, all three novel strains contained major amounts of LL-diaminopimelic acid but lacked characteristic sugars in whole-organism hydrolysates (wall chemotype I, *sensu* Lechevalier & Lechevalier, 1970). They all possessed *N*-acetylated muramic acid, and all had hexa- and octa-hydrogenated menaquinones with nine isoprene units (MK-9 [H_6, H_8]) as predominant isoprenologues, in ratios of 1 : 3, 1 : 1 and 4 : 3, respectively. The cellular fatty acid profiles consisted mainly of saturated straight-chain and iso- and anteiso-branched-chain components (Table S1, available in IJSEM Online) and were of the 2c fatty acid type (*sensu* Kroppenstedt, 1985). None of the strains contained mycolic acids. All of these properties support the classification of all three novel strains in the genus *Streptomyces* (Lechevalier & Lechevalier, 1970; Manfio *et al.*, 1995, 2003; Anderson & Wellington, 2001).

The novel strains were examined for cultural and morphological features following growth, for 3 weeks at 28 °C, on ISP 2, ISP 3, inorganic salts-starch (ISP 4), glycerol-asparagine (ISP 5), tyrosine (ISP 7) and modified Bennett's agars (Jones, 1949; Shirling & Gottlieb, 1966). Spore arrangement and spore surface ornamentation were observed by examining gold-coated, dehydrated preparations from the ISP3 plates, using a scanning electron microscope (Stereoscan 240; Cambridge). On their aerial mycelia, strains BK119^T and BK138^T formed straight to flexuous chains (*Rectiflexibiles*) of spiny and smooth-surfaced spores, respectively, whereas strain BK128^T produced spiral chains of smooth-surfaced spores (Fig. 2). The cultural characteristics of the strains are summarized in Table 1.

The novel strains were also examined for a range of phenotypic properties, using the media and methods described by Williams *et al.* (1983). The results were compared with those from the same tests carried out on the type strains of species classified in the *S. prasinus* subclade (Sun *et al.*, 2007). The novel strains could be distinguished from each other, and from the type strains of the species forming the *S. prasinus* subclade, using a combination of phenotypic features (Table 2). For example, strains BK128^T and BK138^T, unlike strain BK119^T, formed smooth-surfaced spores, degraded adenine and hypoxanthine, and grew on dextrin, *myo*-inositol and methyl α-D-glucopyranoside. Similarly, strains BK128^T and BK138^T can be readily separated since, of these two strains, only strain BK128^T degraded elastin, grew on L-arabinose, D-ribose and D-sorbose as sole carbon sources, and was resistant to rifampicin (at 16 μg ml^{-1}) and lysozyme (at 0.05 %, w/v). Strain BK119^T was also resistant to rifampicin and lysozyme. All three novel strains were susceptible to (μg ml^{-1}, unless indicated otherwise) gentamicin sulphate (8), kanamycin sulphate (8) and streptomycin sulphate (4) but resistant to ampicillin (4), clindamycin (8), ciprofloxacin (2), lincomycin hydrochloride (8), tetracycline hydrochloride (8) and penicillin G (2 IU ml^{-1}). In addition, all three novel strains, unlike the type strains of the species forming the *S. prasinus* subclade, degraded xanthine.

On the basis of the genotypic and phenotypic data, the three novel strains can be distinguished both from one another and from their nearest phylogenetic neighbours. Strains BK119^T, BK128^T and BK138^T therefore represent three novel *Streptomyces* species, for which the names *Streptomyces herbaceus* sp. nov., *Streptomyces incanus* sp. nov. and *Streptomyces pratens* sp. nov., respectively, are proposed.

Description of *Streptomyces herbaceus* sp. nov.

Streptomyces herbaceus (her.ba′ce.us. L. masc. adj. *herbaceus* grass-coloured, grass-green, referring to the green colour of the aerial mycelium).

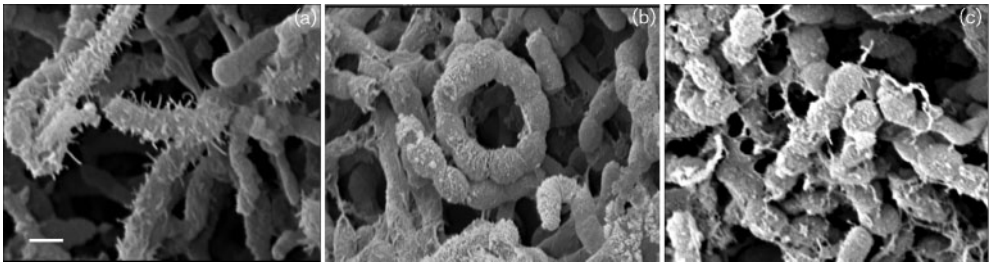

Fig. 2. Scanning electron micrographs of strains BK119^T (a), BK128^T (b) and BK138^T (c) showing the spore arrangement and spore ornamentation following growth on oatmeal agar at 28 °C for 3 weeks. Bar, 2 μm.

Table 1. Culture characteristics of strains BK119^T, BK128^T and BK138^T

All strains were examined after growing on each of six different agars for 3 weeks at 28 °C. All strains produced a dark brown soluble pigment on oatmeal agar. +, Weak or sparse; ++, moderate; +++, abundant. G-YE-ME, Glucose-yeast extract-malt extract.

Strain	Characteristic	Agar					
		Modified Bennett's	G-YE-ME	Oatmeal	Inorganic salts-starch	Glycerol-asparagine	Tyrosine
BK119^T	Growth:	++	++	++	++	+	+++
	Aerial mycelium:	+, white	++, light grey	++, green	++, white	+, dark grey	+++, brown–grey
	Reverse colour:	Light yellow	Light yellow	Dark brown	Light yellow	Yellowish green	Dark brown
BK128^T	Growth:	+	+++	+++	++	+	++
	Aerial mycelium:	None	+++, light grey	+++, light grey	None	None	+, white
	Reverse colour:	Light yellow	Light yellow	Brown	Light yellow	Light yellow	Yellow
BK138^T	Growth:	++	+++	++	++	++	+++
	Aerial mycelium:	+, white	+++, grey–green	++, green	+, white	+, white	+++, white
	Reverse colour:	Light green–yellow	Light green–yellow	Dark green	Light grey	Light grey	Light grey

Aerobic, Gram-staining-positive, non-acid-alcohol-fast actinomycete that, on oatmeal agar, forms a branched substrate mycelium that bears aerial hyphae that differentiate into straight chains of spiny-surfaced spores (0.7–0.8 × 0.7–0.9 μm). Grows at 10–37 °C and at pH 5.0–10.0 but not in the presence of 7.0 % (w/v) NaCl. Degrades casein, DNA, gelatin, starch and L-tyrosine but not chitin, guanine, tributyrin or uric acid. L-Arabitol, maltose, melibiose and D-sorbitol are used as sole carbon sources for energy and growth (at 1 %, w/v) but not oxalic acid (at 0.1 %, w/v). Additional properties are cited in the text and in Tables 1 and 2. Chemotaxonomic properties are typical of the genus *Streptomyces*.

The type strain, BK119^T (=KACC 21001^T =CGMCC 4.5797^T), was isolated from the soil of a hay meadow. The genomic DNA G+C content of the type strain is 70.2 mol%. The species description is based on a single strain and hence serves as the description of the type strain.

Description of *Streptomyces incanus* sp. nov.

Streptomyces incanus (in.ca′nus. L. masc. adj. *incanus* light grey, referring to the colour of the aerial mycelium).

Aerobic, Gram-staining-positive, non-acid-alcohol-fast actinomycete that, on oatmeal agar, forms an extensively branched substrate mycelium bearing aerial hyphae that differentiate into spiral or hooked spore chains of smooth-surfaced spores (0.8–0.9 × 0.8–0.9 μm). Grows at 10–37 °C and at pH 5.0–10.0 but not in the presence of 7.0 % (w/v) NaCl. Degrades casein, DNA, gelatin, starch, L-tyrosine and uric acid but not cellulose, chitin, guanine or tributyrin. L-Arabitol, maltose, melibiose and D-sorbitol (at 1 %, w/v) are used as sole carbon sources for energy and growth but D-salicin (at 1 %, w/v) or oxalic acid (at 0.1 %, w/v) are not. Additional properties are cited in the text and in Tables 1 and 2. Chemotaxonomic properties are typical of the genus *Streptomyces*.

The type strain, BK128^T (=KACC 21002^T =CGMCC 4.5799^T), was isolated from the soil of a hay meadow. The genomic DNA G+C content of the type strain is 69.2 mol%. The species description is based on a single strain and hence serves as the description of the type strain.

Description of *Streptomyces pratens* sp. nov.

Streptomyces pratens (pra′tens. L. masc. adj. *pratens* green, meadow-green, referring to the green colour of the aerial and substrate mycelium).

Aerobic, Gram-staining-positive, non-acid-alcohol-fast actinomycete that, on oatmeal agar, forms a branched substrate mycelium bearing aerial hyphae that differentiate into straight to flexuous chains of smooth-surfaced spores (0.7–0.8 × 0.7–0.8 μm). Grows at 10–37 °C and at pH 5.0–9.0 but not in the presence of 7.0 % (w/v) NaCl. Degrades casein, DNA, gelatin, starch and L-tyrosine but not cellulose, guanine, tributyrin or uric acid. L-Arabitol, maltose, melibiose and D-sorbitol are

Table 2. Phenotypic characteristics of strains BK119 ^{T}, BK128 ^{T} and BK138^{T} and their closest phylogenetic relatives in the genus *Streptomyces*

Strains: 1, BK119^{T}; 2, BK128^{T}; 3, BK138^{T}; 4, *S. bambergiensis* DSM 40590^{T}; 5, *S. cyanoalbus* DSM 40198^{T}; 6, *S. emeiensis* DSM 41884^{T}; 7, *S. hirsutus* DSM 40095^{T}; 8, *S. prasinopilosus* DSM 40098^{T}; 9, *S. prasinus* DSM 40099^{T}. Data for the reference strains in the *S. prasinus* subclade were taken from Sun *et al.* (2007). All of the strains were positive for the assimilation of cellobiose, D-fructose, D-galactose, D-glucose, D-mannose, L-rhamnose, sodium citrate, sucrose, trehalose and D-xylose and negative for the degradation of guanine. +, Positive; −, negative; W, weakly positive.

Characteristic	1	2	3	4	5	6	7	8	9
Aerial spore mass colour on oatmeal agar	Green	Light grey	Greyish green	Green	Green or grey	Greyish green	Green	Green	Green
Spore surface	Spiny	Smooth	Smooth	Hairy	Spiny	Spiny	Spiny	Hairy	Spiny
Production of diffusible pigments	+	+	+	+	−	−	−	−	−
Degradation of:									
Adenine	−	+	+	−	+	+	−	−	−
Aesculin	+	+	+	−	+	+	+	+	−
Elastin	−	+	−	+	+	+	+	+	+
Hypoxanthine	−	+	+	−	−	−	−	−	−
Tween 20	−	−	−	W	+	+	+	+	+
Tween 80	−	−	−	+	+	+	+	+	+
Xanthine	+	+	+	−	−	−	−	−	−
Growth on sole carbon sources (1.0 %, w/v):									
L-Arabinose	−	+	−	W	+	+	+	+	+
Dextrin	−	+	+	+	+	+	−	+	+
myo-Inositol	−	+	+	+	+	+	+	−	+
Inulin	+	+	+	+	+	−	+	+	+
Methyl α-D-glucopyranoside	−	+	+	−	+	−	W	W	+
Raffinose	+	+	+	+	−	+	+	−	−
D-Ribose	−	+	−	−	−	+	−	−	−
D-Sorbose	−	+	−	+	+	−	+	+	+

used as sole carbon sources for energy and growth (at 1 %, w/v) but not oxalic acid (at 0.1 %, w/v). Additional properties are cited in the text and in Tables 1 and 2. Chemotaxonomic properties are typical of the genus *Streptomyces*.

The type strain, BK138^{T} (=KACC 20904^{T}=CGMCC 4.5800^{T}), was isolated from the soil of a hay meadow. The genomic DNA G+C content of the type strain is 73.2 mol%. The species description is based on a single strain and hence serves as the description of the type strain.

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