Amycolatopsis regifaucium sp. nov., a novel actinomycete that produces kigamicins

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The taxonomic position of seven soil actinomycetes provisionally assigned to the genus *Amycolatopsis* was established in a polyphasic study. The isolates, which had identical 16S rRNA gene sequences, had closest similarity to the type strain of *Amycolatopsis orientalis*. A representative isolate, strain GY080^T, had chemotaxonomic properties that were typical of the genus *Amycolatopsis* and could be distinguished from the type strain of *A. orientalis* using DNA–DNA relatedness data. All of the isolates shared a phenotypic profile that distinguished them from representatives of phylogenetically closely related species. Amplified rDNA restriction analysis showed that the isolates formed a homogeneous group that was distinctly separate from single-membered groups consisting of representative *Amycolatopsis* type strains, including that of *A. orientalis*. Based on the combined genotypic and phenotypic evidence, it is proposed that the seven isolates be classified as representatives of a novel species for which the name *Amycolatopsis regifaucium* sp. nov. is proposed. The type strain is GY080^T (=DSM 45072^T =NCIMB 14277^T).

The genus *Amycolatopsis* is well defined mainly due to the application of chemotaxonomic, molecular systematic and numerical phenetic procedures (Goodfellow *et al.*, 2001; Saintpierre-Bonaccio *et al.*, 2005; Tan *et al.*, 2006a). It currently encompasses 37 species with validly published names that form a distinct phyletic line within the *Pseudonocardiaceae* 16S rRNA gene tree (Lee *et al.*, 2006; Majumdar *et al.*, 2006). The genus can be distinguished from the other genera classified in the family *Pseudonocardiaceae* by using a combination of chemical and morphological properties (Kim & Goodfellow, 1999). Similarly, *Amycolatopsis* species can be separated from one another using a range of phenotypic properties (Labeda

et al., 2003; Tan *et al.*, 2006a). The improved classification of the genus provides a sound basis for the circumscription of additional *Amycolatopsis* species, as exemplified by the description of novel species of clinical (Labeda *et al.*, 2003; Huang *et al.*, 2004), ecological (Goodfellow *et al.*, 2001; Lee *et al.*, 2006) and biotechnological (Goodfellow *et al.*, 1997; Wink *et al.*, 2003) interest, notably as a source of novel antibiotics (Tan *et al.*, 2006a).

The aim of the present study was to establish the taxonomic position of seven *Amycolatopsis* isolates that produce a light-grey aerial spore mass, dark yellow–brown substrate mycelium and dark grey–brown diffusible pigments on modified Bennett's agar supplemented with mannitol and soybean flour (Tan *et al.*, 2006b). The seven test strains were the subject of a polyphasic taxonomic study, which showed that they merited recognition as representatives of a novel species.

Strains GY080^T, GY091, GY246, GY248, GY249, GY250 and GY293 were isolated on SM2 agar plates that had been inoculated with tenfold dilutions of a composite Australian soil sample and incubated at 28 °C for 21 days, as described by Tan *et al.* (2006b). The organisms were maintained on modified Bennett's agar slopes (Jones, 1949) at room temperature and as suspensions of mycelial fragments in glycerol (20 %, v/v) at –20 °C. Biomass for molecular systematic studies was grown and prepared according to Tan *et al.* (2006b). All of the isolates produced

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains GY080^T, GY091, GY246, GY248, GY249, GY250 and GY293 are AY129760, AY129761, AY129766, AY129768, AY129767, AY129763 and AY129769, respectively.

A consensus dendrogram showing relationships between the *A. regifaucium* isolates and *A. orientalis* based on S_J-UPGMA analysis of restriction endonuclease-generated fingerprints and an extended 16S rRNA gene sequence-based neighbour-joining tree are available as supplementary material with the online version of this paper.

an amplification product of the expected size when treated with a set of genus-specific 16S rRNA oligonucleotide primers (Tan *et al.*, 2006b).

Extraction of chromosomal DNA, PCR amplification and sequencing of 16S rRNA genes from the isolates was achieved using established procedures (Kim *et al.*, 2002; Tan *et al.*, 2006a). The resultant 16S rRNA gene sequences were aligned manually using the program PHYDIT (available at <http://plaza.snu.ac.kr/~jchun/phydit/>) against corresponding sequences of representatives of genera in the family *Pseudonocardiaceae* as retrieved from the GenBank/EMBL/DDBJ databases. Phylogenetic trees were inferred by using the least-squares (Fitch & Margoliash, 1967), maximum-parsimony (Fitch, 1971) and neighbour-joining (Saitou & Nei, 1987) algorithms drawn from the PHYLIP suite of programs (Felsenstein, 1993); evolutionary distance matrices were generated for the least-squares and neighbour-joining algorithms after Jukes & Cantor (1969). Topologies of the resultant trees were evaluated by bootstrap analysis (Felsenstein, 1985) of the neighbour-joining method based upon 1000 replicates using the programs CONSENSE and SEQBOOT from the PHYLIP package. *Prauserella rugosa* DSM 43194^T was used as the outgroup organism to root the neighbour-joining tree. Phylogenetic comparison of almost complete 16S rRNA gene sequences of the tested strains with corresponding data on representatives of the family *Pseudonocardiaceae* showed that they belonged to the genus *Amycolatopsis* (data not shown).

It is apparent from Fig. 1 that the seven isolates share identical 16S rRNA gene sequences and form a distinct phyletic line in the *Amycolatopsis* tree together with the type strains of *Amycolatopsis japonica* and *Amycolatopsis orientalis*. The relationship between these organisms is supported by all of the tree-making algorithms and by a bootstrap value of 81 % in the neighbour-joining analysis. The seven isolates and *A. orientalis* IMSNU 20058^T shared

99.86 % 16S rRNA gene sequence similarity, a value that corresponds to 2 nt differences at 1437 locations. Similarly, the isolates and *A. japonica* DSM 44213^T had a 16S rRNA gene sequence similarity of 99.45 %, equivalent to 8 nt differences at 1453 sites. High 16S rRNA gene sequence similarities were also found between the isolates and the type strains of *Amycolatopsis alba* (99.04 %), *Amycolatopsis azurea* (98.96 %), *Amycolatopsis coloradensis* (98.68 %), *Amycolatopsis decaplanina* (99.10 %), *Amycolatopsis keratiniphila* subsp. *nogabecina* (99.10 %) and *Amycolatopsis lurida* (99.05 %).

The extent of DNA–DNA relatedness between isolate GY080^T and *A. orientalis* IMSNU 20058^T was estimated using the fluorometric microplate method (Ezaki *et al.*, 1989), as modified by Goris *et al.* (1998). Photobiotin-labelled DNA from the isolate was hybridized with single-stranded unlabelled DNA from *A. orientalis* IMSNU 20058^T that had been non-covalently bound to microtitre wells. The hybridization experiments were carried out under stringent conditions in 50 % formamide at 43 °C. Fluorescence intensities were measured using a Labtech F1 fluorescent plate reader (Labtech International) at a wavelength of 360 nm for excitation and 450 nm for emission. The DNA–DNA relatedness between the two strains based on triplicate samples was 42.5 ± 4.0 % (mean $\pm$ SD), a value well below the 70 % cut-off point recommended for assignment of strains to the same genomic species (Wayne *et al.*, 1987).

Isolate GY080^T was examined for key chemical markers following preparation of biomass as described by Tan *et al.* (2006a). Standard HPLC and TLC procedures were used to determine the predominant menaquinones (Collins, 1994), muramic acid residue type (Uchida *et al.*, 1999), major polar lipids (Minnikin *et al.*, 1984) and diagnostic whole-organism sugars (Schaal, 1985) of the test strain and appropriate control organisms. Strain GY080^T, which had been shown to contain *meso*-diaminopimelic acid but not

Fig. 1. Neighbour-joining tree (Saitou & Nei, 1987) based on nearly complete 16S rRNA gene sequences showing relationships between the novel isolates and representatives of the genus *Amycolatopsis*. Asterisks indicate branches of the tree that were also found using the least-squares (Fitch & Margoliash, 1967) and maximum-parsimony (Fitch, 1971) tree-making algorithms. Numbers at nodes indicate levels of bootstrap support (%) based on neighbour-joining analysis of 1000 resampled datasets. A larger phylogenetic tree showing relationships between the novel isolates and all representatives of the genus *Amycolatopsis* with validly published names is available as Supplementary Fig. S2. Bar, 0.01 substitutions per site.

mycolic acids (Tan *et al.*, 2006a), contained the following: arabinose and galactose in whole-organism hydrolysates; *N*-acetylated muramic acid; diphosphatidylglycerol, phosphatidylethanolamine (taxonomically significant phospholipid), phosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannosides and phosphatidylmethylethanolamine as major polar lipids [phospholipid pattern type II *sensu* Lechevalier *et al.* (1977)]; and tetrahydrogenated menaquinones with nine isoprene units [MK-9(H₄)] as the predominant isoprenologue (71% of total menaquinone composition), with the balance formed by MK-9(H₆). This chemical profile distinguished isolate GY080^T from members of all other wall chemotype IV actinomycetes with the exception of those classified in the genus *Amycolatopsis* (Kim & Goodfellow, 1999).

Genomic DNA was extracted from the isolates and from type strains of representative *Amycolatopsis* species with validly published names using a standard protocol (Sambrook *et al.*, 1989). Enzymically amplified 16S rRNA genes obtained by PCR (Tan *et al.*, 2006a) were analysed by restriction digestion using *EcoRV*, *HhaI*, *RsaI* and *TaqI* (MBI Fermentas). The restriction patterns of the strains were normalized and the combined patterns were examined with BIONUMERICS version 2 software (Applied Maths) using the Jaccard coefficient (*S_J*) and the unweighted pair group method with arithmetic averages (UPGMA) clustering algorithm. The isolates were assigned to a homogeneous cluster in a numerical analysis of the combined amplified rDNA restriction analysis patterns, a taxon that was distinctly separate from the *Amycolatopsis* reference strains, including the type strains of *A. japonica* and *A. orientalis* (Supplementary Fig. S1, available in IJSEM Online).

Colony and pigmentation properties of the isolates were observed following growth on modified Bennett's agar supplemented with mannitol and soybean flour and on peptone-yeast extract-iron and tyrosine agars (Shirling & Gottlieb, 1966) for 14 days at 28 °C using a Nikon Optiphot binocular light microscope fitted with long-working-distance objectives. In addition, a range of phenotypic tests known to be of value in *Amycolatopsis* systematics were carried out using established procedures (de Boer *et al.*, 1990; Goodfellow *et al.*, 1997, 2001). The isolates shared a broad range of phenotypic properties that enabled them to be distinguished from representatives of phylogenetically closely related species, including *A. japonica* and *A. orientalis* (Table 1).

While the use of secondary metabolites is not universally applicable to classification, in genera that produce highly diagnostic metabolites such as *Amycolatopsis*, their presence offers additional phenotypic support. Strains GY080^T, GY091, GY246, GY248, GY249, GY250 and GY293 were grown on modified Bennett's (Jones, 1949) and yeast extract-malt extract (ISP2; Shirling & Gottlieb, 1966) agars at 28 °C for 7 days. The agar was excised, sectioned and extracted with methanol to provide a crude

extract that was analysed by gradient HPLC. The analytical gradient HPLC system consisted of an SCL-10AVP system controller, SIL-10ADVP auto-injector, LC-10ADVP pump, FCV-10ALVP mixer, DGU-14A degasser, CTO-10AVP column oven and SPD-10AVP diode-array detector. HPLC analysis was undertaken by injection of a sample (50 µl) onto a platinum EPS C18 rocket reverse phase column (Alltech Associates Australia). The solvent gradient was 10 to 100% acetonitrile/water containing 0.01% trifluoroacetic acid at 3 ml min⁻¹ over 13 min. The electronically stored data were compiled and analysed using COMET, a metabolite recognition software package (Lacey & Tennant, 2003). This program takes the data generated by diode array detection-HPLC and creates a compact database of the chromatogram and UV spectra. The relationship between these profiles and the type strains of *Amycolatopsis* species was analysed using an in-house database comprising over 6000 type strains.

All of the strains produced a family of secondary metabolites with highly characteristic UV spectra. The presence of these metabolites was a consistent phenotypic feature of all the strains and they produced them on both media. The metabolite pattern was unique to these strains and was not shared by members of the genus *Amycolatopsis* with validly published names. Furthermore, in the analysis of over 2000 type species and published strains of actinobacteria, no cultures exhibited metabolites with comparable elution and UV spectral characteristics. The major analogue of the family of metabolites was isolated by preparative HPLC and identified as kigamicin C. The kigamicins are a family of metabolites that have been isolated and identified recently from an *Amycolatopsis* isolate (Kunimoto *et al.*, 2003).

The extracts of all strains exhibited potent antibacterial and antitumour activity due to the presence of the kigamicins. Antitumour activity was determined in a microtitre plate cell proliferation assay. Briefly, murine NS-1 cells in RPMI 1640 medium (200 µl, 5 × 10⁴ cells ml⁻¹), supplemented with 1 mM sodium pyruvate and 5% (v/v) newborn calf serum, were added to the wells of a microtitre plate containing serial twofold dilutions of the test compound. The plates were incubated at 37 °C in the presence of 5% CO₂. A qualitative assessment of cell proliferation was made at 72 h, with the LD₉₉ determined as the lowest concentration of the test compound at which no cell proliferation was observed.

Antibacterial activity was determined in an agar-based microtitre plate assay. An aliquot of an overnight fermentation of *Bacillus subtilis* ATCC 6633 was applied to the surface of an agar matrix that contained the test compound, which was then incubated at 28 °C. Qualitative assessment of bacterial growth was made at 24 h, with the MIC determined as the lowest concentration of the test compound at which no growth of bacteria was observed.

It is apparent from this polyphasic study that the seven isolates form a homogeneous taxon that can be distinguished

Table 1. Phenotypic characteristics that are useful for differentiation of *Amycolatopsis regifaucium* sp. nov. from representatives of phylogenetically closely related *Amycolatopsis* species

Strains: 1, *A. regifaucium* GY080^T, GY091, GY246, GY248, GY249, GY250 and GY293; 2, *A. alba* KCTC 9611^T; 3, *A. azurea* KCTC 9154^T; 4, *A. coloradensis* KCTC 9818^T; 5, *A. decaplanina* NRRL B-24209^T; 6, *A. japonica* KCTC 9817^T; 7, *A. keratiniphila* subsp. *keratiniphila* NRRL B-24117^T; 8, *A. keratiniphila* subsp. *nogabecina* NRRL B-24206^T; 9, *A. lurida* NRRL 2430^T (data from Mertz & Yao, 1993); 10, *A. orientalis* KCTC 9412^T. Data were obtained in this study unless indicated. +, Positive; –, negative; ND, no soluble pigment produced. All strains hydrolysed aesculin and produced acid from D-cellobiose.

Characteristic	1	2	3	4	5	6	7	8	9	10
Aerial spore mass colour	Light grey	White	Blue	White	Sand yellow	White	Light grey	Sand yellow	White	White
Colour of soluble pigment	Dark grey–brown	ND	Brown	ND	ND	Brown	ND	ND	ND	ND
Hydrolysis of:										
Allantoin	–	+	–	–	+	–	–	–	–	+
Arbutin	+	+	+	–	+	+	+	+	–	+
Urea	–	+	+	–	–	–	–	–	+	+
Nitrate reduction	+	–	+	+	+	–	+	–	+	+
H ₂ S production	+	+	+	–	+	–	+	+	+	+
Degradation of:										
Elastin	+	+	+	+	+	+	+	–	+	+
Hypoxanthine	+	+	–	+	+	+	+	+	+	+
Starch	+	+	–	+	–	+	–	–	–	+
L-Tyrosine	+	+	+	+	+	+	–	–	–	+
Tween 40	+	+	+	+	+	+	+	+	–	+
Uric acid	+	+	+	–	–	+	–	–	–	+
Xanthine	+	+	–	–	–	+	–	–	+	+
Xylan	+	+	+	+	–	+	–	–	+	+
Acid production from:										
Adonitol	–	+	+	–	–	+	+	+	+	+
L-Arabinose	+	+	+	–	+	+	+	+	+	+
D-Arabitol	+	+	+	+	+	+	+	+	–	+
Dextrin	–	–	+	+	+	+	+	+	+	+
Dulcitol	–	–	+	–	–	–	–	–	–	–
meso-Erythritol	–	+	+	–	+	+	+	–	+	+
D-Galactose	+	+	+	+	–	+	–	–	+	+
Glycogen	–	–	–	–	–	+	+	–	–	+
myo-Inositol	+	+	+	+	+	+	+	–	+	+
α-Lactose	–	+	+	–	+	+	–	+	+	–
D-Mannose	+	+	+	+	+	+	+	+	–	+
D-Melezitose	–	–	+	–	–	–	–	–	–	+
D-Melibiose	–	+	+	–	–	+	–	–	–	+
D-Raffinose	–	+	+	–	–	+	+	–	–	–
Sucrose	–	–	+	+	–	+	+	+	+	+
Turanose	–	–	–	–	+	–	+	–	–	–
Xylitol	–	–	–	–	–	–	–	–	–	–
D-Xylose	+	+	+	+	–	–	+	+	+	+
Growth at:										
10 °C	+	–	+	+	+	+	+	+	+	+
37 °C	+	–	–	–	+	+	+	+	+	+
45 °C	–	–	–	–	–	+	–	–	–	–
pH 5.0	+	+	–	–	+	+	+	+	–	–
pH 9.0	+	+	+	–	+	+	+	+	+	+
pH 10.0	+	+	+	–	+	+	+	+	+	+
Growth in presence of 5 % NaCl	+	+	+	–	–	+	+	+	+	+
Growth on:*										
Sodium citrate	+	+	–	–	–	+	–	–	–	–
Sodium oxalate	–	–	+	–	–	+	–	–	–	–
Sodium propionate	–	+	+	+	+	+	–	+	–	+

*Substrates used as sole carbon sources at 0.2 % (w/v).

from representatives of all known species of *Amycolatopsis*. It is proposed that this taxon be recognized as a novel *Amycolatopsis* species, namely *Amycolatopsis regifaucium* sp. nov.

Description of *Amycolatopsis regifaucium* sp. nov.

Amycolatopsis regifaucium (re'gi.fau'ci.um. L. n. *rex*, *regis* king; L. gen. pl. n. *faucium* of a defile; N.L. gen. pl. n. *regifaucium* of King's Canyon, Australia, the source of the soil from which the first strains were isolated).

Aerobic, Gram-positive, non-acid-alcohol-fast, non-motile, catalase-positive actinomycete that forms an extensively branched substrate mycelium that fragments into squarish, rod-like elements. Abundant, light-grey aerial hyphae and dark yellow-brown substrate mycelium with filamentous edges are formed on modified Bennett's agar supplemented with mannitol and soybean flour; a dark grey-brown diffusible pigment is produced on this medium. Melanin pigments are not formed on peptone-yeast extract-iron or tyrosine agars. Good growth occurs between 28 and 37 °C, from pH 5 to 9 and in the presence of sodium chloride (5%, w/v). Aesculin and arbutin are hydrolysed, but not urea. Nitrate is reduced to nitrite and hydrogen sulfide is produced. Grows on L-arabinose, D-arabitol, D-cellobiose, dextrin, D-galactose, D-glucose, glycerol, glycogen, *myo*-inositol, maltose, D-mannitol, methyl α -D-glucoside, D-ribose, sucrose, trehalose and xylitol as sole carbon sources for energy and growth, but not on adonitol, *meso*-erythritol, D-melezitose, D-melibiose, D-raffinose or D-sorbitol. Resistant to (μ g ml⁻¹) gentamicin sulfate (5), neomycin sulfate (8), novobiocin (10), penicillin G (20), polymyxin B sulfate (50), rifampicin (10), streptomycin sulfate (16) and tobramycin sulfate (8). Additional phenotypic properties are shown in Table 1. Chemotaxonomic characteristics are typical of members of the genus *Amycolatopsis*.

The type strain is GY080^T (=DSM 45072^T =NCIMB 14277^T), isolated from Australian arid soil.

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