Micromonospora tulbaghia sp. nov., isolated from the leaves of wild garlic, *Tulbaghia violacea*

Bronwyn M. Kirby† and Paul R. Meyers

Correspondence

Paul R. Meyers

paul.meyers@uct.ac.za

Department of Molecular and Cell Biology, University of Cape Town, Private Bag X3, Rondebosch 7701, Cape Town, South Africa

A novel actinomycete, strain TVU1^T, was isolated from leaves of the indigenous South African plant *Tulbaghia violacea*. Applying a polyphasic approach, the isolate was identified as a member of the genus *Micromonospora*. Phylogenetic analysis of the 16S rRNA gene sequence showed that strain TVU1^T was most closely related to *Micromonospora echinospora* DSM 43816^T. However, phylogenetic analysis based on *gyrB* gene sequences showed that strain TVU1^T was most closely related to the type strains of *Micromonospora aurantiaca* and *Micromonospora chalybeata*. DNA–DNA relatedness values between strain TVU1^T and the type strains of *M. echinospora*, *M. aurantiaca* and *M. chalybeata* were 7.6 ± 4.5 , 45.9 ± 2.0 and 60.9 ± 4.5 %, respectively. Strain TVU1^T could be distinguished from the type strains of all three of these species by several physiological characteristics, such as colony colour, NaCl tolerance, growth temperature range and sole carbon source utilization pattern. Strain TVU1^T (=DSM 45142^T=NRRL B-24576^T) therefore represents a novel species for which the name *Micromonospora tulbaghia* sp. nov. is proposed.

The genus *Micromonospora* was proposed by Ørskov (1923) and is the type genus of the family *Micromonosporaceae* Krasil'nikov 1938 emend. Stackebrandt *et al.* 1997 (Kawamoto, 1989). Distinguishing features of members of the genus *Micromonospora* include the formation of single, non-motile spores on the vegetative mycelium, which does not fragment, and the absence of aerial mycelium (Kawamoto, 1989). Although the first antibacterial activity by a member of this genus was reported in 1942, it was the isolation of the aminoglycoside gentamicin from *Micromonospora purpurea* (reclassified as *Micromonospora echinospora* by Kasai *et al.*, 2000) in 1963 that sparked the widespread screening of this genus for antibiotics (Wagman & Weinstein, 1980). After *Streptomyces*, the genus *Micromonospora* is probably the second most prolific producer of antibiotics (Wagman & Weinstein, 1980). *Micromonosporae* appear to be widely distributed in nature and have been isolated from a number of diverse sources, including a Thai peat swamp forest (Thawai *et al.*, 2005), water samples (Trujillo *et al.*, 2005) and Antarctic sandstone

(Hirsch *et al.*, 2004). Four recently described species are plant endophytes: *Micromonospora coriariae* was isolated from root nodules of the Mediterranean shrub *Coriaria myrtifolia* (Trujillo *et al.*, 2006); *Micromonospora lupini* and *Micromonospora saelicesensis* were isolated from root nodules of the Mediterranean shrub *Lupinus angustifolius* (Trujillo *et al.*, 2007); and *Micromonospora pisi* was isolated from root nodules of a pea plant, *Pisum sativum* (Garcia *et al.*, 2010).

The Cape Floral Kingdom hosts more than 9000 plant species. Even though over 3000 of these indigenous plant species have ethnobotanical applications (Van Wyk *et al.*, 1997), indigenous South African plants have not been systematically screened for novel endophytic micro-organisms. A novel actinomycete, TVU1^T, was isolated from leaves of the bulbous plant *Tulbaghia violacea*, which is a member of the onion family, *Alliaceae* (Manning, 2003), during a screening programme of indigenous plants.

Leaves from *T. violacea* (wild garlic) were collected from an indigenous suburban garden in Wellington, approximately 60 km from Cape Town. The leaves were placed in sterile plastic bags, stored at 4 °C and processed within 24 h of collection. The identity of the plant species was confirmed from Joffe (1993). Leaves were initially surface-sterilized by placing them in 70 % ethanol for 1 min, then soaking them in 1 % (v/v) NaOCl for 3 min, followed by rinsing twice in sterile distilled water. The surface-sterilized leaves were cut into 1 cm segments with sterile flamed scissors (Okazaki, 2003) and placed in a test tube containing 20 ml quarter-strength sterile phosphate buffer [full-strength phosphate

†Present address: Institute for Microbial Biotechnology and Metagenomics, University of the Western Cape, Private Bag X17, Bellville 7535, Cape Town, South Africa.

The GenBank/EMBL/DBJ accession numbers for the 16S rRNA and *gyrB* gene sequences of *Micromonospora tulbaghia* sp. nov. TVU1^T are EU196562 and EU434806, respectively.

Unrooted phylogenetic trees based on 16S rRNA gene sequences of TVU1^T and all species in the genus *Micromonospora* and the *gyrB* gene sequences of selected species in the genus *Micromonospora* are available as supplementary figures with the online version of this paper.

buffer (0.05 M) contained 5.62 g K_2HPO_4 and 2.13 g KH_2PO_4 , distilled water to 1 l, adjusted to pH 7, autoclaved (Demain & Davies, 1999)], vortexed briefly and incubated at 30 °C for 30 min with agitation. The phosphate buffer leaf extract was serially diluted with sterile distilled water and a 100 µl aliquot of each dilution was spread-plated onto plant extract agar (400 ml plant extract, 8.5 g glucose, 4.5 g yeast extract and 17.5 g agar, water to 1 l, pH 6.6–6.8, autoclaved). The plant extract was prepared by placing 160 g cut-up *T. violacea* plant leaves in 1.2 l distilled water, bringing it to the boil and simmering for 30 min. The liquid was cooled, filtered through two coffee filters (House of Coffees, size 1 × 6; Perco) and stored in a sterile glass bottle at –20 °C (method adapted from Demain & Davies, 1999). The isolation medium was supplemented with nalidixic acid (10 µg ml^{–1}) and cycloheximide (50 µg ml^{–1}). The isolation plates were incubated at 28 °C for 14 days. Isolates were selected based on morphology and subcultured onto plant extract agar (without antibiotics) and incubated at 30 °C for 7 days.

Morphological and physiological testing was performed according to the methods of Shirling & Gottlieb (1966) and Williams *et al.* (1989). Unless stated otherwise, all plates were incubated at 30 °C for the recommended periods. The colours of the substrate mycelium and spore mass, as well as the production of diffusible pigments, were determined on yeast extract-malt extract agar (International *Streptomyces* Project medium no. 2, ISP 2; Shirling & Gottlieb, 1966). Utilization of carbon sources was tested according to the method of Shirling & Gottlieb (1966). All carbon sources were filter-sterilized and tested at 1 % (w/v). Salt tolerance was tested on ISP 2; plates were incubated at 30 °C for 14 days. Antibiotic susceptibility was tested by incorporating the filter-sterilized antibiotics into sterile Bennett's medium (Atlas, 1993) to the final concentrations stated in the species description.

To test growth on potato slices, one small potato was cut into 1 × 1 cm blocks and half of the blocks were placed in a glass bottle and autoclaved. The remaining blocks were placed in a bottle containing approximately 5 g $CaCO_3$, shaken to mix and then autoclaved. Potato slices were placed in sterile Petri dishes and inoculated with 50 µl spore suspension. Plates were sealed with Parafilm to prevent dehydration of the potato slices and incubated at 28 °C for 14 days.

Growth under anaerobic conditions was tested on carbon utilization agar (ISP 9; Shirling & Gottlieb, 1966) with glucose as the sole carbon source and on ATCC medium 172 (<http://www.lgcstandards-atcc.org/Attachments/2915.pdf>) with the exception that the N-Z amine type A was substituted with Casitone (Difco). When the plates were initially placed in the anaerobic chamber, they were each opened for 5 s to ensure that all residual O_2 was removed before being sealed in a plastic bag. Plates were incubated anaerobically at 25 °C for 21 days (model 1024 anaerobic chamber; Forma Scientific) in an atmosphere comprising 5 % H_2 : 10 % CO_2 : 85 % N_2 .

For scanning electron microscopy, cultures were grown on ISP 2 agar for 14 days at 28 °C. Agar blocks containing sporulating vegetative mycelium were cut from the growth medium and frozen in liquid nitrogen prior to viewing by cryo-scanning electron microscopy.

Antimicrobial activity against *Enterococcus faecium* (VanA, clinical isolate), *Escherichia coli* ATCC 25922, *Mycobacterium aurum* A+ and *Staphylococcus aureus* ATCC 25923 was assessed on 11-day-old stab-inoculated actinomycete cultures by standard agar overlays. Strain TVU1^T was stab-inoculated into ISP 2 agar, Czapek's solution agar (Atlas, 1993) and Difco Middlebrook 7H9 agar (Becton Dickinson) supplemented with 10 mM glucose (ADC supplement omitted) and incubated at 30 °C for 11 days.

For chemotaxonomic analysis, the isolate was grown in 100 ml ISP 2 broth for 5 days at 30 °C with shaking. The cell mass was collected by centrifugation, washed twice with 100 ml sterile distilled water and freeze-dried. For diamino-pimelic acid analysis, samples were prepared according to the method of Stanek & Roberts (1974) and analysed according to the method of Hasegawa *et al.* (1983). A modified version of the Hasegawa *et al.* (1983) method was used for sugar analysis. The solvent system used for sugar analysis was ethyl acetate/pyridine/water (100:35:25, by vol.).

Genomic DNA was extracted as described previously (Wood *et al.*, 2007). 16S rRNA gene amplification was performed as described by Cook & Meyers (2003). For rapid genus identification, the resulting amplicons were restricted singly with *Mbo*I (isoschizomer of *Sau*3AI), *Vsp*I (isoschizomer of *Asn*I), *Sph*I, *Sna*BI and *Sal*I according to the method of Cook & Meyers (2003). The amplified 16S rRNA gene was purified with an MSB Spin PCRapace kit (Invitex). Sequencing reactions were performed using a Big Dye terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and analysed on an Applied Biosystems 3130 Genetic Analyzer. Local alignments were obtained by performing a standard nucleotide-nucleotide BLAST search (BLASTN) (Altschul *et al.*, 1997) of GenBank. For phylogenetic analysis, sequences were aligned using CLUSTAL_X version 1.81 (Thompson *et al.*, 1997). Neighbour-joining (Saitou & Nei, 1987), minimum-evolution (Rzhetsky & Nei, 1993) and maximum-parsimony (Fitch, 1971) trees were constructed using MEGA version 3.1 (Kumar *et al.*, 2004). DNA–DNA hybridization was performed by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) GmbH Identification Service (Braunschweig, Germany) using the spectrophotometric method of De Ley *et al.* (1970), incorporating the modifications described by Huß *et al.* (1983). The results reported are the mean values from two hybridization experiments ± standard deviation.

The *gyrB* gene was amplified as two overlapping fragments (covering a total of approximately 1.5 kb) using primers 7G-*gyrB*-F (le Roes *et al.*, 2008) + KgyrB-R and GgyrB-F2 + 7G-*gyrB*-R (Everest & Meyers, 2009). Primer KgyrB-R

(5'-CGATCCGGGCCTTCTCGACGTTTCAG-3') was based on the partial *gyrB* sequences from *Kribbella alba* DSM 15500^T (EU434820), *Kribbella antibiotica* DSM 15501^T (EU434819) and *Kribbella solani* CIP 108508^T (EU434813); primer GgyrB-F2 (5'-CAGTTCGAGGGHCAGACSAAGAC-3') was modified from primer GYRBF2 of Shen *et al.* (2006). To ensure that none of the primers would amplify the *gyrB* paralogue (*parE*), all primers were aligned with the *parE* gene sequences from *Corynebacterium glutamicum* ATCC 13032^T (BA000036), *Streptomyces avermitilis* MA-4680^T (BA000030) and *Streptomyces griseus* subsp. *griseus* NBRC 13350 (AP009493). PCR was carried out in 50 µl reaction volumes. Each reaction contained 4 mM MgCl₂, 0.1 U SuperTherm *Taq* polymerase (JMR Holdings), 150 µM of each dNTP, 0.5 µM of each primer and 500–1000 ng template DNA. The PCR programme used was an initial denaturation at 96 °C for 2 min, followed by 30 cycles of denaturation (96 °C for 45 s), annealing (56 °C for 30 s) and extension (72 °C for 2 min), and a final extension at 72 °C for 5 min. PCR products were electrophoresed on 1% agarose gels containing 0.8 µg ethidium bromide ml⁻¹ and visualized on a GelDoc XR System (Bio-Rad). The resulting amplicons were purified and sequenced as described above.

According to the rapid genus identification method of Cook & Meyers (2003), the isolate was identified as belonging to the genus *Micromonospora*. A standard 16S rRNA gene BLASTN search of GenBank showed that strain TVU1^T (1435 bp) was most closely related to *M. echinospora* ATCC 15837^T (99.4% similarity), but the phylogenetic tree showed that there was low bootstrap support (42%) for this association (Fig. 1; a phylogenetic tree containing all recognized species of the genus *Micromonospora* is shown in Supplementary Fig. S1, available in IJSEM Online). The 16S rRNA gene sequence similarities between strain TVU1^T and *Micromonospora chalicea* NBRC 13503^T, *Micromonospora endolithica* DSM

44398^T and *Micromonospora rosaria* DSM 803^T were 99.28, 99.20 and 99.06%, respectively. Sequence similarities to all other type strains of species of the genus *Micromonospora* were <99%. DNA–DNA hybridization studies showed that the DNA–DNA relatedness between strain TVU1^T and *M. echinospora* DSM 43816^T was 7.6 ± 4.5%. Surprisingly, phylogenetic analysis of the *gyrB* gene showed that strain TVU1^T was most closely related to *Micromonospora aurantiaca* NBRC 16155^T and *M. chalicea* NBRC 13503^T, supported by a bootstrap value of 100% (Fig. 2). The overall topologies of the 16S rRNA gene and *gyrB* gene trees were different, with only the close relationship between *M. aurantiaca* NBRC 16155^T and *M. chalicea* NBRC 13503^T being maintained. As the overall topology of a tree can be affected by the number of sequences included in the analysis, a second 16S rRNA gene tree (Supplementary Fig. S2, available in IJSEM Online) was constructed using only those strains included in the *gyrB* gene sequence analysis shown in Fig. 2. The topology of this smaller 16S rRNA gene tree was similar to the 16S rRNA gene tree that included all type strains of the genus *Micromonospora*, thus indicating that the change in the closest relatives of strain TVU1^T between the 16S rRNA and *gyrB* gene trees was not a technical effect (i.e. not due to the number of sequences used in the analysis). This suggests that there has been horizontal transfer of the *gyrB* and/or 16S rRNA genes between members of the genus *Micromonospora*. A previous study by Kasai *et al.* (2000) found that within the genus *Micromonospora*, phylogenetic trees based on *gyrB* gene sequences were different from those based on the 16S rRNA gene. They determined that a *gyrB*-based genetic distance of 0.014 would correspond to 70% DNA–DNA relatedness and that the *gyrB* gene was superior at inferring intragenomic relationships (Kasai *et al.*, 2000). However, if there has been horizontal transfer of the *gyrB* gene in this genus, the conclusion

Fig. 1. Unrooted 16S rRNA gene-based phylogenetic tree showing the relationship between strain TVU1^T and its closest phylogenetic neighbours. The phylogenetic tree is based on 1398 bp and was constructed using the neighbour-joining method. Bootstrap values are based on 1000 resampled datasets and only values >40% are shown. Bar, 0.001 nt substitutions per nucleotide site. The hash (#) denotes the cluster that was conserved using the neighbour-joining (Saitou & Nei, 1987), minimum-evolution (Rzhetsky & Nei, 1993) and maximum-parsimony (Fitch, 1971) methods to construct the phylogenetic trees. The asterisk (*) denotes the cluster that was conserved using the neighbour-joining and minimum-evolution methods to construct the phylogenetic trees.

Fig. 2. Unrooted *gyrB* gene phylogenetic tree showing the relationship between strain TVU1^T and all species of the genus *Micromonospora* with published *gyrB* gene sequences. The phylogenetic tree is based on 935 bp and was constructed using the neighbour-joining method. Bootstrap values are based on 1000 resampled datasets and only values >40% are shown. Bar, 0.01 nt substitutions per nucleotide site. The hash (#) denotes the cluster that was conserved using the neighbour-joining (Saitou & Nei, 1987), minimum-evolution (Rzhetsky & Nei, 1993) and maximum-parsimony (Fitch, 1971) methods to construct the phylogenetic trees.

that the *gyrB* gene is more useful for determining inter-species relationships than the 16S rRNA gene must be questioned. Indeed, it seems that multilocus sequence analysis of the entire genus will be necessary to gauge the extent of horizontal gene transfer in this genus and to assess the relationships between the recognized species. The *gyrB*-based genetic distance, determined using Kimura's two-parameter model (Kimura, 1980), between strain TVU1^T and *M. aurantiaca* NBRC 16155^T was 0.022, whereas the distance between strain TVU1^T and *M. chalcea* NBRC 13503^T was 0.034 (i.e. well above the 0.014 threshold and therefore indicating that the DNA–DNA relatedness between these pairs of strains would be expected to be <70%). To determine whether strain TVU1^T does indeed represent a distinct genomic species, DNA–DNA hybridization studies were performed between strain TVU1^T and the type strains of *M. aurantiaca* and *M. chalcea*. The DNA–DNA relatedness values of strain TVU1^T with *M. aurantiaca* DSM 43813^T and *M. chalcea* DSM 43026^T were 45.9 ± 2.0 and 60.9 ± 4.5 %, respectively. These findings clearly distinguish strain TVU1^T as a separate genomic species.

Strain TVU1^T was characterized by the presence of *meso*-diaminopimelic acid as the diagnostic diaminoacid of the cell-wall peptidoglycan and by the presence of glucose, ribose and xylose in its whole-cell hydrolysates. Morphologically, strain TVU1^T was characterized by the formation of medium brown vegetative mycelium on ISP 2 agar. The colonies became dark brown upon sporulation. Strain TVU1^T formed spores with smooth surfaces, which were on sporophores borne on the branching hyphae (Fig. 3). Selected physiological features that distinguish strain TVU1^T from *M. aurantiaca* NBRC 16155^T, *M. chalcea*

NBRC 13503^T, *M. echinospora* DSM 43816^T and *M. endolithica* DSM 44398^T are shown in Table 1. Additional physiological characteristics are reported in the species description.

Description of *Micromonospora tulbaghiaie* sp. nov.

Micromonospora tulbaghiaie (tul.ba.ghi'ae. N.L. gen. fem. n. *tulbaghiaie* of/from *Tulbaghia*, the genus name of *Tulbaghia violacea*, the host of the organism).

Gram-stain-positive. Aerial mycelium is not formed. Substrate mycelium is medium brown on ISP 2 agar. Colonies become dark brown upon sporulation. Diffusible pigments are not produced. Grows on ISP 2 agar in the presence of 5% (w/v) NaCl. Degrades casein, gelatin, starch and Tween 80. H₂S is produced, but nitrate is not reduced. Grows at 4–37 °C, but not at 45 °C. Growth only

Fig. 3. Scanning electron micrograph of cells of strain TVU1^T grown on ISP 2 for 14 days at 28 °C. Bar, 1 μm.

Table 1. Selected phenotypic characteristics that distinguish strain TVU1^T from related species of the genus *Micromonospora*

Strains: 1, *Micromonospora tulbaghia* sp. nov. TVU1^T (data from this study); 2, *M. echinospora* DSM 43816^T (data from Kawamoto, 1989); 3, *M. aurantiaca* ATCC 27029^T (Thawai *et al.*, 2004); 4, *M. chalicea* IFO 13503^T (Kawamoto, 1989); 5, *M. endolithica* DSM 44398^T (Hirsch *et al.*, 2004). ND, Not determined; +, growth or positive reaction; w, weak; –, no growth or negative reaction; v, variable results.

Characteristic	1	2	3	4	5
NaCl tolerance (% w/v)	5	2*	4	5	7
Growth on potato	+	–	ND	+	ND
Growth on potato + CaCO ₃	–	–	ND	ND	ND
H ₂ S production	+	–	ND	ND	–
Nitrate reduction	–	–	+	v	–
Starch hydrolysis	+	+	–	+	+
Degradation of:					
L-Tyrosine	–	–	+	–	+
Xylan	w	–*	ND	ND	–
Growth temperature (°C)	4–37	27–37	12–40	27–45†	8–39
Colony colour	Yellow–brown	Maroon–purple	ND	Red–orange†	Orange to olive
Colour of diffusible pigment	None	Purple	ND	Yellow	None
Spore colour	Dark brown	Brown–black	ND	ND	Black
Utilization as sole carbon source:					
(+)–L-Arabinose	–	+	w	w	+
(–)-D-Fructose	+	–	+	w	+
Glycerol	–	–	–	–	ND
<i>myo</i> -Inositol	w	–	ND	–	–
Lactose	–	–	–	+	+
(–)-D-Mannitol	+	–	–	–	w
Melibiose	–	–	+	+	+
Raffinose	–	–	w	+	+
(+)–L-Rhamnose	+	+	–	–	–
(–)-D-Ribose	+	–	–	–	–
(+)–D-Xylose	–	+	+	+	+
Resistance to:					
Lincomycin hydrochloride (100 µg ml ^{–1})	+	–	ND	ND	ND
Rifampicin (50 µg ml ^{–1})	+	–	ND	ND	ND

*Results determined in this study.

†Data from Horan & Brodsky (1986).

occurs at pH 7–9 on ISP 2. (–)-D-Fructose, (+)-D-glucose, *myo*-inositol (weakly), (–)-D-mannitol, (+)-L-rhamnose and (–)-D-ribose are utilized as sole carbon sources. (+)-L-Arabinose, cellobiose, glycerol, (–)-D-lactose, melibiose, raffinose, salicin and (+)-D-xylose are not utilized. Susceptible to (ml^{–1}) cephaloridine (100 µg) and vancomycin hydrochloride (50 µg), but resistant to kanamycin (10 µg), lincomycin hydrochloride (100 µg), oleandomycin phosphate (100 µg), penicillin G (10 U) and rifampicin (50 µg). Utilizes (NH₄)₂HPO₄, L-cysteine, gelatin, L-glutamine, L-histidine, L-isoleucine, L-leucine, L-methionine and L-valine as sole nitrogen sources. Does not utilize DL- α -alanine, β -alanine, DL- α -amino-*n*-butyric acid, L-arginine, L-aspartic acid, L-glutamic acid, L-hydroxyproline, D-leucine, DL- β -phenylalanine, KNO₃, L-proline, NaNO₃ or L-serine as sole nitrogen sources. Weak growth occurs on ATCC 172 medium under anaerobic conditions

over 21 days at 25 °C; no growth occurs on ISP 9 under anaerobic conditions. Moderate antibiosis is observed against *Mycobacterium aurum* A+.

The type strain, TVU1^T (=DSM 45142^T=NRRL B-24576^T), was isolated from leaves of wild garlic, *Tulbaghia violacea*.

Acknowledgements

The authors wish to thank Di James for DNA sequencing and Miranda Waldron of the Electron Microscope Unit, University of Cape Town (UCT), for assistance with scanning electron microscopy. We thank Professor Hans G. Trüper for his assistance in deriving the specific name for the isolate and Associate Professors Shez Reid and Val Abratt for use of the anaerobic chamber. B. K. held a Scarce Skills Scholarship from the National Research Foundation (NRF) and a UCT Postgraduate Research Associateship. This work was supported

by research grants to P. R. M. from the Medical Research Council of South Africa, the NRF (grant number 2073133) and the University Research Committee (UCT).

References

- Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.
- Atlas, R. M. (1993). *Handbook of Microbiological Media*. Edited by L. C. Parks. Boca Raton, FL: CRC Press.
- Cook, A. E. & Meyers, P. R. (2003). Rapid identification of filamentous actinomycetes to the genus level using genus-specific 16S rRNA gene restriction fragment patterns. *Int J Syst Evol Microbiol* **53**, 1907–1915.
- De Ley, J., Cattoir, H. & Reynaerts, A. (1970). The quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem* **12**, 133–142.
- Demain, A. L. & Davies, J. E. (editors) (1999). *Manual of Industrial Microbiology and Biotechnology*, 2nd edn. Washington, DC: American Society for Microbiology.
- Everest, G. J. & Meyers, P. R. (2009). The use of *gyrB* sequence analysis in the phylogeny of the genus *Amycolatopsis*. *Antonie van Leeuwenhoek* **95**, 1–11.
- Fitch, W. M. (1971). Toward defining the course of evolution: minimum change for a specific tree topology. *Syst Zool* **20**, 406–416.
- Garcia, L. C., Martínez-Molina, E. & Trujillo, M. E. (2010). *Micromonospora pisi* sp. nov., isolated from root nodules of *Pisum sativum*. *Int J Syst Evol Microbiol* **60**, 331–337.
- Hasegawa, T., Takizawa, M. & Tanida, S. (1983). A rapid analysis for chemical grouping of aerobic actinomycetes. *J Gen Appl Microbiol* **29**, 319–322.
- Hirsch, P., Mevs, U., Kroppenstedt, R. M., Schumann, P. & Stackebrandt, E. (2004). Cryptoendolithic actinomycetes from Antarctic sandstone rock samples: *Micromonospora endolithica* sp. nov. and two isolates related to *Micromonospora coerulea* Jensen 1932. *Syst Appl Microbiol* **27**, 166–174.
- Horan, A. C. & Brodsky, B. C. (1986). *Micromonospora rosaria* sp. nov., nom. rev., the rosaramicin producer. *Int J Syst Bacteriol* **36**, 478–480.
- Huß, V. A. R., Festl, H. & Schleifer, K. H. (1983). Studies on the spectrophotometric determination of DNA hybridization from denaturation rates. *Syst Appl Microbiol* **4**, 184–192.
- Joffe, P. (1993). *The Gardener's Guide to South African Plants*. Cape Town, South Africa: Tafelberg.
- Kasai, H., Tamura, T. & Harayama, S. (2000). Intrageneric relationships among *Micromonospora* species deduced from *gyrB*-based phylogeny and DNA relatedness. *Int J Syst Evol Microbiol* **50**, 127–134.
- Kawamoto, I. (1989). Genus *Micromonospora* Ørskov 1923, 147^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 4, pp. 2442–2450. Edited by S. T. Williams, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins.
- Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.
- Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* **5**, 150–163.
- le Roes, M., Goodwin, C. M. & Meyers, P. R. (2008). *Gordonia lacunae* sp. nov., isolated from an estuary. *Syst Appl Microbiol* **31**, 17–23.
- Manning, J. (2003). *Photographic Guide to the Wildflowers of South Africa*. Pretoria, South Africa: Briza Publications.
- Okazaki, T. (2003). Studies on actinomycetes isolated from plant leaves. In *Selective Isolation of Rare Actinomycetes*, chapter 4. Edited by I. Kurtböke. Queensland, Australia: University of the Sunshine Coast.
- Ørskov, J. (1923). *Investigations into the Morphology of the Ray Fungi*. Copenhagen: Levin and Munksgaard.
- Rzhetsky, A. & Nei, M. (1993). Theoretical foundation of the minimum-evolution method of phylogenetic inference. *Mol Biol Evol* **10**, 1073–1095.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Shen, F.-T., Lu, H.-L., Lin, J.-L., Huang, W.-S., Arun, A. B. & Young, C.-C. (2006). Phylogenetic analysis of members of the metabolically diverse genus *Gordonia* based on proteins encoding the *gyrB* gene. *Res Microbiol* **157**, 367–375.
- Shirling, E. B. & Gottlieb, D. (1966). Methods for characterization of *Streptomyces* species. *Int J Syst Bacteriol* **16**, 313–340.
- Staneck, J. L. & Roberts, G. D. (1974). Simplified approach to identification of aerobic actinomycetes by thin-layer chromatography. *Appl Microbiol* **28**, 226–231.
- Thawai, C., Tanasupawat, S., Itoh, T., Suwanborirux, K. & Kudo, T. (2004). *Micromonospora aurantionigra* sp. nov., isolated from a peat swamp forest in Thailand. *Actinomycetologica* **18**, 8–14.
- Thawai, C., Tanasupawat, S., Itoh, T., Suwanborirux, K. & Kudo, T. (2005). *Micromonospora siamensis* sp. nov., isolated from Thai peat swamp forest. *J Gen Appl Microbiol* **51**, 229–234.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.
- Trujillo, M. E., Fernández-Molinero, C., Velázquez, E., Kroppenstedt, R. M., Schumann, P., Mateos, P. F. & Martínez-Molina, E. (2005). *Micromonospora miobrigensis* sp. nov. *Int J Syst Evol Microbiol* **55**, 877–880.
- Trujillo, M. E., Kroppenstedt, R. M., Schumann, P., Carro, L. & Martínez-Molina, E. (2006). *Micromonospora coriariae* sp. nov., isolated from root nodules of *Coriaria myrtifolia*. *Int J Syst Evol Microbiol* **56**, 2381–2385.
- Trujillo, M. E., Kroppenstedt, R. M., Fernández-Molinero, C., Schumann, P. & Martínez-Molina, E. (2007). *Micromonospora lupini* sp. nov., and *Micromonospora saelicesensis* sp. nov., isolated from root nodules of *Lupinus angustifolius*. *Int J Syst Evol Microbiol* **57**, 2799–2804.
- Van Wyk, B.-E., Van Oudtshoorn, B. & Gericke, N. (1997). *Medicinal Plants of South Africa*. Pretoria, South Africa: Briza Publications.
- Wagman, G. H. & Weinstein, M. J. (1980). Antibiotic from *Micromonospora*. *Annu Rev Microbiol* **34**, 537–557.
- Williams, S. T., Goodfellow, M. & Alderson, G. (1989). Genus *Streptomyces* Waksman and Henrici 1943, 339^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 4, pp. 2452–2492. Edited by S. T. Williams, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins.
- Wood, S. A., Kirby, B. M., Goodwin, C. M., le Roes, M. & Meyers, P. R. (2007). PCR screening reveals unexpected antibiotic biosynthetic potential in *Amycolatopsis* sp. strain UM16. *J Appl Microbiol* **102**, 245–253.