Mycobacterium seoulense sp. nov., a slowly growing scotochromogenic species

Ho-Suk Mun,[1] Hyun-Ju Kim,[1] Eun-Ju Oh,[1] Hong Kim,[1] Gil-Han Bai,[2] Hee-Kyung Yu,[2] Young-Gil Park,[2] Chang-Yong Cha,[1] Yoon-Hoh Kook[1] and Bum-Joon Kim[1]

Correspondence
Bum-Joon Kim
kbumjoon@snu.ac.kr

[1]Department of Microbiology and Immunology, Cancer Research Institute and Liver Research Institute, College of Medicine, Seoul National University, Seoul 110-799, Korea

[2]The Korean Institute of Tuberculosis, The Korean National Tuberculosis Association, Seoul 137-140, Korea

A previously undescribed, slowly growing, scotochromogenic mycobacterium was isolated from a patient with symptomatic pulmonary infection during *hsp65* sequence-based identification of Korean clinical isolates. Phenetic characteristics of this strain were generally similar to those of *Mycobacterium nebraskense* and *Mycobacterium scrofulaceum*. However, some phenetic characteristics differentiated it from these two species. Its 16S rRNA gene sequences were unique and phylogenetic analysis based on 16S rRNA gene sequences placed the organism in the slowly growing *Mycobacterium* group close to *M. nebraskense* and *M. scrofulaceum*. Its unique mycolic acid profiles and the results of phylogenetic analysis based on two independent alternative chronometer molecules, *hsp65* and *rpoB*, confirmed the taxonomic status of this strain as representing a novel species. These data support the conclusion that this strain represents a novel mycobacterial species, for which the name *Mycobacterium seoulense* sp. nov. is proposed. The type strain is strain 03-19[T] (=DSM 44998[T]=KCTC 19146[T]).

Mycobacteria are widely distributed in the environment; some are pathogenic to humans and animals and, of these, a number are saprophytes. Recently, the application of molecular techniques to the taxonomy and identification of isolates from environmental sources and clinical specimens has led to an increased awareness of the diversity within the genus *Mycobacterium* (Kirschner *et al.*, 1993). Conventional biochemical tests have been performed extensively to detect and identify *Mycobacterium tuberculosis* and clinically important non-tuberculous mycobacteria (Goodfellow & Magee, 1998; Wayne & Kubica, 1986). However, because of the increasing number of newly defined taxa and the recognition of 'difficult-to-identify' variants of known species, tests sometimes fail to provide precise identification (Kirschner *et al.*, 1993). To overcome the limitations of conventional methods, sequencing methods targeting several chronometer molecules have been developed (Kim *et al.*, 1999, 2005; Roth *et al.*, 1998; Stahl & Urbance, 1990;

Stone *et al.*, 1995). Moreover, the combination of molecular assays and conventional methods provides conclusive identification of infrequently encountered species and allows the delimitation of novel taxa. In the present study, a novel non-tuberculous mycobacterial species was isolated from a patient with general symptoms of pulmonary infection. The organism was discovered during an exercise designed to identify Korean slowly growing mycobacteria (SGM) clinical isolates using the recently developed *hsp65* sequencing method (Kim *et al.*, 2005).

The subject strain of the present study, strain 03-19[T], was one of the 'difficult-to-identify' isolates submitted to the Korean Institute of Tuberculosis by mycobacteriology laboratories in Korea during 2003. This strain was isolated from sputum samples of a 52-year-old female who had been experiencing general pulmonary symptoms. The same strain was isolated from sputum specimens obtained from this patient on three successive occasions; no other mycobacterium was observed.

The phenetic characteristics of strain 03-19[T] and eight mycobacteria reference strains were analysed and compared (Table 1). Colony morphology, pigment production in the dark, photoinduction and the ability to grow at temperatures ranging from 25 to 45 °C were examined during a 6 week incubation on Lowenstein–Jensen (LJ) medium and Middlebrook 7H10 agar. Acid–alcohol-fastness was

Table 1. Cultural and biochemical characteristics that differentiate 03-19[T] from other closely related SGM species

Strains: 1, strain 03-19[T]; 2, *M. nebraskense* ATCC BAA-837[T]; 3, *M. scrofulaceum* ATCC 19981[T]; 4, *Mycobacterium avium* ATCC 25291[T]; 5, *Mycobacterium terrae* ATCC 15755[T]; 6, *M. kansasii* ATCC 12478[T]; 7, *Mycobacterium phlei* ATCC 11758[T]; 8, *Mycobacterium gordonae* ATCC 14470[T]; 9, *Mycobacterium triviale* ATCC 23292[T]. +++, Highest level of growth; ++, higher level of growth; +, positive result or modest growth; −, negative result or no growth; ±, variable. All strains showed negative results in niacin and EMB tests and positive results in catalase and pyrazinamidase tests.

Characteristic	1	2	3	4	5	6	7	8	9
Growth at:									
25 °C	+++	++	+++	++	+++	+++	++	+++	+++
37 °C	+++	++	+++	+++	+++	+++	+++	+++	+++
45 °C	−	−	−	+	−	−	+++	−	−
Growth rate									
<7 days	−	−	−	−	±	−	+	−	−
>7 days	+	+	+	+	++	+	++	+	+
Morphology*	SO	SO	SY	SWY	IWY	IW	RWY	SY	RW
Pigmentation†	S	S	S	N	N	P	N	S	N
Nitrate reductase	+	+	−	−	+	+	+	−	+
Arylsulfatase									
3 days	−	−	−	−	−	±	−	−	+
14 days	−	−	−	−	−	+	−	−	+
Tellurite reductase	+	±	±	±	+	+	+	−	−
Tween hydrolysis									
<5 days	−	−	−	−	+	+	±	+	+
<10 days	−	−	−	+	+	+	+	+	+
Urease	−	−	+	−	−	+	+	−	−
Growth on:									
TCH (10 mg ml^{-1})	+++	−	+++	++	+++	+++	++	+++	+++
PNB (500 mg ml^{-1})	++	−	++	++	+++	+++	+++	+++	+++
5 % NaCl	−	−	−	−	−	−	+	−	+++
MacConkey agar	−	−	−	−	−	−	+	−	++
Picric acid	−	−	−	−	−	−	+	−	−

*I, Intermediate in roughness; R, rough; S, smooth; O, orange; W, white; Y, yellow.

†N, Non-photochromogenic; P, photochromogenic; S, scotochromogenic.

determined by Ziehl–Neelsen and auramine O staining. The following biochemical features (Kent & Kubica, 1985) were investigated: niacin accumulation, nitrate reductase, arylsulfatase on days 3 and 14, heat-stable catalase (pH 7, 68 °C), tellurite reductase, Tween 80 hydrolysis, urease and pyrazinamidase. Inhibition tests included tolerance of thiophene-2-carboxylic acid hydrazide (TCH), *p*-nitrobenzoate (PNB), 5 % sodium chloride, ethambutol (EMB) and picric acid and ability to grow on MacConkey agar without crystal violet.

HPLC was used to analyse mycolic acids from strain 03-19[T] and the most phenotypically similar strain, *Mycobacterium nebraskense* ATCC BAA-837[T], as described by Butler *et al.* (1992) or as described in the guidelines of the CDC National Center for HIV, STD, and TB Prevention Division of Tuberculosis Elimination (http://www.cdc.gov/nchstp/tb/Laboratory_Services/Liquid_Chroma.htm). Low- and high-molecular-mass standards (Ribi ImmunoChem) were added for peak identification. To identify and quantify mycolic

acids and assign these to *Mycobacterium* species based on mycolic acid patterns, the Microbial Identification system (MIDI Inc.) was used.

Chromosomal DNA for molecular taxonomy was extracted using the bead-beater phenol extraction method as reported previously (Kim *et al.*, 2005). Purified DNA was used as a template for PCR amplifications of three independent genes, the 16S rRNA gene, *hsp65* (encoding heat-shock protein 65) and *rpoB* (encoding a subunit of RNA polymerase). The nearly complete 16S rRNA gene sequence (1523 bp) and partial sequences of *hsp65* (644 bp) and *rpoB* (352 bp) were amplified as described previously (Springer *et al.*, 1996; Kim *et al.*, 1999, 2005). PCR amplicons of all target genes were cloned directly using Topo TA cloning kits (Invitrogen) and sequenced (Kim *et al.*, 2005). To obtain sequence information on the *rpoB* and *hsp65* genes of *M. nebraskense*, which were not available in GenBank, these sequences were also analysed from *M. nebraskense* ATCC BAA-837[T], purchased from the ATCC. The 16S rRNA gene sequence of 03-19[T]

obtained in the present study was compared with sequences from GenBank using the BLAST analysis program (http://www.ncbi.nlm.nih.gov/blast/).

Multiple alignments of sequences of the three genes of 03-19[T] and reference strains of a wide range of both slowly and rapidly growing mycobacteria were created using the multiple-alignment algorithm in MEGALIGN as described previously (Kim *et al.*, 1999, 2005). All three trees were inferred by neighbour joining (Saitou & Nei, 1987) and maximum parsimony (Fitch, 1971) using *Tsukamurella paurometabola* strain NCTC 10741 (16S rRNA gene) or strain KCTC 9821[T] (*hsp65*) or *Rhodococcus equi* ATCC 10146[T] (*rpoB*) as an outgroup. Evolutionary distance matrices were generated according to the model described by Jukes & Cantor (1969). The neighbour-joining and maximum-parsimony methods were carried out using MEGA version 2.1 (Kumar *et al.*, 2001) and the resulting trees and topologies were evaluated by bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings.

Acid-fast microscopy showed generally rod-shaped and frequently bent acid-fast bacilli. Occasional coccid forms were noted. Spores and filaments were not present. The optimal growth temperature was 37 °C. No growth was observed at 45 °C. On Middlebrook 7H10 medium, mature growth developed in 3 weeks at 25 and 37 °C. Microcolonies developed in 2 weeks at the same temperature. However, 4 weeks or more was required for mature colonies to form on LJ medium. Colonies grown on Middlebrook 7H10 agar were usually larger, smooth, occasionally rough, and always orange in appearance under both dark and photoinduction conditions. Cells on LJ medium were film-like and produced an orange pigment. No growth was observed on MacConkey agar, after the addition of 5 % NaCl to the culture medium or after adding 5 mg EMB ml^{-1} or picric acid to the medium. However, strain 03-19[T] showed tolerance against 10 mg TCH ml^{-1} and against 500 mg PNB ml^{-1}. The strain was negative for urease activity, arylsulfatase, niacin accumulation and Tween 80 hydrolysis and positive for nitrate reductase, heat-stable catalase, pyrazinamidase and tellurite reductase. Generally, the biochemical profile of 03-19[T] was most like those of *M. nebraskense*, another scotochromogenic species. However, since a difference was found between these two species in terms of tolerance against TCH and PNB, such tests might be used to differentiate the two taxa. Cultural and biochemical characteristics that differentiate 03-19[T] from other closely related SGM species are shown in Table 1.

In HPLC analysis of mycolic acids, the profile of strain 03-19[T] showed two clusters of peaks that did not overlap with any previously reported mycobacterial profile. The closest match was with the *Mycobacterium avium/intracellulare/scrofulaceum* complex, with a similarity index of 0.052. In a comparison with the mycolic acids of *M. nebraskense* ATCC BAA-837[T] (Fig. 1), differences were found in the relative heights of peaks as follows: peak 1, 2.70 % (strain 03-19[T]) and 10.45 % (*M. nebraskense* ATCC BAA-837[T]); peak 2, 1.53
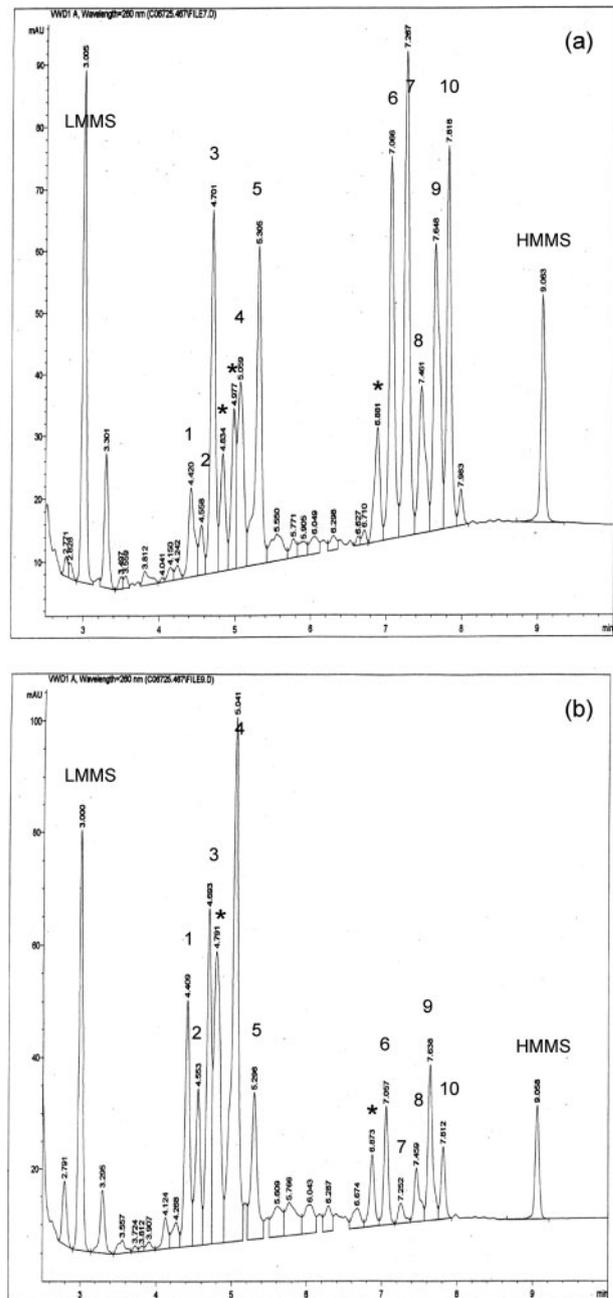

Fig. 1. Mycolic acid patterns of strain 03-19[T] (a) and *M. nebraskense* ATCC BAA-837[T] (b) obtained by HPLC analysis. The relative retention time is indicated for each peak. LMMS, Low-molecular-mass standard; HMMS, high-molecular-mass standard. Asterisks (*) indicate peaks specific to either 03-19[T] or *M. nebraskense* ATCC BAA-837[T].

and 6.64 %; peak 3, 11.16 and 14.21 %; peak 4, 5.67 and 22.20 %; peak 5, 9.74 and 6.23 %; peak 6, 11.74 and 5.06 %, peak 7, 14.88 and 0.84 %; peak 8, 4.48 and 2.27 %; peak 9, 8.79 and 6.64 %, and peak 10, 11.76 and 3.08 %. Such differences in HPLC peak heights have been reported to be species-specific for mycobacteria (CDC, 1996, 1999; Duffey

et al., 1996; Floyd et al., 1996). Furthermore, three unique peaks (retention times 4.834, 4.977 and 6.881 min) distinguished strain 03-19[T] from *M. nebraskense* ATCC BAA-837[T].

A BLAST search of the GenBank database using 16S rRNA gene sequences of strain 03-19[T] gave a closest match (99 %) to *Mycobacterium* sp. IWGMT 90160 and the second best match to *M. nebraskense* UNMC-MY 1349[T] (= ATCC BAA-837[T]) (99 %). The 16S rRNA gene of 03-19[T] differed from that of *Mycobacterium* sp. IWGMT 90160 by one deletion and eight substitutions and from that of *M. nebraskense* UNMC-MY 1349[T] by one deletion and 11 substitutions. In hypervariable region 'A' of the 16S rRNA gene, five and four differences were observed, respectively, between 03-19[T] and *M. nebraskense* UNMC-MY 1349[T] and *Mycobacterium* sp. IWGMT 90160. In hypervariable region 'B', a unique 1 bp deletion distinguished strain 03-19[T] from these closely related strains (Fig. 2). Perhaps these polymorphisms in the 16S rRNA gene could be used to detect this strain in the future.

A neighbour-joining tree based on the aligned 16S rRNA gene sequences of strain 03-19[T] and 42 other *Mycobacterium* strains indicated a close relationship between strain 03-19[T] and *M. nebraskense*, *Mycobacterium scrofulaceum* and *Mycobacterium* sp. IWGMT 90160 within the SGM. The high bootstrapping values and topology of the maximum-parsimony tree strongly supported the grouping of these species (Fig. 3).

Trees based on *hsp65* and *rpoB* gene sequences showed different groupings of strain 03-19[T], compared with the 16S rRNA gene sequences. In the *hsp65* neighbour-joining tree (Supplementary Fig. S1 available in IJSEM Online), strain 03-19[T] was closely related to *Mycobacterium interjectum*, rather than to *M. nebraskense* or *M. scrofulaceum*, and this

was strongly supported by high bootstrap values and the recovery of this grouping in the maximum-parsimony tree. In the *rpoB* neighbour-joining tree (Supplementary Fig. S2), strain 03-19[T] was closely related to *Mycobacterium xenopi*. In spite of low bootstrap values, the same grouping was observed in the maximum-parsimony tree. Overall, phylogenetic analysis based on the three different gene sequences showed slightly different results for the relationship of strain 03-19[T] to known species. The distinct grouping of 03-19[T] among different trees confirmed the taxonomic status of this strain as a member of a novel species. It also strongly supported the notion that the description of the novel species could not have been achieved with results from a single gene. Sequence alignments of partial *hsp65* and *rpoB* genes for strain 03-19[T] and closely related strains are available as Supplementary Fig. S3(a, b) in IJSEM Online.

The distinct sequences of these three gene targets together with the uniqueness of its mycolic acid profile and phenetic traits confirm the taxonomic status of strain 03-19[T] as a member of a novel mycobacterial species rather than a variant of a previously described species. Moreover, the successive isolations of this strain from sputum samples of a patient at different stages in the absence of other mycobacteria strongly support the possibility that it may be a causative agent of pulmonary disease.

Description of *Mycobacterium seoulense* sp. nov.

Mycobacterium seoulense (seo.ul.en′se. N.L. neut. adj. *seoulense* pertaining to Seoul, Republic of Korea, the geographical origin of the type strain).

The bacillus stains acid–alcohol-fast. Cells are generally rod-shaped and frequently bent. Occasional coccid forms are noted. Spores and filaments are not present. The optimal

```
Region A           80                          125
M. tuberculosis (X58890)    AGG TCT CTT CGG AGA TAC TCG   TGG GTG ATC TGC CCT GCA CTT CGG GAT AAG CCT GGG AAA CTG GGT CTA ATA CCG GAT
M. scrofulaceum (AJ536034)  ... C.C ... ... G.G ... ...   ... .CA ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
M. nebraskense (AY368456)   ... ... ... ... ... ... ...   ... ..A ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... A..
03-19ᵀ (DQ536403)           ... C.C ... ... G.G ... ...   ... .CA ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... A..


                   177
M. tuberculosis (X58890)    CGG ATA GG-ACCA CGG GAT GCA TGT CT- TGT GGA
M. scrofulaceum (AJ536034)  ... ... ..-.... .TT .GC ... ..C ..- ... ...
M. nebraskense (AY368456)   ..A ... ..-.... .TT .GC ... ..C ..- ... ...
03-19ᵀ (DQ536403)           ..A ... ..-.... .TT .GC ... ..C ..- ... ...


Region B           445
M. tuberculosis (X58890)    TCG ACG AAG G-- TCC GGG TTC TCT CGG ATT GAC GGT AGG TGG AGA AGA AGC ACC GGC CAA CTA CGT GCC AGC
M. scrofulaceum (AJ536034)  ... ... ... .-- CT. A-- -CT .TG T.. G.. ... ... ... ... ... ... ... ... ... ... ... ... ...
M. nebraskense (AY368456)   ... ... ... .-- ... ... ..T ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
03-19ᵀ (DQ536403)           ... ... ... .-- ... ... ..- ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
```

Fig. 2. Alignment of hypervariable regions A and B of the 16S rRNA gene from strain 03-19[T] and genotypically similar reference strains. Nucleotide positions are indicated according to the *Escherichia coli* sequence. Species are represented by the corresponding type strains. The sequence of 03-19[T] was determined in the present study; other sequences were obtained from GenBank.
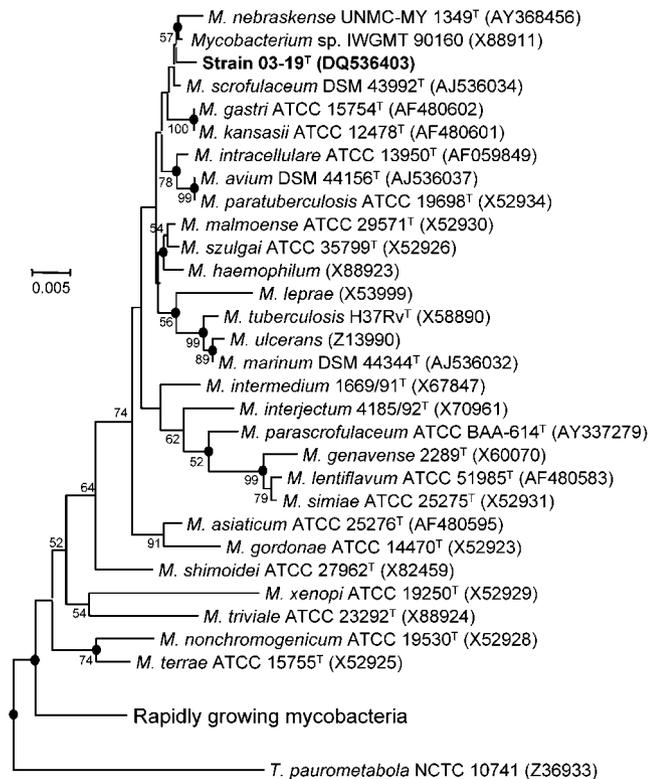
Fig. 3. Phylogenetic relationships of strain 03-19[T] among other *Mycobacterium* species based on 16S rRNA gene sequences. The tree was constructed using the neighbour-joining method. Percentages indicated at nodes represent bootstrap levels supported by 1000 resampled datasets. Bootstrap values of <50 % are not shown. Shaded circles indicate that the corresponding nodes (groupings) were also recovered in the maximum-parsimony tree (not shown). *T. paurometabola* NCTC 10741 was used as an outgroup in both trees. Bar, 0.5 % sequence difference.

growth temperature is 37 °C. No growth is observed at 45 °C. On Middlebrook 7H10 medium, mature growth develops in 3 weeks at 25 and 37 °C. Microcolonies develop in 2 weeks at the same temperature. However, 4 weeks or more is required for mature colonies to form on LJ medium. Colonies grown on Middlebrook 7H10 agar are usually larger and smooth, although occasionally rough, but always orange in appearance, under both dark and photoinduction conditions. Cells on LJ medium grow in a film-like manner and produce an orange pigment. No growth is observed on MacConkey agar or on culture medium containing 5 % NaCl or 5 mg EMB ml^{-1}. Negative for urease activity, arylsulfatase and Tween 80 hydrolysis and positive for nitrate reductase, heat-stable catalase, pyrazinamidase and tellurite reductase. HPLC analysis shows a unique mycolic acid profile. Genetically, the organism has sequences unique amongst species of *Mycobacterium* for the 16S rRNA, *hsp65* and *rpoB* genes. Phylogenetic analysis using 16S rRNA gene sequences shows that *M. seoulense* belongs to the SGM and is

closely related to *M. nebraskense*, *M. scrofulaceum* and *Mycobacterium kansasii*.

The type strain is 03-19[T] (= DSM 44998[T] = KCTC 19146[T]), isolated from human sputum samples in Seoul, Republic of Korea.

Acknowledgements

References

Butler, W. R., Thibert, L. & Kilburn, J. O. (1992). Identification of *Mycobacterium avium* complex strains and some similar species by high-performance liquid chromatography. *J Clin Microbiol* **30**, 2698–2704.

CDC (1996). *Standardized method for HPLC identification of mycobacteria.* Atlanta, GA: Centers for Disease Control and Prevention. http://www.cdc.gov/ncidod/publications/hplc.pdf

CDC (1999). *Mycolic acid pattern standards for HPLC identification of mycobacteria.* Atlanta, GA: Centers for Disease Control and Prevention. http://www.cdc.gov/nchstp/tb/Laboratory_Services/maps _tagged.pdf

Duffey, P. S., Guthertz, L. S. & Evans, G. C. (1996). Improved rapid identification of mycobacteria by combining solid-phase extraction with high-performance liquid chromatography analysis of BACTEC cultures. *J Clin Microbiol* **34**, 1939–1943.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.

Fitch, W. M. (1971). Toward defining the course of evolution: minimum change for a specific tree topology. *Syst Zool* **20**, 406–416.

Floyd, M. M., Guthertz, L. S., Silcox, V. A., Duffey, P. S., Jang, Y., Desmond, E. P., Crawford, J. T. & Butler, W. R. (1996). Characterization of an SAV organism and proposal of *Mycobacterium triplex* sp. nov. *J Clin Microbiol* **34**, 2963–2967.

Goodfellow, M. & Magee, J. G. (1998). Taxonomy of mycobacteria. In *Mycobacteria*, vol. 1, *Basic Aspects*, pp. 1–71. Edited by P. R. J. Gangadharam & P. A. Jenkins. New York & London: Chapman & Hall.

Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.

Kent, P. T. & Kubica, G. P. (1985). *Public Health Mycobacteriology. Guide for the level III Laboratory.* US Department of Health and Human Service publication. Atlanta, GA: Centers for Disease Control and Prevention.

Kim, B. J., Lee, S. H., Lyu, M. A., Kim, S. J., Bai, G. H., Chae, G. T., Kim, E. C., Cha, C. Y. & Kook, Y. H. (1999). Identification of mycobacterial species by comparative sequence analysis of the RNA polymerase gene (*rpoB*). *J Clin Microbiol* **37**, 1714–1720.

Kim, H., Kim, S.-H., Shim, T.-S., Kim, M.-n., Bai, G.-H., Park, Y.-G., Lee, S.-H., Chae, G.-T., Cha, C.-Y. & other authors (2005). Differentiation of *Mycobacterium* species by analysis of the heat-shock protein 65 gene (*hsp65*). *Int J Syst Evol Microbiol* **55**, 1649–1656.

Kirschner, P., Springer, B., Vogel, U., Meier, A., Wrede, A., Kiekenbeck, M., Bange, F. C. & Bottger, E. C. (1993). Genotypic identification of mycobacteria by nucleic acid sequence determination:

report of a 2-year experience in a clinical laboratory. *J Clin Microbiol* **31**, 2882–2889.

Kumar, S., Tamura, K., Jakobsen, I.-B. & Nei, M. (2001). MEGA2: molecular evolutionary genetics analysis software. Tempe, AZ: Arizona State University.

Roth, A., Fischer, M., Hamid, M. E., Michalke, S., Ludwig, W. & Mauch, H. (1998). Differentiation of phylogenetically related slowly growing mycobacteria based on 16S-23S rRNA gene internal transcribed spacer sequences. *J Clin Microbiol* **36**, 139–147.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Springer, B., Stockman, L., Teschner, K., Roberts, G. D. & Bottger, E. C. (1996). Two-laboratory collaborative study on identification of

mycobacteria: molecular versus phenotypic methods. *J Clin Microbiol* **34**, 296–303.

Stahl, D. A. & Urbance, J. W. (1990). The division between fast- and slow-growing species corresponds to natural relationships among the mycobacteria. *J Bacteriol* **172**, 116–124.

Stone, B. B., Nietupski, R. M., Breton, G. L. & Weisburg, W. G. (1995). Comparison of *Mycobacterium* 23S rRNA sequences by high-temperature reverse transcription and PCR. *Int J Syst Bacteriol* **45**, 811–819.

Wayne, L. G. & Kubica, G. P. (1986). The mycobacteria. In *Bergey's Manual of Determinative Bacteriology*, 9th edn, pp. 1435–1457. Edited by R. E. Buchanan & N. E. Gibbons. Baltimore: Williams & Wilkins.