Streptomyces durmitorensis sp. nov., a producer of an FK506-like immunosuppressant

Miloje Savic,[†] Ivana Bratic[‡] and Branka Vasiljevic

Correspondence
Branka Vasiljevic
vasiljb@eunet.yu

Institute of Molecular Genetics and Genetic Engineering, Vojvode Stepe 444a, PO Box 23, 11 010 Belgrade, Serbia

Screening of soil samples from the Durmitor National Park, Serbia and Montenegro, for strains producing immunosuppressants with a similar mechanism of action to FK506 resulted in the isolation of the actinomycete strain MS405^T. Isolate MS405^T was found to have morphological and phenotypic properties that were consistent with its classification as a *Streptomyces* strain. The DNA G + C content of strain MS405^T was 72 mol%. 16S rRNA gene sequence data confirmed the taxonomic position of the strain, following the generation of phylogenetic trees by using various treeing algorithms. On the basis of 16S rRNA gene sequence similarity, strain MS405^T was shown to belong to the *Streptomyces albidoflavus* 'supercluster', being related to *Streptomyces aureus* DSM 41785^T (99.59 % similarity) and *Streptomyces kanamyceticus* DSM 40500^T (99.32 %). The 16S–23S rRNA internally transcribed spacer (ITS) region exhibited variations in length and sequence composition, showing limited usefulness in phylogenetic analyses. However, DNA relatedness values support the classification of this isolate within a novel species. A number of physiological and biochemical tests distinguished strain MS405^T from its closest phylogenetic neighbours. Therefore, strain MS405^T represents a novel species, for which the name *Streptomyces durmitorensis* sp. nov. is proposed, with the type strain MS405^T (=DSM 41863^T =CIP 108995^T).

Actinomycetes are distributed in terrestrial environments and have been a source of useful bioactive molecules. By conventional isolation methods, members of the genus *Streptomyces* comprise more than 95 % of the filamentous actinomycete population of soil (Elander, 1987). Therefore, an organized taxonomic system to identify novel strains is needed in order to preclude reisolation of already known species. The 16S rRNA gene sequence is highly conserved within living cells and has been widely used for evolutionary studies in bacteria (Woese, 1987), placing organisms in the framework of phylogenetic relationships (Stackebrandt *et al.*, 1997). However, 16S rRNA gene

sequences may be insufficient to define phylogenetic relationships among closely related species and among strains belonging to a species because of evolutionary conservation of the 16S rRNA gene (Woese, 1987). It has been suggested that the 16S–23S rRNA internally transcribed spacer (ITS) region is a powerful tool for phylogenetic analysis of Gram-negative bacteria, but not of Gram-positive bacteria, especially *Streptomyces* species (Gurtler & Stanisich, 1996; Hain *et al.*, 1997; Song *et al.*, 2004). To overcome these problems, it has now become common practice to delineate novel *Streptomyces* species using a combination of genotypic and phenotypic data (Kim *et al.*, 1998, 2000; Sembiring *et al.*, 2000) in a so-called polyphasic taxonomic study, which is expected to lead to well-described species and a stable nomenclature (Goodfellow *et al.*, 1997).

Actinomycete strain MS405^T was isolated by a serial dilution method (<http://www.bio.com/protocolstools/protocol.jhtml?id=p2181>) from soil samples collected at the Durmitor National Park, Serbia and Montenegro, as a producer of a secondary metabolite exhibiting an FK506-like immunosuppressant mechanism of action (Skoko *et al.*, 2005). The taxonomic status of strain MS405^T was investigated using a combination of phenotypic and molecular systematic means, which were indispensable in placing strain MS405^T within the genus *Streptomyces*. Polyphasic study of strain MS405^T showed that this strain

[†]Present address: Faculty of Life Sciences, University of Manchester, Manchester Interdisciplinary Biocentre, 131 Princess Street, Manchester M1 7DN, UK.

[‡]Present address: Division of Metabolic Diseases, Department of Laboratory Medicine, Karolinska Institute, Novum, SE-14186 Stockholm, Sweden.

Abbreviations: DAP, diaminopimelic acid; ITS, internally transcribed spacer.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence of strain MS405^T and the 16S–23S rDNA ITS region sequences of eight clones of strain MS405^T are respectively DQ067287 and DQ067288–DQ067295.

Sequences of ITS variable regions in strain MS405^T and electron micrographs of the strain are available as supplementary material with the online version of this paper.

should be formally recognized as representing a novel species of the genus *Streptomyces*.

To investigate the phylogenetic relationships of strain MS405^T, its almost-complete 16S rRNA gene sequence (1517 nt) was determined. Bacterial DNA was extracted by a method described previously (Hopwood *et al.*, 1985). The extracted DNA was subjected to PCR amplification with the bacteria-specific 16S rRNA primers 27f (Lane, 1991) and 1392rev (Marchesi *et al.*, 1998). PCR amplification was performed as described by Marchesi *et al.* (1998). The 16S–23S ITS region, including the 3' end of the 16S rRNA gene, was amplified by PCR using primers AM45 (Mehling *et al.*, 1995) and L1 (Jensen *et al.*, 1993). PCR products were excised from the gel and purified using a QIAEXII gel extraction kit (Qiagen) according to the manufacturer's instructions. The purified product was then ligated to pMOSBlue vector according to the manufacturer's instructions (Amersham Pharmacia Biotech). Recombinant plasmid constructs were isolated using Qiagen minicolumns (QIAprep Spin Miniprep kit) and sequenced on an ALF Express sequencer using a Cy5-AutoRead kit (Amersham Biosciences) and the universal sequencing primers M13f and M13r.

16S rRNA gene sequence analysis was conducted using the BLAST network services provided by the NCBI (Altschul *et al.*, 1997) and the Ribosomal Database Project (RDP; <http://rdp.cme.msu.edu>) (Maidak *et al.*, 2001). Alignment was performed with the CLUSTAL W program (Thompson *et al.*, 1994). 16S rRNA gene sequences and 16S–23S ITS sequences were aligned against published sequences available in the DDBJ/GenBank/EMBL databases by the K_{nuc} value of Kimura (1980), and phylogenetic trees were constructed by the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971; Felsenstein, 1993) algorithms contained in the PHYLIP package (version 3.5c; <http://www.pasteur.fr>). The topology of the phylogenetic tree was evaluated by the bootstrap resampling method of Felsenstein (1985) with 1000 replicates. 16S rRNA gene sequence similarities were calculated using the EMBOSS:needle (global) method available at <http://www.ebi.ac.uk>.

Phylogenetic analysis, at the RDP (Maidak *et al.*, 2001), placed the strain within the evolutionary radiation encompassed by the genus *Streptomyces*, where the 16S rRNA gene sequence of *Streptomyces aureus* DSM 41785^T was identified with the highest probability (0.969) as the closest matching sequence to MS405^T. The analysis was supported by the neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1993) methods. Comparing *Streptomyces* 16S rRNA gene sequences by phylogenetic tree algorithms, it was obvious that a subclade was formed (Fig. 1) containing sequences of *Streptomyces seoulensis* IMSNU 21266^T, *S. aureus* DSM 41785^T, *Streptomyces kanamyceticus* DSM 40500^T and strain MS405^T. Within this subclade, strain MS405^T formed a monophyletic line. This relationship was evident in evolutionary trees based on

Fig. 1. Phylogenetic tree derived from 16S rRNA gene sequences showing the relationship of strain MS405^T and related *Streptomyces* species. Asterisks indicate branches that were recovered using the maximum-parsimony algorithm (Fitch, 1971; Felsenstein, 1993); black dots indicate branches recovered using the maximum-likelihood (Felsenstein, 1993) and neighbour-joining (Saitou & Nei, 1987) algorithms at the RDP server. The phylogenetic tree based on BIONJ algorithm (Gascuel, 1997) had the same topology as the tree shown here (data not shown). Numbers at nodes exhibit levels of bootstrap support based on neighbour-joining analyses of 1000 resampled datasets; only values above 600 are given. Accession numbers are given in parentheses. Bar, 0.002 nucleotide substitutions per position.

different treeing algorithms: neighbour-joining and DNADIST contained in the PHYLIP package (Felsenstein, 1993), the maximum-parsimony algorithm (Fitch, 1971; Felsenstein, 1993), the maximum-likelihood (Felsenstein, 1993) and BIONJ (Gascuel, 1997) (Fig. 1). The 16S rRNA gene sequence similarity values were 99.59 and 99.32 % between strain MS405^T and *S. aureus* DSM 41785^T and *S. kanamyceticus* DSM 40500^T, over 1448 and 1477 nucleotides compared, respectively.

The high degree of sequence divergence of the 16S–23S ITS region among streptomycetes has been shown to be of limited usefulness during phylogenetic positioning of strains (Song *et al.*, 2004), though the utility of this region for inferring phylogenetic relationships has been demonstrated for *Streptomyces albidoflavus* strains (Hain *et al.*, 1997). Sequencing of the 16S–23S ITS region of strain MS405^T, from eight randomly selected clones, revealed length and sequence composition heterogeneity. The analysed sequences were 283 to 303 nt in length. Within the sequenced spacer regions, five variable regions and six conserved regions were identified. Highly conserved regions C1 to C6 were respectively 14, 99, 12, 11, 9 and 53 nucleotides long. For each of the variable regions V1–V5, more than one sequence was found (see Supplementary Table S1 in IJSEM Online). Consistent with previous observations for other streptomycetes, no tRNA-like sequences were found in any of the 16S–23S ITS regions.

Phylogenetic analyses based on the 16S–23S ITS regions placed strain MS405^T close to *Streptomyces scabiei* isolate 87.79 (clone 79) (Fig. 2), showing the limited usefulness of the 16S–23S ITS region in inferring the phylogenetic position of strain MS405^T. The branching of MS405^T 16S–23S ITS sequences in two closely related clades is probably due to indel events contained within sequences ITS2, ITS4, ITS6 and ITS7 that are absent in the other four sequences.

DNA relatedness analysis was performed with strain MS405^T and the type strains *S. aureus* DSM 41785^T and *S. kanamyceticus* DSM 40500^T by the method of De Ley *et al.* (1970), under consideration of the modifications described by Huß *et al.* (1983), using a 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). Levels of DNA relatedness between strain MS405^T and the type strains of two closely related species (based on the phylogenetic data), *S. aureus* DSM 41785^T and *S. kanamyceticus* DSM 40500^T, were respectively 15.5 and 13.3 %. These values clearly indicate that isolate MS405^T does not belong to the species *S. aureus* or *S. kanamyceticus*, as these values are well below the threshold value of 70 % for definition of bacterial species according to Wayne *et al.* (1987). Additionally, DNA relatedness values below 80 % have been recommended for the recognition of novel genomic species of *Streptomyces* (Labeda, 1993, 1996, 1998).

The morphological characteristics of strain MS405^T were assessed by transmission (model CM12; Philips) and scanning (model JSM-6460; JEOL) electron microscopy of 14-day-old cultures grown on NE medium at 30 °C (Skeggs *et al.*, 1985), following the procedure of O'Donnell

et al. (1993). Cultural characteristics of strain MS405^T were recorded after 14 days incubation according to the International *Streptomyces* Project (ISP) methods (Shirling & Gottlieb, 1966). Colours were described according to the NBS-ISCC Color System (<http://www.anthus.com>).

Morphological observation of 14-day-old cultures grown on NE agar revealed aerial mycelium which consisted of straight chains of 10 or more rod-shaped, smooth-surfaced spores (0.5–0.9 × 1.0–1.5 µm) (Supplementary Fig. S1). The colour of the substrate mycelium was greenish grey on glycerol-asparagine agar (ISP5), where no diffusible pigment was detected, and medium grey on inorganic salts-starch agar (ISP4). Melanin was not produced on peptone-yeast extract-iron agar (ISP6) or tyrosine agar (ISP7) plates. Soluble dark-grey and dark-olive-green pigments were observed on ISP4 and NE, respectively. The phenotypic characteristics of strain MS405^T and phylogenetically related *Streptomyces* species are shown in Table 1. Biochemical, physiological and polymer degradation tests were performed as described previously (Seeley & VanDemark 1981; Hopwood *et al.*, 1985; Williams *et al.*, 1983).

Sensitivity to antibiotics was tested by growing colonies on NE plates with linear antibiotic gradients ranging from 0 to 100 µg ml⁻¹ for 7 days at 30 °C. Detailed physiological and biochemical characteristics of strain MS405^T are given in the species description.

The isomer type of diaminopimelic acid (DAP) in the peptidoglycan layer was determined by hydrolysing cells with 6 M HCl at 100 °C for 18 h and analysed by TLC as described previously (Rhuland *et al.*, 1955; Becker *et al.*, 1965; Hasegawa *et al.*, 1983), followed by ninhydrin staining (0.1 % w/v ninhydrin in acetone). Analysis showed that the cell wall contains LL-DAP, thereby indicating that strain MS405^T exhibits cell-wall chemotype I (Lechevalier & Lechevalier, 1970). Utilization of sugars or sugar alcohols as sole carbon sources was monitored in minimal medium containing M9 salts (6 g Na₂HPO₄, 3 g K₂HPO₄, 0.5 g NaCl, 1 g NH₄Cl per litre, filter-sterilized) supplemented with 1 ml 1 M MgSO₄, 1 ml 0.1 M CaCl₂ and 1 % carbon source (w/v) (Miller, 1992). Plates were incubated for 7 days at 30 °C. All media were adjusted to pH 7 prior to seeding.

The chemical, morphological and phylogenetic data suggest that strain MS405^T represents a novel species when compared with type strains of species with validly published names within the genus *Streptomyces*. Thus, on the basis of this polyphasic taxonomic study, strain MS405^T merits classification as the type strain of a novel species within the genus *Streptomyces*, and the name *Streptomyces durmitorensis* sp. nov. is proposed.

Description of *Streptomyces durmitorensis* sp. nov.

Streptomyces durmitorensis (dur.mi.tor.en'sis. N.L. masc. adj. *durmitorensis* pertaining to Durmitor, Serbia and Montenegro, where the type strain was isolated).

Fig. 2. Unrooted neighbour-joining tree based on 16S–23S ITS region sequences. Bootstrap values indicated at branching points were based on neighbour-joining analyses of 1000 resampled datasets (only values above 600 are shown). Asterisks indicate branches that were recovered using the maximum-parsimony algorithm. Accession numbers are given in parentheses. Bar, 0.02 nucleotide substitutions per position.

Table 1. Phenotypic characteristics that differentiate strain MS405^T from its closest neighbours

Strains: 1, MS405^T (*S. durmitorensis* sp. nov.); 2, *S. aureus* DSM 41785^T (data from Atalan *et al.*, 2000; Manfio *et al.*, 2003); 3, *S. kanamyceticus* DSM 40500^T (Umezawa *et al.*, 1957); 4, *S. seoulensis* IMSNU 21266^T (Chun *et al.*, 1997). All strains are positive for growth on 1 % (w/v) D-(+)-mannitol.

Characteristic	1	2	3	4
Colour of aerial spore mass on ISP5	Greenish yellow	Grey	Colourless to yellow	Grey
Colour of soluble pigment on:				
ISP4	Dark grey	Reddish orange	Faint brown	—
ISP3	—	Golden	Yellowish pink	—
Growth on sole carbon sources (1 % w/v)				
Dextran	+	—	—	—
<i>myo</i> -Inositol	—	+	—	—
D-Lactose	—	+	—	+
Growth on sole nitrogen sources (0.1 % w/v)				
L-Histidine	+	—	+	+
L-Hydroxyproline	+	+	+	—
Degradation of xanthine	+	+	+	—
Nitrate reduction	+	+	+	—
Maximum NaCl concentration for growth (% w/v)	9	7	7	7
Growth in the presence of thallos acetate (0.001 % w/v)	+	+	—	—
Antibiosis against <i>Micrococcus luteus</i> NCIMB 196	+	—	+	—

Gram-positive, non-acid-fast streptomycete that produces a yellowish-grey and a greenish-grey substrate mycelium and a greenish-yellow aerial spore mass on yeast extract-malt extract and glycerol-asparagine agars. Soluble pigments are not formed on oatmeal, yeast extract-malt extract or glycerol-asparagine agars, while dark-grey pigment is formed on inorganic salts-starch agar. Melanoid pigments are not formed on peptone/yeast extract/iron or tyrosine agars. Spore chains are *rectiflexibiles*, with 10 or more rod-shaped, smooth-surfaced spores (0.5–0.9 × 1.0–1.5 µm) per chain. Temperature range for growth is 10–37 °C with an optimum between 28 and 32 °C. The cell wall contains LL-DAP. Cellobiose, dextran, D-(–)-fructose, D-(+)-galactose, D-(+)-glucose, glycerol, D-(–)-mannitol, D-(+)-mannose, α-melibiose, D-(+)-raffinose, L-(+)-rhamnose, sucrose, α-trehalose and D-(+)-xylose are utilized for growth, but L-(+)-arabinose, *myo*-inositol, α-lactose, β-lactose, maltose and D-sorbitol are not utilized. Acid is produced from glucose. L-Alanine, L-arginine, L-cysteine, L-glycine, L-histidine, L-hydroxyproline, L-methionine, L-proline and L-valine are utilized as sole nitrogen sources, but L-asparagine, L-lysine, ornithine hydrochloride, L-phenylalanine and thiamine are not utilized. Nitrate is reduced to nitrite, gelatin is liquefied, xanthine is degraded, starch is not hydrolysed and aesculin and DNA are not degraded. Positive for nitrate reduction, catalase, extracellular protease, haemolysin (β-haemolysis) and urease. Negative for lecithinase and H₂S and indole are not produced. Grows in the presence of NaCl (9 %, w/v) and thallos acetate (0.001 %, w/v), but not sodium azide (0.01 %, w/v), phenol (0.1 %, w/v) or potassium tellurite (0.001 %, w/v). Susceptible to apramycin (10 µg ml^{–1}), kanamycin (5 µg ml^{–1}), gentamicin (5 µg ml^{–1}), tetracycline (10 µg ml^{–1}), thiostrepton (10 µg ml^{–1}), chloramphenicol (35 µg ml^{–1}) and spectinomycin (90 µg

ml^{–1}), but resistant to ampicillin (100 µg ml^{–1}), erythromycin (100 µg ml^{–1}) and FK506 (100 µg ml^{–1}). The type strain, MS405^T, produces an immunosuppressant with an FK506-like mechanism of action. The G + C content of the DNA is 72 mol%. The type strain shows antimicrobial activity against *Micrococcus luteus* NCIMB 196 and *Saccharomyces cerevisiae* FAV 20, but not against *Bacillus subtilis* NCIMB 3610^T, *Candida albicans* CBS 562, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Saccharomyces cerevisiae* FAS 20 or *Staphylococcus aureus* ATCC 25923.

The type strain, MS405^T (=DSM 41863^T =CIP 108995^T), was isolated from a soil sample taken at the Durmitor National Park, Serbia and Montenegro.

Acknowledgements

This work was supported by grant 143056 from the Ministry of Science of the Republic of Serbia. We would like to thank Dr Graeme Conn for carefully reading and correcting the manuscript, Dr Aleksandra Korac for help with electron microscopy and Mr Dusan Mistic and Lidija Djokic for help with physiological tests. We thank the DSMZ staff for their technical assistance with DNA relatedness analysis.

References

- Altschul, S. F., Madden, T. L., Schaffer, 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.
- Atalan, E., Manfio, G. P., Ward, A. C., Kroppenstedt, R. M. & Goodfellow, M. (2000). Biosystematic studies on novel streptomycetes from soil. *Antonie van Leeuwenhoek* **77**, 337–353.

- Becker, B., Lechevalier, M. P. & Lechevalier, H. A. (1965). Chemical composition of cell-wall preparations from strains of various formgenera of aerobic actinomycetes. *Appl Microbiol* **13**, 236–243.
- Chun, J., Youn, H. D., Yim, Y. I., Lee, H., Kim, M. Y., Hah, Y. C. & Kang, S. O. (1997). *Streptomyces seoulensis* sp. nov. *Int J Syst Bacteriol* **47**, 492–498.
- De Ley, J., Cattoir, H. & Reynaerts, A. (1970). The quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem* **12**, 133–142.
- Elander, R. P. (1987). Microbial screening, selection and strain improvement. In *Basic Biotechnology*, pp. 217–251. Edited by J. Bu'Lock & B. Kristiansen. London: Academic Press.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Felsenstein, J. (1993). PHYLIP (phylogenetic inference package), version 3.5c. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle, USA.
- Fitch, W. M. (1971). Toward defining the course of evolution: minimum change for a specific tree topology. *Syst Zool* **20**, 406–416.
- Gascuel, O. (1997). BIONJ: an improved version of the NJ algorithm based on a simple model of sequence data. *Mol Biol Evol* **14**, 685–695.
- Goodfellow, M., Manfio, G. P. & Chun, J. (1997). Towards a practical species concept for cultivable bacteria. In *Species: The Units of Diversity*, pp. 25–29. Edited by M. F. Claridge, H. A. Dawah & M. R. Wilson. London: Chapman & Hall.
- Gurtler, V. & Stanisich, V. A. (1996). New approaches to typing and identification of bacteria using the 16S–23S rDNA spacer region. *Microbiology* **142**, 3–16.
- Hain, T., Ward-Rainey, N., Kroppenstedt, R. M., Stackebrandt, E. & Rainey, F. A. (1997). Discrimination of *Streptomyces albidoflavus* strains based on the size and number of 16S–23S ribosomal DNA intergenic spacers. *Int J Syst Bacteriol* **47**, 202–206.
- Hasegawa, T., Takizawa, M. & Tanida, S. (1983). A rapid analysis for chemical grouping of aerobic actinomycetes. *J Gen Appl Microbiol* **29**, 319–322.
- Hopwood, D. A., Bibb, M. J., Chater, K. F., Kieser, T., Bruton, C. J., Kieser, H. M., Lydiate, C. P., Smith, C. P., Wards, J. M. & Shrempf, H. (1985). *Genetic Manipulation of Streptomyces: a Laboratory Manual*. Norwich: John Innes Foundation.
- Huß, V. A. R., Festl, H. & Schleifer, K. H. (1983). Studies on the spectrophotometric determination of DNA hybridisation from renaturation rates. *Syst Appl Microbiol* **4**, 184–192.
- Jensen, M. A., Webster, A. J. & Straus, N. (1993). Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. *Appl Environ Microbiol* **59**, 945–952.
- Kim, S. B., Falconer, C., Williams, E. & Goodfellow, M. (1998). *Streptomyces thermocarboxydovorans* sp. nov. and *Streptomyces thermocarboxydis* sp. nov., two moderately thermophilic carboxydophilic species from soil. *Int J Syst Bacteriol* **48**, 59–68.
- Kim, B., al-Tai, A. M., Kim, S. B., Somasundaram, P. & Goodfellow, M. (2000). *Streptomyces thermocrophilus* sp. nov., a cellulase-free endoxylanase-producing streptomycete. *Int J Syst Evol Microbiol* **50**, 505–509.
- 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.
- Labeda, D. P. (1993). DNA relatedness among strains of the *Streptomyces lavendulae* phenotypic cluster group. *Int J Syst Bacteriol* **43**, 822–825.
- Labeda, D. P. (1996). DNA relatedness among verticil-forming *Streptomyces* species (formerly *Streptoverticillium* species). *Int J Syst Bacteriol* **46**, 699–703.
- Labeda, D. P. (1998). DNA relatedness among the *Streptomyces fulvissimus* and *Streptomyces griseoviridis* phenotypic cluster groups. *Int J Syst Bacteriol* **48**, 829–832.
- Lane, D. J. (1991). 16S/23S rRNA sequencing. In: *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115–175. Edited by E. Stackebrandt & M. Goodfellow. Chichester: Wiley.
- Lechevalier, M. P. & Lechevalier, H. (1970). Chemical composition as a criterion in the classification of aerobic actinomycetes. *Int J Syst Bacteriol* **20**, 435–443.
- Maidak, B. L., Cole, J. R., Lilburn, T. G., Parker, C. T., Jr, Saxman, P. R., Farris, R. J., Garrity, G. M., Olsen, G. J., Schmidt, T. M. & Tiedje, J. M. (2001). The RDP-II (Ribosomal Database Project). *Nucleic Acids Res* **29**, 173–174.
- Manfio, G. P., Atalan, E., Zakrewska-Czerwinska, J., Mordarski, M., Rodriguez, C., Collins, M. D. & Goodfellow, M. (2003). Classification of novel soil streptomycetes as *Streptomyces aureus* sp. nov., *Streptomyces laceyi* sp. nov. and *Streptomyces sanglieri* sp. nov. *Antonie van Leeuwenhoek* **83**, 245–255.
- Marchesi, J. R., Sato, T., Weightman, A. J., Martin, T. A., Fry, J. C., Hiom, S. J., Dymock, D. & Wade, W. G. (1998). Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Appl Environ Microbiol* **64**, 795–799.
- Mehling, A., Wehmeier, F. U. & Piepersberg, W. (1995). Nucleotide sequences of streptomycete 16S ribosomal DNA: towards a specific identification system for streptomycetes using PCR. *Microbiology* **141**, 2139–2147.
- Miller, J. H. (1992). *A Short Course in Bacterial Genetics: a Laboratory Manual and Handbook for Escherichia coli and Related Bacteria*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- O'Donnell, A. G., Falconer, C., Goodfellow, M., Ward, A. C. & Williams, E. (1993). Biosystematics and diversity amongst novel carboxydophilic *Actinomycetes*. *Antonie van Leeuwenhoek* **64**, 325–340.
- Rhuland, L. E., Work, E., Denman, R. F. & Hoare, D. S. (1955). The behavior of the isomers of α,ϵ -diaminopimelic acid on paper chromatograms. *J Am Chem Soc* **77**, 4844–4846.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Seeley, H. W., Jr & VanDemark, P. J. (1981). *Microbes in Action. A Laboratory Manual of Microbiology*, 3rd edn. San Francisco: W. H. Freeman.
- Sembiring, L., Ward, A. C. & Goodfellow, M. (2000). Selective isolation and characterisation of members of the *Streptomyces violaceusniger* clade associated with the roots of *Paraserianthes falcata*. *Antonie van Leeuwenhoek* **78**, 353–366.
- Shirling, E. B. & Gottlieb, D. (1966). Methods for characterization of *Streptomyces* species. *Int J Syst Bacteriol* **16**, 313–340.
- Skeggs, P. A., Thompson, J. & Cundliffe, E. (1985). Methylation of 16S ribosomal RNA and resistance to aminoglycoside antibiotics in clones of *Streptomyces lividans* carrying DNA from *Streptomyces tenjimaniensis*. *Mol Gen Genet* **200**, 415–421.
- Skoko, N., Vujovic, J., Savic, M., Papic, N., Vasiljevic, B. & Ljubijankic, G. (2005). Construction of *Saccharomyces cerevisiae* strain FAV20 useful in detection of immunosuppressants produced by soil actinomycetes. *J Microbiol Methods* **61**, 137–140.
- Song, J., Lee, S.-C., Kang, J.-W., Baek, H.-J. & Suh, J.-W. (2004). Phylogenetic analysis of *Streptomyces* spp. isolated from potato scab lesions in Korea on the basis of 16S rRNA gene and 16S–23S rDNA

intergenic transcribed spacer sequences. *Int J Syst Evol Microbiol* **54**, 203–209.

Stackebrandt, E., Rainey, F. A. & Ward-Rainey, N. L. (1997). Proposal for a new hierarchic classification system, *Actinobacteria* classis nov. *Int J Syst Bacteriol* **47**, 479–491.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

Umezawa, H., Ueda, M., Maeda, K., Yagishita, K., Kondo, S., Okami, Y., Utagahara, R., Osato, Y., Nitta, K. & Takeuchi, T. (1957). Production and

isolation of a new antibiotic: kanamycin. *J Antibiot (Tokyo)* **10**, 181–188.

Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E. & other authors (1987). Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.

Williams, S. T., Goodfellow, M., Alderson, G., Wellington, E. M. H., Sneath, P. H. A. & Sackin, M. J. (1983). Numerical classification of *Streptomyces* and related genera. *J Gen Microbiol* **129**, 1743–1813.

Woese, C. R. (1987). Bacterial evolution. *Microbiol Rev* **51**, 221–271.