Streptomyces scopuliridis sp. nov., a bacteriocin-producing soil streptomycete

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Actinomycete strain RB72^T was isolated from woodland bluff soil in northern Alabama, USA, and shown to produce a broad spectrum bacteriocin. Based on morphological and chemotaxonomic characteristics, the strain was determined to belong to the genus *Streptomyces*. Phylogenetic analysis of the near-complete 16S rRNA gene sequence indicated that it differed from those of the described streptomycetes available in public databases. The distinctive white aerial hyphae and lack of sporulation suggest a deficiency in the *whi* pathway of the organism. A combination of substrate utilization patterns, morphological and chemotaxonomic characteristics and DNA–DNA hybridization results supported the affiliation of strain RB72^T to the genus *Streptomyces* and enabled the genotypic and phenotypic differentiation of strain RB72^T from closely related reference strains. Strain RB72^T therefore represents a novel species of the genus *Streptomyces*, for which the name *Streptomyces scopuliridis* sp. nov. is proposed. The type strain is RB72^T (=DSM 41917^T =NRRL B-24574^T).

The genus *Streptomyces*, with more than 500 species with validly published names, contains the largest number of species of any genus in the domain *Bacteria* (Hain *et al.*, 1997). The genus, first proposed by Waksman & Henrici (1943), includes aerobic, Gram-positive, high G+C content (69–78 mol%) bacteria. Most members of the genus *Streptomyces* possess LL-diaminopimelic acid in the ultrastructure of their peptidoglycan cell wall and produce extensively branching networks of substrate mycelia that give rise to the vertical projection of branching aerial hyphae (Williams *et al.*, 1983; Embley & Stackebrandt, 1994). Maturity of the aerial hyphae typically culminates in a sporulation event, resulting in the formation of chains of uninucleoidal spores from the multinucleoidal, filamentous hyphae (Kwak & Kendrick, 1996). The erection of aerial hyphae generally requires a minimum of 48 h of substrate mycelium growth, while the maturation of the spores can take an additional 2 to 4 days (Lawlor *et al.*, 1987; Willey *et al.*, 1991; Kieser *et al.*, 2000). Mutations in the regulatory genes guiding this process can result in alterations of phenotype. Mutations in the *bld* cascade and/or the proposed sky pathway cause early termination of aerial hyphae production with the differentiation of the colony arrested at the substrate mycelium growth stage (Claessen *et al.*, 2006). Mutation within the *whi* cascade results in the production of an aerial mycelium that does not generate mature spores and remains white in colour (Willey *et al.*, 1991; Chater, 2001). Bacteriocin production within the genus *Streptomyces* has been previously reported, with bactericidal spectra described as species-specific (Zhang *et al.*, 2003) or genus-specific (Roelants & Naudts, 1964).

In the present study, we isolated strain RB72^T from a soil sample collected at Rainbow Bluff, a woodland bluff in Lynn, Alabama. Soil-extract medium, developed from a cold-water extraction of the native soil of the organism supplemented with 10 μg cycloheximide ml^{-1}, 20 μg nalidixic acid ml^{-1} and 100 U catalase ml^{-1}, was seeded with a soil sample suspension and incubated at 25 °C for 14 days (Farris & Olson, 2007). Strain RB72^T was selected for its appearance as a characteristic streptomycete colony producing a leathery substrate mycelium and developing aerial hyphae with colony maturity. Colour production within the substrate mycelium and aerial hyphae was evaluated according to the *Colour Harmony Manual* as described by Tresner & Backus (1963) and Shirling & Gottlieb (1966). The isolate was maintained on nutrient agar slants at 25 °C and as suspensions in nutrient broth (Difco) with glycerol (20 %, v/v) at −20 °C. Biomass for the chemotaxonomic and molecular systematic studies was prepared as described previously (Li *et al.*, 2002). Mannitol soya flour agar (Hobbs *et al.*, 1989) was used for maintenance growth, and nutrient broth with 0.4 % glucose (w/v) was used for biomass growth.

The morphological characteristics of strain RB72^T were examined using light and scanning electron microscopy of

Abbreviation: ISP, International *Streptomyces* Project.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain RB72^T is EF657884.

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

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colonies grown on mannitol soya flour agar, nutrient agar with 0.4 % glucose (w/v), yeast extract-malt extract agar [International *Streptomyces* Project (ISP) medium 2; Shirling & Gottlieb, 1966] and oatmeal agar (ISP medium 3) after 7, 14 and 21 days at 25 °C. The coverslip method of Hopwood (1960) was used to observe hyphal characters by phase-contrast light microscopy with a Nikon Eclipse E600 microscope equipped with a Spot RT Colour imaging system (version 3.4 imaging software; Diagnostic Instruments). For high-resolution scanning electron microscopy, agar blocks containing mycelium were fixed with osmium tetroxide (1 %, w/v, in 0.1 M cacodylate buffer, pH 7.2) for 2 h, passed through increased concentrations of acetone (25, 50, 75, 90 and 100 %) and dried to critical point with a Denton DCP-1 critical point drying apparatus. The dried samples were mounted on graphite-coated aluminium stubs, coated with gold/palladium alloy by a Technics Hummer sputter coater, and examined with a Hitachi S2500 scanning electron microscope.

Colony morphology of strain RB72^T was observed on several standard media [ISP2, ISP3, inorganic salts-starch agar (ISP4), glycerol-asparagine agar (ISP5)] after 14 days of incubation at 25 °C. Examination of strain RB72^T for a range of biochemical and physiological characters was as described by Shirling & Gottlieb (1966), Williams *et al.* (1983) and Kämpfer *et al.* (1991). Tolerance to salt, temperature and pH was tested on nutrient agar with 0.4 % (w/v) glucose plates incubated for 7–14 days.

Liquid cultures of strain RB72^T, *Streptomyces hachijoensis* NRRL B-3106^T and *Streptomyces kentuckensis* NRRL B-1831^T were grown under identical conditions (nutrient broth with 0.4 %, w/v, glucose, 225 r.p.m., 30 °C) until late exponential phase (8 days), washed, lyophilized and whole-cell fatty acid profiles determined for triplicate samples following standard protocols (Sasser, 2001) except that fatty acids were identified by co-elution with known standards and mass spectral analysis of their methyl and picolinyl esters (Christie, 1998).

Genomic DNA was extracted from biomass of actively growing cultures on nutrient agar supplemented with 0.4 % glucose (w/v) as described by Olson *et al.* (2002). PCR amplification using universal primers 24f and 1492r was performed as described by Farris & Olson (2007). Amplified fragments were ligated into pCR2.1 cloning vector (TA cloning kit; Invitrogen) and used to transform *Escherichia coli* DH10B (Invitrogen) according to the manufacturer's instructions. Plasmids with inserts of the correct size were sequenced at the Macrogen (Korea) sequencing facility. Genomic DNA isolated from strain RB72^T using the method of Bollet *et al.* (1991) was sent to the HudsonAlpha Genomic Services Lab (Huntsville, AL) for Illumina Genome Analyser IIx sequencing.

16S rRNA gene sequence data were aligned using Sequencher version 4.5 (Gene Codes) and relatedness to gene sequences of type strains of characterized species of the genus *Streptomyces* was determined via NCBI BLAST searches (Altschul *et al.*, 1997). The reference sequences and strain RB72^T sequence (GenBank accession number EF657884) were aligned in BioEdit Sequence Alignment Editor, version 7.0.5.3 (Hall, 1999), using CLUSTAL W (Thompson *et al.*, 1994). The neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony algorithms of PAUP* version 4.0b 10 (Swofford, 2002) were used to infer the phylogenetic relatedness of the sequences. The method of Kimura (1980) was used to generate evolutionary distance matrices for the neighbour-joining algorithm. Tree topologies were calculated by bootstrap analyses based on 1000 resamplings.

DNA–DNA relatedness experiments were performed between strain RB72^T and two closely related strains, *Streptomyces hachijoensis* NRRL B-3106^T and *Streptomyces kentuckensis* NRRL B-1831^T (=*Streptomyces netropsis*; Hatano *et al.* 2003), using the fluorometric method described by Gonzalez & Saiz-Jimenez (2005). Briefly, strains RB72^T, *Streptomyces hachijoensis* NRRL B-3106^T and *Streptomyces kentuckensis* NRRL B-1831^T were grown in either nutrient broth (Difco) or SYZ (15 g soluble starch, 2 g yeast extract, 4 g NZ amine, 2 g glucose, 1 l deionized H_2O; pH 6.2) medium. Genomic DNA was isolated from the above strains using the method of Bollet *et al.* (1991). The purified genomic DNA samples possessed A_{260}/A_{280} ratios between 1.8 and 2.0. Homoduplex and heteroduplex DNA–DNA hybridizations were performed as described by Gonzalez & Saiz-Jimenez (2005) using a T_{or} of 82.7 °C. Thermal denaturation experiments contained 0.2 μg duplex DNA μl^{-1}, 0.1 × SSC (pH 8.0) and SYBR Green nucleic acid stain diluted 1 : 100 000. Melting curve analysis was performed using a MyiQ Real-time PCR Detection System (Bio-Rad). T_m values for homoduplex and heteroduplex genomic DNA solutions were calculated as the temperatures corresponding to a 50 % decrease in fluorescence. ΔT_m values were calculated as the difference between the T_m of the heteroduplex genomic DNA solution and the T_m of the reference strain homoduplex genomic DNA solution.

The organism exhibited a range of chemotaxonomic and phenotypic characters typical of the members of the genus *Streptomyces* (Table 1 and Supplementary Table S1, available in IJSEM Online). Strain RB72^T formed an extensively branched substrate mycelium and aerial hyphae on several standard growth media (Supplementary Figs S1 and S2). The organism produced white aerial hyphae with no spores and a golden brown substrate mycelium on all standard morphological media tested with the exception of ISP2, on which the extent of the aerial hyphae formation was reduced and the substrate mycelium did not produce pigment. Sporulation of the aerial hyphae was not detected after 14 days, and the aerial hyphae remained white in colour, typical of other *Streptomyces* strains that do not sporulate (Hopwood *et al.*, 1970; Chater, 1972, 1993; Aínsa *et al.*, 2000; Gehring *et al.*, 2000). Interestingly, analysis of the genomic sequencing failed to identify highly conserved (within the genus *Streptomyces*) primers for the bacterial signal recognition particle receptor FtsY, which has been

Table 1. Comparison of morphological, cultural and physiological characteristics of strain RB72^T and related species of the genus *Streptomyces*

Strains: 1, RB72^T; 2, *S. hachijoensis* NRRL B-3106^T; 3, *S. kentuckensis* NRRL B-1831^T. All data were determined in the laboratory under the same growth conditions. ND, Not determined; d, variable; +, positive; −, negative. All strains were positive for growth on D-glucose and *myo*-inositol and negative for growth on sucrose.

Characteristic	1	2	3
Morphology and pigmentation			
Aerial mass on oatmeal agar	White	Beige	Red–white
Spore-chain arrangement	−	ND	ND
Spore surface	−	ND	ND
Melanin production	−	−	+
Production of diffusible pigments	−	−	−
Growth on sole carbon sources (1 %, w/v)			
L-Arabinose	+	−	−
D-Fructose	−	d	−
D-Galactose	+	−	+
D-Mannitol	−	−	−
D-Raffinose	+	−	−
L-Rhamnose	+	−	−
D-Xylose	+	−	−
Sorbitol	−	+	+
Cellobiose	+	−	+
Melibiose	+	−	+
L-Sorbose	−	−	−
Maltose	+	+	+
Adonitol	+	+	+
Lactose	+	−	+
D-Mannose	+	+	+
Dextrin	+	+	+
Inulin	−	+	+

shown to regulate sporulation in *Streptomyces coelicolor* through interaction with *whiH* (Shen *et al.*, 2008). These results suggest a deficiency (or silent transcription) in the *whi* pathway of the organism.

In addition to the characters in Table 1, strain RB72^T reduced nitrate to nitrite and growth occurred at sodium chloride concentrations of 4 and 7 %, but not at 10 or 13 % (w/v). Growth occurred at pH 6.0–11.0 (optimum, pH 7.0) and 15–37 °C (optimum, 25 °C). Strain RB72^T hydrolysed adenine, casein, aesculin, gelatin, hypoxanthine, L-tyrosine, starch and xanthine but not cellulose.

The whole-cell fatty acid profiles of all of the species of the genus *Streptomyces* analysed were mainly comprised of iso-branched, even- and odd-chain, saturated and monounsaturated fatty acids (Supplementary Table S1). Strain RB72^T was unique in that the iso-branched, odd-chain, unsaturated fatty acid iso-$C_{17:1}\omega8$ comprised greater than 10 % of its total fatty acids.

A near-complete 16S rRNA gene sequence (1376 nt) was determined for strain RB72^T. Comparison of the sequence with sequences of reference micro-organisms confirmed that the unknown isolate was closely related to species of the genus *Streptomyces*. Phylogenetic analysis showed that strain RB72^T formed a sister grouping with *Streptomyces kentuckensis* NRRL B-1831^T (98.47 % similarity), *Streptomyces netropsis* NBRC 12893^T (98.45 %), *Streptomyces stramineus* NBRC 16131^T (98.45 %), *Streptomyces hachijoensis* NRRL B-3106^T (98.61 %) and *Streptomyces celluloflavus* NBRC 13780^T (98.59 %) (Fig. 1). *Streptomyces kentuckensis* NRRL B-1831^T is considered a subjective synonym of *Streptomyces netropsis* (Labeda, 1996; Hatano *et al.*, 2003). Genomic sequencing revealed 70.3 mol% DNA G+C content for strain RB72^T.

Analysis of DNA–DNA relatedness was performed between strain RB72^T and the closely related strains *Streptomyces hachijoensis* NRRL B-3106^T and *Streptomyces kentuckensis* NRRL B-1831^T using the fluorometric method described by Gonzalez & Saiz-Jimenez (2005). This method measures the difference in the thermal denaturation midpoints of homoduplex versus heteroduplex genomic DNA (ΔT_m) and has been used successfully in the characterization of other species of the genus *Streptomyces* (Kumar & Goodfellow, 2008). Distinct genomic species have a ΔT_m equal to or greater than 5 °C (e.g. Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994; Rosselló-Mora & Amann, 2001). For each species–species comparison, two independent experiments were performed. The following temperature differences represent the means and single standard deviations (given in parentheses) of these experimental sets. We found a difference in melting temperature of *Streptomyces hachijoensis* homoduplex genomic DNA versus RB72^T–*Streptomyces hachijoensis* heteroduplex genomic DNA of 12.2 °C (1.0 °C), confirming a definite species delineation. Likewise, the melting temperature difference between *Streptomyces kentuckensis* homoduplex genomic DNA and RB72^T–*Streptomyces kentuckensis* heteroduplex genomic DNA was 7.6 °C (1.9 °C), also corroborating species delineation.

Strain RB72^T demonstrated a broad spectrum of bacteriolytic activity. The purified bacteriocin (data not shown) was active against the Gram-positive bacteria *Streptomyces avermitilis* MA-4680^T, *Streptomyces coelicolor* A3(2), '*Streptomyces lividans*' 66, *Streptomyces venezuelae* NRRL-ISP 5230^T, *Nocardia salmonicida* NRRL B-2778^T, *Nocardia vaccinii* NRRL WC-3500^T, *Rhodococcus marinonascens* DSM 43752^T, *Bacillus megaterium* ATCC 14581^T, *Bacillus subtilis* 168, *Staphylococcus aureus* FDA209, *Streptococcus pyogenes* ATCC 14289, *Enterococcus faecalis* ATCC 29212 and *Micrococcus luteus* strain 85W0996, and the Gram-negative bacteria *Escherichia coli* DH10B and *Klebsiella pneumoniae* ATCC 13883^T.

Strain RB72^T warrants classification as the type strain of a novel species of the genus *Streptomyces* based on comparison of its 16S rRNA gene sequence with other known species of the genus *Streptomyces* and the phenotypic characters of sole carbon source utilization, genomic DNA

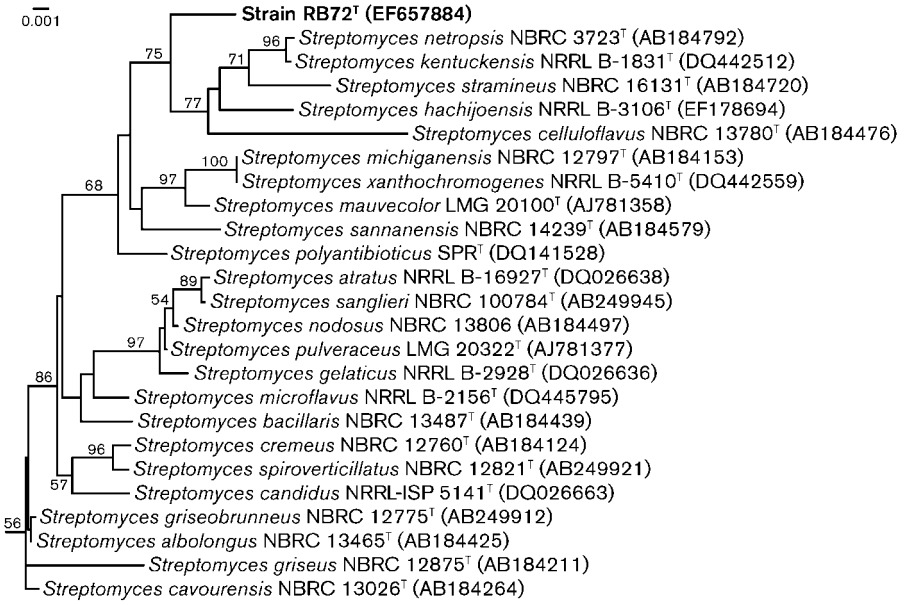

Fig. 1. Neighbour-joining phylogenetic tree based on near-complete 16S rRNA gene sequences showing the relationship between strain RB72^T and 24 species of the genus *Streptomyces*. Numbers at nodes indicate levels of bootstrap support (%) based on analysis of 1000 resampled datasets; only values above 50 % are given. NCBI accession numbers for each sequence are in parentheses. Bar, 1 substitution per 1000 nt.

hybridization/thermal denaturation experiments, chemotaxonomic characters, broad spectrum bacteriocin production and lack of sporulation that set it apart from other described species of the genus *Streptomyces*. For strain RB72^T, we propose the name *Streptomyces scopuliridis* sp. nov.

Description of *Streptomyces scopuliridis* sp. nov.

Streptomyces scopuliridis (scop.ul.i′rid.is. L. masc. n. *scopulus* cliff, bluff, crag; L. gen. n. *Iridis* of or belonging to the goddess of the rainbow; N.L. gen. n. *scopuliridis* from a rainbow cliff, referring to the location of isolation, Rainbow Bluff, a woodland bluff in Lynn, Alabama).

Aerobic, Gram-positive, non-motile, non-spore-forming actinomycete. Substrate and aerial mycelia are produced; however, the aerial hyphae fail to undergo the sporulation process. The substrate and aerial hyphae branch extensively, and the aerial hyphae remain white upon maturation. The reverse side of the substrate mycelium produces a golden brown pigment on ISP3, ISP4 and ISP5 media. In addition to the characters described in Table 1, nitrate is reduced to nitrite and growth occurs at sodium chloride concentrations of 4–7 % (w/v), at pH 6.0–11.0 and at temperatures of 15–37 °C. Hydrolyses adenine, casein, aesculin, gelatin, hypoxanthine, L-tyrosine, starch and xanthine, but not cellulose. The four most abundant fatty acids are iso-$C_{16:0}$, iso-$C_{17:1}\omega8$, iso-$C_{15:0}$ and anteiso-$C_{15:0}$ and the G+C content of the genomic DNA of the type strain is 70.3 mol%. Produces a broad spectrum bacteriocin with activity against Gram-positive and Gram-negative bacteria.

The type strain, RB72^T (=DSM 41917^T =NRRL B-24574^T), was isolated from a soil sample collected from Rainbow Bluff, a woodland bluff in Lynn, Alabama.

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