Streptomyces radiopugnans sp. nov., a radiation-resistant actinomycete isolated from radiation-polluted soil in China

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The taxonomic position of an actinomycete isolated from radiation-polluted soil collected in Xinjiang Province, north-west China, was determined by using a polyphasic approach. The isolate, designated strain R97^T, had chemical and morphological properties characteristic of streptomycetes. An almost-complete 16S rRNA gene sequence of the isolate was generated and compared with corresponding sequences of representative streptomycetes. The 16S rRNA data not only supported the classification of the strain in the genus *Streptomyces* but also showed that it represented a distinct phyletic line that was most closely, albeit loosely, associated with three other thermotolerant organisms, namely *Streptomyces macrosporus* NBRC 14748^T, *Streptomyces megasporus* NBRC 14749^T and *Streptomyces thermolineatus* NBRC 14750^T. Strain R97^T could be distinguished from these organisms based on a range of phenotypic properties. It is proposed that R97^T (=CGMCC 4.3519^T =DSM 41901^T) be classified as the type strain of a novel species in the genus *Streptomyces*, *Streptomyces radiopugnans* sp. nov. The organism was shown to be resistant to ⁶⁰Co gamma radiation at a dose of 15 kGy.

The first radiation-resistant micro-organism to be described, designated '*Micrococcus radiodurans*', was isolated from irradiated meat (Anderson *et al.*, 1956) and was subsequently reclassified as *Deinococcus radiodurans* (Brooks & Murray, 1981). Other notable radiation-resistant bacteria include *Bacillus nealsonii* (Venkateswaran *et al.*, 2003), *Hymenobacter actinoscleris* (Collins *et al.*, 2000), *Trueperia radiovictrix* (Albuquerque *et al.*, 2005) and the actinobacteria *Kineococcus radiotolerans* (Phillips *et al.*, 2002) and *Rubrobacter taiwanensis* (Chen *et al.*, 2004). During a study on the bioremediation of radiation-contaminated soils in Xinjiang Province, China, a radiation-resistant streptomycete-like strain, designated R97^T, was isolated. The present study was designed to determine the taxonomic status of this organism by using genotypic and phenotypic procedures. The resultant data show that the strain should be classified as representing a novel species of the genus *Streptomyces*.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain R97^T is DQ912930.

A figure showing the gamma-radiation survival curves of strain R97^T, *Deinococcus radiodurans* DSM 20539^T and *Escherichia coli* K12 is available as supplementary material with the online version of this paper.

Strain R97^T was isolated from a modified Bennett's agar plate (Jones, 1949), which had been inoculated with a soil suspension and incubated at 28 °C for 14 days. The soil sample was collected from radiation-contaminated soil in the Xinjiang Uigur Autonomous Region of north-west China. The organism was maintained on inorganic salts-starch agar slopes (ISP medium 4; Difco) (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 modified Bennett's broth for up to 7 days at 28 °C, harvested by centrifugation and washed twice in distilled water; cells for chemical studies were freeze-dried.

Extraction of genomic DNA, PCR-mediated amplification of the 16S rRNA gene and purification of the products from isolate R97^T were achieved following the procedures of Chun & Goodfellow (1995). The PCR products were sequenced directly by using a *Taq* DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems) and an Applied Biosystems 373A DNA sequencer. The resultant sequence (1442 nt) was aligned manually with corresponding sequences of available streptomycetes drawn from the

DDBJ/EMBL/GenBank databases. The program MEGA version 3.1 (Kumar *et al.*, 2004) was used for both multiple alignment and phylogenetic analyses. Phylogenetic trees were generated via the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) tree-making algorithms; evolutionary distances for the neighbour-joining algorithm were calculated with the Kimura two-parameter model (Kimura, 1980). The topologies of the resultant trees were evaluated in a bootstrap analysis (Felsenstein, 1985) based on 1000 replicates. It was apparent from the initial neighbour-joining analysis, including more than 500 related sequences of *Streptomyces* type strains, that isolate R97^T represented a bona fide member of the genus *Streptomyces* (data not shown).

The new isolate was examined for a range of chemotaxonomic and morphological properties known to be characteristic of members of the genus *Streptomyces* (Williams *et al.*, 1989). To this end, hyphal and spore chain arrangement were observed on modified Bennett's and inorganic salts-starch agar plates after incubation at 28 °C for 14 days, by using the coverslip technique of Kawato & Shinobu (1959). Spore chain morphology and spore surface ornamentation were observed by examining gold-coated dehydrated specimens under an FEI QUANTA scanning electron microscope. The isomers of diaminopimelic acid and whole-organism sugars were analysed by using the procedures developed by Hasegawa *et al.* (1983) and Lechevalier & Lechevalier (1980). Polar lipids were examined and identified according to the method of Minnikin *et al.* (1984), and fatty acids were extracted, methylated and analysed via GC by using the standard Sherlock MIDI (Microbial Identification) system (Sasser, 1990; Kämpfer & Kroppenstedt, 1996). Menaquinones were extracted and purified following the method of Collins (1985) and then analysed by HPLC. The DNA

G + C content of the strain was determined by using the thermal denaturation method of Marmur & Doty (1962) with *Escherichia coli* K12 as a control.

Morphological and chemical characteristics of isolate R97^T were in line with its assignment to the genus *Streptomyces* (Williams *et al.*, 1989; Manfio *et al.*, 1995). The organism formed an extensively branched substrate mycelium, with aerial hyphae that differentiated into rough to warty, oval-shaped spores (0.70–1.0 × 0.92–1.4 µm) in spiral spore chains (Fig. 1). It contained major amounts of LL-diaminopimelic acid in whole-organism hydrolysates, hexahydrogenated and octahydrogenated menaquinones with nine isoprene units [MK-9(H₆, H₈)] as predominant isoprenologues and diphosphatidylglycerol and phosphatidylethanolamine as major polar lipids (phospholipid type II *sensu* Lechevalier *et al.*, 1977), but lacked mycolic acids and characteristic sugars. The fatty acid profile was rich in saturated straight-chain and iso- and anteiso-branched components (fatty acid type 2c *sensu* Kroppenstedt, 1985). The DNA G + C content was 72.7 mol%.

Strain R97^T was compared with its closest phylogenetic neighbours by using the procedures described above. It was evident from the phylogenetic tree thus constructed (Fig. 2) that the organism formed a distinct phyletic line together with the type strains of *Streptomyces macrosporus* and *Streptomyces megasporus*, an association that was supported by both of the tree-making algorithms employed and by a 99 % bootstrap value in the neighbour-joining analysis. Strain R97^T shared highest 16S rRNA gene sequence similarity with *S. macrosporus* NBRC 14748^T (97.5 %), which corresponds to 35 nt differences at 1444 locations with gaps, and lower values with *S. megasporus* NBRC 14749^T (96.7 %) and *Streptomyces thermolineatus* NBRC 14750^T (97.1 %). DNA–DNA relatedness studies were not

Fig. 1. Scanning electron micrographs of strain R97^T showing spiral chains of rough to warty spores after growth on inorganic salts-starch agar for 14 days at 28 °C. Bars, 5 µm.

Fig. 2. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences showing relationships between strain R97^T and the type strains of phylogenetically close *Streptomyces* species. Asterisks indicate branches of the tree that were also recovered with the maximum-parsimony tree-making algorithm. Numbers at nodes are percentage bootstrap values based on 1000 resampled data sets; only values above 50 % are given. Bar, 0.005 substitutions per nucleotide position.

carried out between strain R97^T and these organisms as representatives of several *Streptomyces* species with much higher 16S rRNA gene sequence similarities have DNA–DNA relatedness values well below the 70 % cut-off point recommended for the delineation of genomic species (Wayne *et al.*, 1987), as exemplified in a study on neutrotolerant acidophilic streptomycetes (Xu *et al.*, 2006).

The organism was also examined for a range of phenotypic properties. Colonial and pigmentation features were observed on inorganic salts-starch agar (ISP medium 4), and yeast extract-malt extract agar (ISP medium 2; Difco), and on modified Bennett's agar, Gauze's synthetic medium no. 1 agar (DSMZ medium no. 1048) and yeast-starch agar (DSMZ medium no. 1027) after incubation for 14 days at 28 °C. Similarly, peptone-yeast extract-iron and tyrosine

Table 1. Phenotypic properties that differentiate strain R97^T from representatives of phylogenetically close *Streptomyces* species

All strains grew at 45 °C. Data are taken from the present study and from Goodfellow *et al.* (1987). +, Positive; –, negative; +^w, weakly positive.

Characteristic	Strain R97 ^T	<i>S. macrosporus</i> NBRC 14748 ^T	<i>S. megasporus</i> NBRC 14749 ^T	<i>S. thermolineatus</i> NBRC 14750 ^T
Spore chain morphology	Spiral	Spiral	Spiral	Rectiflexibiles
Spore surface ornamentation	Rough to warty	Warty	Warty	Smooth
Degradation of:				
Elastin	–	–	+	–
Xanthine	+	–	–	–
Growth on sole carbon sources				
Inositol	–	+	–	–
Inulin	+	+ ^w	–	–
Mannitol	+	–	–	+ ^w
L-Rhamnose	+	+	+ ^w	–
Sucrose	+	–	–	–
Trehalose	–	+	–	+
Xylitol	+	–	–	–
D-Xylose	+	+	–	–
Sodium citrate	+	–	–	–
Growth at:				
pH 5	+	–	–	–
pH 11	+	+	–	+
Growth on sole nitrogen sources				
KNO ₃	–	+	–	+
L-Cysteine	+	–	–	–
L-Phenylalanine	–	+	–	+

agars (Shirling & Gottlieb, 1966) were examined for the production of melanin pigments. Additional phenotypic properties were determined by using established media and methods (Williams *et al.*, 1983; Kämpfer *et al.*, 1991). The various media supported the growth of a white to grey aerial spore mass and a range of substrate mycelial pigments. Melanin pigments were not formed on either peptone-yeast extract-iron or tyrosine agars. It is evident from Table 1 that strain R97^T can be distinguished from its closest phylogenetic neighbours based on a combination of phenotypic properties, although like them it is thermotolerant.

The survival rate of strain R97^T in response to gamma radiation was compared with those of *Deinococcus radiodurans* DSM 20539^T (radiation-resistant organism) and *Escherichia coli* K12 (radiation-sensitive organism) via an established procedure (Ferreira *et al.*, 1997; Chen *et al.*, 2004). The strains were grown in modified Bennett's broth (containing glass beads to prevent mycelium formation) to exponential growth phase, at which point biomass was washed with sodium chloride (0.85 %, w/v), centrifuged at 4 °C and resuspended in saline (0.85 %, w/v) to give a concentration of 1×10^7 – 1×10^8 c.f.u. ml⁻¹. Each suspension was divided into 2-ml aliquots and exposed to a ⁶⁰Co source at a dose rate of 0.167 kGy min⁻¹ at room temperature (1 kGy = 10⁵ rads); the gamma radiation doses were from zero to 20.0 kGy in steps of 2.5 kGy. Treated samples were diluted and plated in triplicate onto modified Bennett's agar plates and then incubated at 28 °C for 15 days, at which point the colony-forming units were counted. Viability was assessed by using non-irradiated suspensions of each strain under the same conditions as the controls.

Strain R97^T was resistant to gamma radiation with a shoulder dose (the dose required before the number of colony-forming units began to decline) of 5 kGy, a result comparable with the shoulder dose for *D. radiodurans* DSM 20539^T. Exposure of the two radiation-resistant strains to 15 kGy resulted in survival rates for strain R97^T and *D. radiodurans* DSM 20539^T of 1 and 2.6 %, respectively (see Supplementary Fig. S1 in IJSEM Online). Consequently, strain R97^T can be added to the taxonomically diverse group of thermotolerant/thermophilic bacteria that are able to resist gamma radiation (Mattimore & Battista, 1996; Ferreira *et al.*, 1997, 1999). It is not clear how such organisms have acquired their ability to resist radiation damage, although there is evidence that it may be due to evolutionary processes resulting from environmental stress, particularly that caused by drought and heat (Mattimore & Battista, 1996).

The genotypic and phenotypic data presented clearly demonstrate that strain R97^T represents a novel species of the genus *Streptomyces*, for which the name *Streptomyces radiopugnans* sp. nov. is proposed.

Description of *Streptomyces radiopugnans* sp. nov.

Streptomyces radiopugnans (ra.di.o.pug'nans. L. n. *radius* a beam or ray; N.L. pref. *radio-* pertaining to radiation; L.

part. adj. *pugnans* fighting or resisting; N.L. part. adj. *radiopugnans* radiation-resisting).

Aerobic, Gram-positive, radiation-resistant actinomycete that forms an extensively branched substrate mycelium which carries aerial hyphae that differentiate into spiral chains of spores with rough to warty surfaces. Moderate to abundant, white to pale-grey aerial spore mass is formed on modified Bennett's, Gauze's synthetic medium no. 1, inorganic salts-starch, yeast extract-malt extract and yeast-starch agars. Substrate mycelium is yellowish brown on modified Bennett's, Gauze's no. 1 and yeast-starch agars and light pinkish yellow on inorganic salts-starch and yeast extract-malt extract agars. Diffusible pigments are not formed on any of the media, and melanin pigments are not formed on peptone-yeast extract-iron or tyrosine agars. Growth occurs between 20 and 50 °C, but not at 55 °C. Growth also occurs in the presence of 0.1 % phenol, but not in the presence of 7 % NaCl. Tween 60 is degraded, but Tweens 20 and 80 are not. L-Arabinose, L-melezitose and L-ribose are used as sole carbon sources for energy and growth, but not L-cellobiose, L-lactose, L-lactulose, L-melibiose, D-raffinose or trehalose (all at 1 %, w/v). Similarly, L-cysteine, L-glycine, D-glutamate, sodium azelate, sodium isobutyrate and sodium malonate are used as sole carbon sources for energy and growth, but not D-glutamic acid, hydroxy-L-proline, DL-isoleucine, L-leucine, methyl D-glucopyranoside, methyl α-D-mannopyranoside, L-phenylalanine, sodium propionate, sodium pyruvate, sodium suberate or spermidine (all at 0.1 %, w/v). Additional phenotypic properties are given in Table 1. The fatty acid profile consists of iso-C_{16:0} (34.5 %), anteiso-C_{15:0} (15.4 %), iso-H-C_{16:1} (14.2 %), anteiso-C_{17:0} (9.1 %), iso-C_{14:0} (5.6 %) and anteiso-C_{17:1}ω9c (5.2 %). The DNA G + C content is 72.7 mol%.

The type strain, R97^T (=CGMCC 4.3519^T =DSM 41901^T), was isolated from a radiation-contaminated soil sample collected from Xinjiang Province, north-west China. The species description is based on a single strain and hence serves as a description of the type strain.

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