Streptomyces lunalinharesii sp. nov., a chitinolytic streptomycete isolated from cerrado soil in Brazil

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A novel chitinolytic actinomycete isolated from a Brazilian cerrado soil, designated strain RCQ1071^T, was assigned to the genus *Streptomyces* on the basis of chemical and morphological characteristics. The almost-complete nucleotide sequence of the 16S rRNA gene of strain RCQ1071^T was determined and also placed this strain in the genus *Streptomyces*. Phylogenetic analyses of 16S rRNA gene sequences showed that strain RCQ1071^T formed a long branch in a group related to *Streptomyces albulus*, sharing approximately 98 % sequence similarity. Levels of DNA–DNA relatedness between strain RCQ1071^T and members of this group, namely *S. albulus* DSM 40492^T, *Streptomyces noursei* DSM 40635^T and *Streptomyces yunnanensis* DSM 41793^T, were 38.3, 27.8 and 46 %, respectively, strongly indicating that strain RCQ1071^T was not a member of any of these species. The relatively long branch length within a stable clade together with the phenotypic data strongly supported that strain RCQ1071^T represented a novel species. Based on the combination of physiological, phylogenetic and genomic data, strain RCQ1071^T is suggested to represent a novel species of the genus *Streptomyces*, for which the name *Streptomyces lunalinharesii* sp. nov. is proposed. The type strain is RCQ1071^T (=ATCC BAA-1231^T =CIP 108852^T =DSM 41876^T).

Actinomycetes are filamentous, Gram-positive bacteria widely distributed in terrestrial environments, where they are responsible for the degradation and recycling of many natural biopolymers. Together with their broad metabolic capabilities and their ability to produce pigments, antibiotics and hydrolytic enzymes, this makes them a fascinating group to study and to be employed in biotechnological applications. The genus *Streptomyces* remains a focus for research, not only because its members are still the most promising source of commercially significant compounds, but also because current molecular biology methods are having an increasing impact on conventional systematics of streptomycetes (Williams *et al.*, 1983a, b; Goodfellow *et al.*, 1992; Huang *et al.*, 2004). Streptomycetes represent a considerable proportion of actinomycete communities in soils and are intrinsically linked with soil quality and function. Our

laboratory has been isolating actinomycete strains from different Brazilian soils for many years and selecting those which have shown significant enzyme production and therefore biotechnological potential (Coelho & Drozdowicz, 1978; Gomes *et al.*, 2000a, b; Nascimento *et al.*, 2003; Semêdo *et al.*, 2004). Strain RCQ1071^T was isolated from an acid orthic ferralsol from central Brazil, under cerrado vegetation, and was selected for further study because of its chitinolytic activity detected on colloidal chitin agar medium (Gomes *et al.*, 2001). When utilizing glycol chitin as a substrate, this isolate exhibited high endochitinase activity at 40 °C and pH 8.0. Purified enzyme was stable at temperatures up to 70 °C and pH 4.0–9.0. Broad-range enzyme stability is promising for biotechnological purposes, specifically the degradation of chitin-containing waste (Sakai *et al.*, 1998). The purified endochitinase also showed antifungal activity when tested against seven phytopathogenic fungi (Gomes *et al.*, 2001), suggesting that strain RCQ1071^T has potential in the biological control of plant fungal pathogens.

Preliminary taxonomic characterization, based on analysis of morphological features (aerial hyphae with spiral spore

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain RCQ1071^T is DQ094838.

A table detailing differential phenotypic properties between strain RCQ1071^T and chitinolytic *Streptomyces* species is available as supplementary material with the online version of this paper.

chains) and chemotaxonomic data (presence of LL-diaminopimelic acid and the absence of a diagnostic cell-wall sugar, which characterizes it as cell-wall chemotype I *sensu* Lechevalier & Lechevalier, 1970) revealed that the strain belonged to the genus *Streptomyces* (Gomes *et al.*, 2001). Numerical taxonomic analysis was performed according to Williams *et al.* (1983a). Seventy-five tests (see Supplementary Table S1 in IJSEM Online) were performed and the results were compared with those of other streptomycetes by using the program PIBwin version 1.9.2 (Bryant, 2004). Strain RCQ1071^T did not cluster with described groups within the identification matrix. Chain morphology and spore ornamentation were examined by light and transmission electron microscopy (EM 900; Zeiss) after 14 days of growth on malt extract/yeast extract agar (Shirling & Gottlieb, 1966). Aerial spore-mass colour, substrate mycelial pigmentation and production of diffusible pigments were recorded after incubation at 28 °C for 14 days on inorganic salts/starch agar and glycerol/asparagine agar (Shirling & Gottlieb, 1966). Melanin production was tested on tyrosine agar medium and peptone/yeast extract/iron agar (Shirling & Gottlieb, 1966).

The ability of strain RCQ1071^T to grow in the presence of ($\mu\text{g ml}^{-1}$ unless indicated) neomycin sulfate (50), oleandomycin (100), penicillin G sodium salt (10 IU ml⁻¹), rifampicin (50), streptomycin sulfate (100), gentamicin sulfate (100), dimethylchlorotetracycline (500), novobiocin (100), methacyclin hydrochloride (100), ampicillin sodium salt (100) and cycloheximide (100) was tested by incubation at 28 °C for 7 days on plates containing modified Bennett's agar (MBA), with glucose replaced by glycerol. Similarly, strain RCQ1071^T was examined to test its ability to grow on MBA supplemented with (% w/v) phenol (0.1), potassium tellurite (0.001), sodium chloride (7) and sodium azide (0.01) and to grow at 45 °C. Antimicrobial activity of strain RCQ1071^T was tested against *Aspergillus niger* LIV 131, *Micrococcus luteus* NCIB 196, *Streptomyces murinus* ISP 5091, *Bacillus subtilis* NCIB 3610 and *Candida albicans* CBS 562. All physiological and biochemical characteristics were determined according to Williams *et al.* (1983a); results that distinguished strain RCQ1071^T from its closest phylogenetic relatives are shown in Table 1.

Cell-wall amino acids were analysed after acid hydrolysis of the cell mass by using 6 M HCl. Hydrolysed material was dried, suspended in the appropriate buffer and injected into an automatic analyser (High Performance Amino Acid Analyser, system 6300; Beckman), according to the manufacturer's instructions. Amino acids were identified automatically by comparison of retention times against a standard mixture. The major amino acids found in cell-wall hydrolysates of strain RCQ1071^T, in order of relative abundance, were alanine and glycine, followed by glutamic acid, leucine, threonine, methionine and lysine, which are consistent with patterns obtained for amino acid analysis of another streptomycete isolated from Brazilian soil (Semêdo *et al.*, 2004).

Table 1. Differential physiological and biochemical properties between strain RCQ1071^T and its closest relatives

Strains: 1, RCQ1071^T; 2, *S. albulus* DSM 40492^T; 3, *S. noursei* ATCC 11455^T; 4, *S. yunnanensis* DSM 41793^T. Data for reference strains were obtained in this study.

Characteristic	1	2	3	4
Spore-mass colour*	G	G/R	G	LGB/PY
Spore surface†	S	S	S	R
Substrate mycelium colour on:*				
Glycerol-asparagine agar	YB	—	—	LOY
Inorganic salts-starch agar	YB	—	—	LGY
Diffusible pigment production on glycerol-asparagine agar*	—	—	—	LY
Degradation of:				
Allantoin	+	—	—	+
Guanine	—	+	—	—
L-Tyrosine	+	—	+	+
Urea	+	+	—	+
Xanthine	—	+	—	—
Growth on sole carbon source				
D-Xylose	—	+	—	—
L-Arabinose	+	+	—	+
L-Rhamnose	+	—	—	+
Raffinose	—	—	—	+
Sucrose	+	—	+	—
Growth in:				
Sodium azide (0.01 %, w/v)	+	—	+	+
Sodium chloride (7 %, w/v)	+	—	—	—
Growth on sole nitrogen source				
Amino- <i>n</i> -butyric acid	+	—	+	+
L-Arginine	+	—	+	+
L-Methionine	+	—	+	+

*G, Grey; LGB, light grey-brown; LGY, light grey-yellow; LOY, light orange-yellow; LY, light yellow; PY, pale yellow; R, red; YB, yellowish brown.

†R, Rugose; S, spiny.

Extraction of genomic DNA from strain RCQ1071^T was carried out as described by Rintala *et al.* (2001). PCR amplification of the 16S rRNA gene was carried out by using a Gene Amp PCR System 9700 (Applied Biosystems) thermocycler and primers as described by Edwards *et al.* (1989). Amplified fragments were purified and sequenced directly by using the PureLink PCR purification kit (Invitrogen) and an ABI Prism dye terminator cycle sequencing reaction kit (ABI Prism 310 Genetic Analyzer), respectively. The almost-complete 16S rRNA gene sequence (1480 nt) obtained was subjected to similarity searches against data online at the Ribosomal Database Project (RDP) (Maidak *et al.*, 1997) and GenBank (BLASTN; Altschul *et al.*, 1997). Sequences retrieved were aligned and edited to the same size (1420 bp) by using BioEdit version 7.0.4.1 (Hall, 1999). Phylogenetic analyses were conducted by using MEGA version 4 (Tamura *et al.*, 2007). Evolutionary distances were generated with

Kimura's two-parameter model (Kimura, 1980) and the maximum-likelihood model (Felsenstein, 1981), and trees were constructed by using neighbour-joining (Saitou & Nei, 1987). The type strains of *Streptomyces melanosporofaciens* and *Streptomyces violaceusniger* were used as an outgroup in phylogenetic reconstructions. Phylogenetic inferences depicted as phylograms/trees were subjected to bootstrap analysis based on 5000 resamplings in order to gain a measure of internal branch order and stability.

Based on 16S rRNA gene sequence similarities, strain RCQ1071^T was related most closely to *Streptomyces yunnanensis* YIM 41004^T (97.97%), *Streptomyces albulus* NBRC 13410^T (97.83%), *Streptomyces noursei* NBRC 15452^T (97.83%) and *Streptomyces viridis* NBRC 13373^T (97.76%). Although the 16S rRNA gene sequence of strain RCQ1071^T was most similar to that from *S. yunnanensis* DSM 41793^T, strain RCQ1071^T formed a long branch separated from the above four species in the neighbour-joining tree (Fig. 1).

Genomic DNA–DNA hybridization experiments were performed to compare strain RCQ1071^T with its closest relatives as determined based on 16S rRNA gene sequences. Levels of genomic DNA–DNA relatedness were determined at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ). DNA was isolated by using a French pressure cell (Thermo Spectronic) and purified by chromatography on hydroxyapatite as described by

Cashion *et al.* (1977). DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970) with the modifications described by Huß *et al.* (1983) by using a model Cary 100 Bio UV/Vis spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with *in-situ* temperature probe (Varian). Strain RCQ1071^T shared 38.3, 27.8 and 46% DNA–DNA relatedness with *S. albulus* DSM 40492^T, *S. noursei* DSM 40635^T and *S. yunnanensis* DSM 41793^T, respectively. These results clearly demonstrate that strain RCQ1071^T does not belong to one of the species seen in the 16S rRNA gene sequencing clusters, as values were well below the 70% threshold recommended for the recognition of separate genomic species (Wayne *et al.*, 1987).

Given that strain RCQ1071^T showed high chitinase activity (Gomes *et al.*, 2001), the most similar 16S rRNA gene sequences from the type strains of chitinolytic streptomycetes were also included in our analyses. Phylogenetic trees placed almost all chitinolytic streptomycetes in a unique clade, separated from strain RCQ1071^T (Fig. 1; see strains from *Streptomyces thermoviolaceus* to *Streptomyces halstedii*). Analyses of 16S rRNA gene sequences demonstrated that strain RCQ1071^T is phylogenetically distinct from the most similar type strains that exhibit chitinolytic activity. Phenotypic comparisons amongst these strains with strain RCQ1071^T (Table 1) highlight its uniqueness and support its recognition as representing a novel species. In conclusion, chitinolytic strain RCQ1071^T, isolated from

Fig. 1. Neighbour-joining tree (Saitou & Nei, 1987) showing the phylogenetic relationships between strain RCQ1071^T and related streptomycetes. The phylogeny is based on an alignment of 1408 bases of the 16S rRNA gene sequence. Asterisks indicate branches that were also recovered by using the Kimura (1980) and maximum-likelihood (Felsenstein, 1981) models. Sequence accession numbers are shown in parentheses. *Streptomyces melanosporofaciens* NRRL B-12234^T and *Streptomyces violaceusniger* NRRL-ISP 5563^T were used as the outgroup. Numbers at branch nodes indicate percentage bootstrap values based on 5000 resamplings; only values above 50% are shown. Bar, 0.005 substitutions per nucleotide position.

an acid orthic ferralsol from central Brazil under cerrado vegetation, is clearly distinct from other phylogenetically and phenotypically related recognized *Streptomyces* species and thus represents a novel species. Based on the above we propose that this important chitinolytic strain, RCQ1071^T, be formally recognized as the type strain of a novel species of the genus *Streptomyces*, for which the name *Streptomyces lunalinharesii* sp. nov. is proposed.

Description of *Streptomyces lunalinharesii* sp. nov.

Streptomyces lunalinharesii (lu'na.lin.ha're.si.i. N.L. masc. gen. n. *lunalinharesii* of Luna Linhares, named after Luiz Fernando de Toledo Luna Linhares, a Brazilian soil microbiologist).

An aerobic, Gram-positive, chitinolytic actinomycete that forms a grey-coloured aerial mycelium and yellow-brown substrate mycelium and presents spiny spores in spiral chains. The colour of the substrate mycelium is not pH-sensitive and results from the production of a yellow-brown diffusible pigment the colour of which is also not pH-sensitive. Cell-wall hydrolysates contain LL-diaminopimelic acid. The predominant amino acids in the cell-wall hydrolysate are alanine and glycine, followed by glutamic acid, leucine, threonine, methionine and lysine. Very good growth occurs on yeast extract/malt extract agar and on modified Bennett's agar. Antimicrobial activity is exhibited against *Aspergillus niger* and *Candida albicans* but is not observed against *Kocuria rhizophila*, *Streptomyces murinus*, *Bacillus subtilis* or *Saccharomyces cerevisiae*. Resistant to novobiocin, methacyclin hydrochloride, ampicillin sodium salt, cycloheximide, streptomycin sulfate (all at 100 µg ml⁻¹) and penicillin G sodium salt (10 IU ml⁻¹) and degrades keratin, L-tyrosine, hypoxanthine, allantoin, casein, elastin, gelatin and starch. The species description is based on a single strain and hence serves as the type strain description.

The type strain, RCQ1071^T (=ATCC BAA-1231^T =CIP 108852^T =DSM 41876^T), was isolated from an acid orthic ferralsol from central Brazil under cerrado vegetation.

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