Actinomadura rudentiformis sp. nov., isolated from soil

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As part of an antibiotic-screening programme, the actinomycete strain HMC1^T was isolated from soil collected from the banks of the Gamka River in the Swartberg Nature Reserve, Western Cape Province, South Africa. The isolate produced branching vegetative mycelia, which entwined to form rope-like structures. It exhibited antibiosis against *Enterococcus faecium* VanA (a vancomycin-resistant clinical strain) and *Mycobacterium aurum* A+. Organic solvent extracts of the culture filtrate and mycelial mass of strain HMC1^T exhibited moderate antibiosis against *Mycobacterium aurum* A+, *Mycobacterium bovis* BCG (Tokyo) and *Mycobacterium tuberculosis* H37Rv^T. The chemotaxonomic characteristics of strain HMC1^T corresponded with those of members of the genus *Actinomadura*. Furthermore, phylogenetic analysis based on the 16S rRNA gene showed that the strain was closely related to members of the genus *Actinomadura*. Phenotypic and 16S rRNA gene sequence analyses indicated that strain HMC1^T represents a novel species of the genus *Actinomadura*, for which the name *Actinomadura rudentiformis* sp. nov. is proposed. The type strain is HMC1^T (=DSM 44962^T=NRRL B-24458^T).

Tuberculosis is the leading cause of death from a bacterial infectious disease (Viveiros *et al.*, 2003; Sherman *et al.*, 2001; McKinney *et al.*, 2000; Kochi, 1991), killing 5000 people per day (World Health Organization, 2005). Furthermore, it is estimated that a third of the world's population is infected with the tuberculosis bacillus (Glyn Hewinson, 2005; Tran *et al.*, 2004; World Health Organization, 2004; Zahrt & Deretic, 2001; Bloom & Murray, 1992; Kochi, 1991). South Africa had an incidence of 718 tuberculosis cases per 100 000 people in the population in 2004 – the highest incidence among the 22 high-burden countries (World Health Organization, 2006). To compound the problem, it is estimated that 61 % of all tuberculosis cases are attributable to patients who are also HIV-positive (50 % in 2003; World Health Organization, 2006).

According to Goodfellow (1989), the historical starting point for the genus *Actinomadura* was in 1894 when Vincent isolated and described the causative agent of 'Madura foot disease'. He proposed the name *Streptothrix madurae*, but the combination was considered to be invalid and the strain was subsequently transferred to the genus *Nocardia* and, eventually, to the genus *Streptomyces* (Goodfellow, 1989).

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain HMC1^T is DQ285420.

An extended neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the position of strain HMC1^T amongst its phylogenetic neighbours is available as a supplementary figure in IJSEM Online.

With the introduction of cell-wall analysis, it was soon realized that the strain differed from members of the genus *Streptomyces*, and the genus *Actinomadura* was established by Lechevalier and Lechevalier in 1970 (Goodfellow, 1989; Mertz & Yao, 1986). The genus soon became a 'dumping ground' for actinomycetes containing *meso*-diaminopimelic acid and having no characteristic sugars. The use of the polyphasic approach in bacterial taxonomy and, in particular, 16S rRNA gene sequence analysis, resulted in the clarification of the taxonomy of the genus, which at the time of writing comprises 34 recognized species and two subspecies. The type species is *Actinomadura madurae* (Euzéby, 2006; Quintana *et al.*, 2003). The genus *Actinomadura* belongs to the family *Thermomonosporaceae*, which also includes *Actinocorallia*, *Spirillospora* and *Thermomonospora* (Zhang *et al.*, 1998; Trujillo & Goodfellow, 2003).

As part of a screening programme for antibiotic-producing actinomycetes, an actinomycete with unusual morphology was isolated from soil collected from the banks of the Gamka River, Swartberg Nature Reserve, Western Cape Province, South Africa.

Strain HMC1^T was isolated on MC agar (1⁻¹: 2.0 g glucose, 0.5 g NaNO₃, 0.3 g K₂HPO₄, 0.3 g KCl, 0.3 g MgSO₄·7H₂O, 0.01 g FeSO₄·7H₂O, 0.001 g CuSO₄·5H₂O, 0.001 g ZnSO₄·7H₂O, 0.001 g MnSO₄·7H₂O, 20.0 g agar; pH 7.4; Nonomura & Ohara, 1971) after the soil had been air-dried for 5 days at room temperature and then heat-treated at 120 °C for 1 h. The treated soil (0.1 g) was suspended in

1 ml sterile distilled water, vortexed for 1 min, allowed to settle, serially diluted in sterile distilled water and then spread-plated in duplicate onto MC agar. The plates were incubated at 30 °C for 21 days. Following isolation, strain HMC1^T was maintained on yeast extract-malt extract agar (ISP 2; Shirling & Gottlieb, 1966).

Antimicrobial activity was determined by using the sloppy-agar overlay technique. The isolate was stab-inoculated into Difco Middlebrook 7H9 agar (Becton Dickinson) supplemented with 10 mM glucose (albumin-dextrose-catalase supplement omitted), Czapek Solution agar (Atlas, 1993) and ISP 2 agar plates, in duplicate. The plates were incubated for 10 days at 30 °C (and the duplicate set at 37 °C) and overlaid with 6 ml Luria sloppy agar (Sambrook *et al.*, 1989) containing the test bacterium. Antimicrobial activity was tested against *Enterococcus faecium* VanA (a vancomycin-resistant clinical isolate), *Mycobacterium aurum* A+ and *Escherichia coli* ATCC 25922.

For the organic solvent extraction of antibiotics, strain HMC1^T was cultivated in 100 ml ISP 2 and 100 ml *Nocardia* medium (l⁻¹: 1.0 g glucose, 1.0 g glycerol, 1.0 g bacteriological peptone, 0.5 g beef extract; pH 7.0) (Tanaka *et al.*, 1997) for 4 days at 30 °C on a rocking shaker. The cultures were filtered separately through coffee filters (1 × 4-sized filters; House of Coffees). The two samples of mycelial mass were combined and extracted successively with methanol, chloroform and hexane. The three solvent extracts were combined and concentrated 50 times by evaporating the solvent mixture and redissolving the residue in methanol. The culture filtrates were separately extracted with ethyl acetate and the extracts were combined and concentrated 50 times. Activity was tested against a range of Gram-positive and Gram-negative bacteria by bioautography (Betina, 1973). All tests on *Mycobacterium tuberculosis* H37Rv^T were performed in a Biosafety Level 3 laboratory.

The morphological characteristics of strain HMC1^T were determined using standard methods (Locci, 1989). The isolate was grown on ISP 2 agar for 14 days at 30 °C and the morphology observed under a light microscope and by cryo-scanning electron microscopy.

Standard physiological tests (utilization of carbon sources, utilization of nitrogen sources, degradation of substrates, growth in the presence of inhibitors, and growth at different temperatures and pH values) were performed as described by Locci (1989). ISP media were prepared as described by Shirling & Gottlieb (1966). Antibiotic resistance was determined by incorporation of the antibiotics into Bennett's medium agar plates (Atlas, 1993) at the recommended concentrations (Locci, 1989). Physiological characteristics were determined after growth at 30 °C (unless otherwise stated) for the recommended incubation periods. All carbon sources used for carbon-utilization tests were filter-sterilized and tested at the concentrations recommended by Locci (1989) and Shirling & Gottlieb (1966).

Acid-fast staining (using 1% sulphuric acid for the decolourization step) and acid-alcohol-fast staining (with Ziehl-Neelsen stain) were performed using standard methods.

Freeze-dried cells for the chemotaxonomic tests were obtained from a 500 ml ISP 2 culture of strain HMC1^T, which was cultivated on a rocking shaker at 30 °C for 5 days. The diaminopimelic acid isomer and the whole-cell sugar pattern were determined using the method of Hasegawa *et al.* (1983), with the exception that freeze-dried cells were used instead of colonies from agar plates. The presence of mycolic acids was investigated by using the method of Minnikin *et al.* (1975).

The 16S rRNA gene of strain HMC1^T was amplified by PCR using the universal bacterial primers F1 and R5; the amplified DNA was then subjected to the rapid identification method of Cook & Meyers (2003). For the phylogenetic analysis, reference strains were chosen from the results of a BLAST analysis (Altschul *et al.*, 1997) of the 16S rRNA gene sequence of strain HMC1^T. For the construction of phylogenetic trees, the software packages MEGA, version 2.1 (Kumar *et al.*, 2001), and CLUSTAL_X, version 1.81 (Thompson *et al.*, 1997), were used. Unrooted phylogenetic trees were constructed using the neighbour-joining (Saitou & Nei, 1987), minimum-evolution and maximum-parsimony methods (Takahashi & Nei, 2000) and were evaluated by bootstrap resampling (1000 replications).

Under cryo-scanning electron microscopy (Fig. 1), unusual rope-like or fibre-like growth was clearly visible. This unusual morphology is similar to that reported by Mertz & Yao (1990) for *Actinomadura fibrosa* (96.68% 16S rRNA gene sequence similarity to strain HMC1^T by global alignment using DNAMAN software, version 4.13; Lynnon BioSoft). No sporangia and no spores were detected in the 14-day-old culture. The phenotypic characteristics are given in the species description.

Fig. 1. Cryo-scanning electron micrograph of strain HMC1^T grown on ISP 2 at 30 °C for 14 days. The unusual rope-like mycelium is clearly visible. Bar, 3 μm.

The rapid identification technique of Cook & Meyers (2003) placed strain HMC1^T within a group containing *Actinocorallia*, *Actinomadura*, *Saccharothrix* and *Spirillospora*. The chemotaxonomic characteristics of strain HMC1^T, however, were most consistent with those of members of the genus *Actinomadura*. meso-Diaminopimelic acid was detected in the peptidoglycan of strain HMC1^T and the whole-cell hydrolysates yielded galactose, glucose, madurose, mannose and ribose. No mycolic acids were detected in the cell wall, and the isolate was non-acid-fast and non-acid-alcohol-fast.

A 1433 bp 16S rRNA gene sequence was obtained for strain HMC1^T. A BLAST search showed that HMC1^T was most closely related to *Actinomadura fulvescens* DSM 43923^T (98.0 %), *Actinomadura hibisca* DSM 44148^T (97.0 %), *Actinomadura nitritigenes* DSM 44137^T (96.7 %), *Actinomadura fibrosa* ATCC 49459^T (96.7 %), *Spirillospora albida* NBRC 12248^T (96.4 %), *Actinomadura meyeriae* A288^T (96.2 %) and *Actinomadura viridis* DSM 43175^T (96.2 %) (note that these similarity values were calculated from pairwise global alignments using DNAMAN and are not

the BLAST-analysis figures). A neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing *Actinomadura* species, the type species of the genera *Actinocorallia* and *Spirillospora*, and strain HMC1^T (see Supplementary Fig. S1 available in IJSEM Online) showed that strain HMC1^T clustered with *Spirillospora albida*, *Actinomadura fulvescens*, *Actinomadura nitritigenes* and *Actinomadura fibrosa*. Even though *Spirillospora albida* appears to be the closest phylogenetic neighbour of strain HMC1^T (Supplementary Fig. S1), the clear difference in morphology (lack of sporangia in HMC1^T) and the low level of 16S rRNA gene sequence similarity (96.4 %) clearly differentiate the two strains from each other. Furthermore, when a smaller number of sequences were used in the construction of the phylogenetic tree (Fig. 2), strain HMC1^T clustered with *Actinomadura fulvescens* (the top BLAST hit) rather than *Spirillospora albida*. A comparison of the phenotypic characteristics of strain HMC1^T and its phylogenetic neighbours is shown in Table 1. It is clear from these data that strain HMC1^T is phenotypically different from the most closely related *Actinomadura* species and *Spirillospora albida*.

Fig. 2. Unrooted neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the position of strain HMC1^T amongst its phylogenetic neighbours. The 16S rRNA gene sequence of *Streptosporangium album* NBRC 13900^T was used as an outgroup. All sequences were edited to the longest common region (1395 bp). GenBank accession numbers are given in parentheses. Numbers at nodes show bootstrap percentages of 1000 replications. Asterisks indicate the clades that were conserved when neighbour-joining, minimum-evolution and maximum-parsimony methods were used to construct phylogenetic trees. Bar, 0.005 nucleotide substitutions per position.

Table 1. Phenotypic characteristics that differentiate strain HMC1^T from related type strains

Strains: 1, HMC1^T; 2, *Actinomadura fulvescens* (data from Trujillo & Goodfellow, 2003); 3, *Actinomadura nitritigenes* DSM 44137^T (Lipski & Altendorf, 1995); 4, *Actinomadura fibrosa* ATCC 49459^T (Mertz & Yao, 1990); 5, *Spirillospora albida* NBRC 12248^T (Wink, 2001). ND, Not determined.

Characteristic	1	2	3	4	5
Growth on ISP 2*	SM: cream (extensive wrinkling); AM: –	SM: yellow; AM: –; DP: yellow	SM: colourless; AM: brown	SM: brown; AM: white; DP: brown	SM: beige; AM: white; DP: –
Growth on ISP 4*	SM: white; AM: –	Does not grow	SM: brown; AM: white	SM: brown; AM: pink	SM: beige; AM: white
Production of sporangia	–	–	–	–	+
Degradation of:					
Gelatin	+	+	ND	ND	–
L-Tyrosine	–	–	ND	+	+
Utilization of sole carbon sources:					
(+)–D-Cellobiose	+	+	+	ND	–
<i>myo</i> -Inositol	+	ND	ND	ND	–
(–)-D-Lactose	+	33 %†	–	ND	ND
(–)-D-Mannitol	+	–	ND	ND	+
(+)–D-Melibiose	+	–	ND	ND	ND
Raffinose	+	–	ND	ND	–
(+)–L-Rhamnose	+	33 %†	+	ND	–
(+)–D-Xylose	+	+	+	ND	–
Utilization of sole nitrogen sources:					
L-Arginine	+	–	ND	ND	–
L-Phenylalanine	+	–	ND	ND	ND

*AM, aerial mycelium; DP, diffusible pigment; SM, substrate mycelium.

†Percentage of strains within the group that are positive in the test.

Strain HMC1^T degraded adenine and xylan, whereas *Actinomadura fulvescens* did not. Strain HMC1^T utilized (–)-D-mannitol, (+)-D-melibiose and raffinose as sole carbon sources, and used L-arginine and L-phenylalanine as sole nitrogen sources; none of these was used by *Actinomadura fulvescens*. The growth characteristics of these two strains on ISP 2 and ISP 4 also differed (Table 1) (information on *Actinomadura fulvescens* was obtained from Trujillo & Goodfellow, 2003).

There are five major differences between strain HMC1^T and *Actinomadura nitritigenes*, including differences in growth characteristics on ISP 2 and ISP 4; strain HMC1^T degraded adenine and xylan but not hypoxanthine, and utilized (–)-D-lactose as a sole carbon source. There are also five major differences between strain HMC1^T and *Actinomadura fibrosa*, including differences in growth characteristics on ISP 2 and ISP 4; *Actinomadura fibrosa* reduces nitrate and degrades hypoxanthine and starch, but does not degrade adenine (Mertz & Yao, 1990).

There are ten major differences between strain HMC1^T and *Spirillospora albida*, including differences in growth characteristics on ISP 2 and ISP 4: *Spirillospora albida* produces

sporangia, degrades L-tyrosine (but not gelatin), cannot utilize (+)-D-cellobiose, *myo*-inositol, raffinose, (+)-L-rhamnose and (+)-D-xylose as sole carbon sources, and cannot utilize L-arginine as a sole nitrogen source (Wink, 2001).

On the basis of the phenotypic and genotypic data presented, it is proposed that strain HMC1^T represents a novel species within the genus *Actinomadura*, for which the name *Actinomadura rudentiformis* sp. nov. is proposed.

Description of *Actinomadura rudentiformis* sp. nov.

Actinomadura rudentiformis (ru.den.ti.for'mis. L. masc. n. *rudens* rope; L. adj. suffix *-formis* -like, in the shape of; N.L. fem. adj. *rudentiformis* shaped like a rope).

During incubation for 14 days on oatmeal agar (ISP 3) supplemented with 0.1 % yeast extract, forms cream-coloured substrate mycelium, with sparse white aerial mycelium. Wrinkled, cream-coloured substrate mycelium forms on ISP 2, but no sporulation occurs. White substrate mycelium forms on inorganic salts-starch agar (ISP 4), but

no sporulation occurs. No diffusible pigment is produced on glycerol-asparagine agar (ISP 5) and melanin production on peptone-yeast extract-iron agar (ISP 6) or tyrosine agar (ISP 7) is not observed. Very strong antibiosis is exhibited against *M. aurum* A+ and weak antibiosis is shown against *Enterococcus faecium* VanA (vancomycin-resistant) in agar overlays. Bioautographic analysis of organic solvent extracts of cell mass shows moderate antibiosis against *M. aurum* A+, *Mycobacterium bovis* BCG (Tokyo) and *M. tuberculosis* H37Rv^T. Catalase-positive, oxidase-negative and Gram-positive. Grows in the presence of 0.3 % 2-phenylethanol, weakly in the presence of 0.0001 % crystal violet, but not in the presence of 4 % NaCl, 0.1 % phenol or sodium azide (0.01 %). Growth occurs in the presence of the following antibiotics ($\mu\text{g ml}^{-1}$, unless indicated otherwise): kanamycin (10), neomycin (50), oleandomycin (100), penicillin G (10 IU ml^{-1}), rifampicin (50) and tobramycin (50). Growth is not observed in the presence of the following antibiotics ($\mu\text{g ml}^{-1}$): cephaloridine (100), gentamicin (100), lincomycin (100), streptomycin (100) and vancomycin (50). Growth occurs at 30, 37 and 45 °C, but not at 4 °C or pH 4.3. Utilizes DL- α -amino-n-butyric acid, L-arginine, L-cysteine, L-histidine, L-hydroxyproline, L-methionine, L-phenylalanine, potassium nitrate, L-serine, L-threonine and L-valine as sole nitrogen sources. Utilizes adonitol, (+)-D-cellobiose, (-)-D-fructose, (+)-D-galactose, (+)-D-glucose, *myo*-inositol, inulin, (-)-D-lactose, (-)-D-mannitol, (+)-D-mannose, (+)-D-melibiose, raffinose, (+)-L-rhamnose, sucrose, trehalose and (+)-D-xylose as sole carbon sources; (+)-L-arabinose, (+)-D-melezitose, (-)-D-ribose, salicin, sodium acetate (0.1 %) and xylitol are weakly utilized. Sodium citrate (0.1 %) is not utilized. H₂S production and nitrate reduction do not occur. Lecithinase, lipase and protease activities are not observed on egg-yolk agar. Hippurate is hydrolysed, but pectin is not. Degrades adenine, aesculin, arbutin, casein, cellulose, gelatin, Tween 80 and xylan; guanine and starch are weakly degraded. Allantoin, hypoxanthine, L-tyrosine, urea and xanthine are not degraded.

The type strain, HMC1^T (=DSM 44962^T=NRRL B-24458^T), was isolated from soil collected from the banks of the Gamka River in the Swartberg Nature Reserve, Western Cape Province, South Africa.

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