Mycobacterium arupense sp. nov., a non-chromogenic bacterium isolated from clinical specimens

Joann L. Cloud,¹ Jay J. Meyer,^{1,2} June I. Pounder,¹ Kenneth C. Jost, Jr,³ Amy Sweeney,⁴ Karen C. Carroll⁴ and Gail L. Woods^{1,2†}

Correspondence

Joann L. Cloud
cloudjl@aruplab.com

¹ARUP Institute for Clinical and Experimental Pathology, Salt Lake City, UT 84108, USA

²Department of Pathology, University of Utah, Salt Lake City, UT 84108, USA

³Texas Department of State Health Services, Austin, TX 78756, USA

⁴Microbiology Division, Johns Hopkins University School of Medicine, Baltimore, MD 21087, USA

Several *Mycobacterium*-like organisms related to the *Mycobacterium terrae* complex have been isolated from clinical samples. In the clinical microbiology laboratory, partial 16S rRNA gene sequencing (approximately the first 500 bp) rather than full 16S rRNA gene sequencing is often used to identify *Mycobacterium* species. Partial 16S rRNA gene sequence analysis revealed 100% similarity between 65 clinical isolates and *Mycobacterium* sp. MCRO 6 (GenBank accession no. X93032). Even after sequencing the nearly full-length 16S rRNA gene, closest similarity was only 99.6% to *Mycobacterium nonchromogenicum* ATCC 19530^T. Sequencing of the nearly full-length 16S rRNA gene, the 16S–23S internal transcribed spacer region and the *hsp65* gene did not reveal genotypic identity with the type strains of *M. nonchromogenicum*, *M. terrae* or *Mycobacterium triviale*. Although sequence analysis suggested that these clinical isolates represented a novel species, mycolic acid analysis by HPLC failed to distinguish them from *M. nonchromogenicum*. Therefore, phenotypic analysis including growth characterization, antibiotic susceptibility testing and biochemical testing was performed. These strains from clinical samples should be recognized as representing a novel species of the genus *Mycobacterium*, for which the name *Mycobacterium arupense* sp. nov. is proposed. The type strain is AR30097^T (=ATCC BAA-1242^T=DSM 44942^T).

At the time of writing, the genus *Mycobacterium* comprises 119 species with validly published names, at least 30 of which have been described within the last 5 years (<http://www.bacterio.cict.fr/m/mycobacterium.html>). Despite this rapid increase in the number of newly recognized *Mycobacterium* species, many additional *Mycobacterium* species

remain to be formally described (Pauls *et al.*, 2003; Tortoli, 2003; Turenne *et al.*, 2004). Many of these unnamed species have been isolated from clinical specimens and need to be correctly characterized for appropriate patient management.

In the clinical microbiology laboratory, phenotypic and biochemical testing may not identify *Mycobacterium* species accurately, as the results of these tests may be identical between different species or may vary depending on the growth conditions employed. Sequencing the 16S rRNA gene of *Mycobacterium* species has improved the speed and accuracy of identification (Cloud *et al.*, 2002; Turenne *et al.*, 2001; Patel *et al.*, 2000). Sequencing additional targets such as the *hsp65* gene and the 16S–23S internal transcribed spacer region 1 (ITS1) has increased our ability to describe novel *Mycobacterium* species (Turenne *et al.*, 2004; Tortoli, 2003; Ringuet *et al.*, 1999; Mohamed *et al.*, 2005).

The purpose of this study was to describe a *Mycobacterium*-like organism that appears to be a genotypic match to

†Present address: Department of Pathology and Laboratory Services, University of Arkansas for Medical Sciences, Little Rock, AR 72205, USA.

Abbreviations: FL-HPLC, fluorescence detection HPLC; ITS1, internal transcribed spacer region 1; NTM, non-tuberculous mycobacteria.

The GenBank/EMBL/DDBJ accession numbers for the sequences of the 16S rRNA gene (*E. coli* bases 8–1494), *hsp65* gene and 16S–23S ITS1 region of *M. arupense* AR30097^T are respectively DQ157760, DQ168662 and DQ168663.

Details of PCR primers, a comparison of the 16S rRNA gene V3 region among various *Mycobacterium* species and a sequence chromatogram and sequence alignment showing polymorphisms observed in the ITS1 region of *M. arupense* strains are available as supplementary material in IJSEM Online.

Mycobacterium sp. MCRO 6 (GenBank accession no. X93032). MCRO 6 is genotypically related to the *Mycobacterium terrae* complex, which includes *M. terrae*, *Mycobacterium nonchromogenicum* and *Mycobacterium triviale* (Lee *et al.*, 2004). Over a 5-year period, our laboratory has isolated and identified via partial 16S rRNA gene sequencing (positions 7–530 of the *Escherichia coli* sequence) a genotypic match to *Mycobacterium* sp. MCRO 6 from 65 human clinical samples. Several investigators have reported 'MCRO 6' being isolated from human specimens, suggesting that the organism is clinically relevant (Torkko *et al.*, 1998; Pauls *et al.*, 2003; Lee *et al.*, 2004).

Independent data were generated from four separate patient samples (tendon, bronchial wash, sputum and a finger wound), designated strains AR30097^T, AR31431, AR08316 and AR30818, respectively. Strain AR30097^T shares the same partial 16S rRNA gene sequence and culture characteristics as 64 other isolates recovered from patient specimens in our laboratory, all matching identically with MCRO 6. Strain AR08316 was included as a rare genetic variant of this group, being isolated from sputum and growing more slowly than AR30097^T, AR31431 and AR30818 at room temperature, 30 and 37 °C. Towards the end of the study, AR30818 was isolated from a finger wound of a patient showing signs of infection and therefore was added for sequence comparison and antibiotic susceptibility testing. Because of difficulties in interpretation of the end point of the susceptibility testing procedure, four additional isolates (eight in total) were tested for susceptibility patterns to account for variations that may be observed. The following reference strains were included in the study for comparison: *M. terrae* ATCC 15755^T, *M. nonchromogenicum* ATCC 19530^T and *M. triviale* ATCC 23292^T (= TMC 1453^T). All strains were stored at –70 °C in Middlebrook 7H9 broth with 10% DMSO. Cell mass was obtained after subculture of the isolate in Löwenstein–Jensen agar slants.

The culture characterization and biochemical tests listed in Table 1 were performed on isolates AR30097^T, AR31431 and AR08316 as described by Vincent *et al.* (2003). For comparison, the phenotypic properties of *M. nonchromogenicum*, *M. terrae* and *M. triviale* were retrieved from Wayne & Kubica (1986). The three new isolates showed identical phenotypic properties. Cells of the three strains stained acid-fast, revealing straight to slightly curved bacilli with moderate beading. Growth was observed at 22–37 °C (Table 1) with optimal growth at 30 °C. There was no growth at 42 °C. Growth was considered to be rapid at 30 °C, with colonies appearing after 5–6 days, whereas growth was slow at 37 °C, with colonies appearing after 10–11 days. Results of pyrazinamidase testing were negative among the three novel strains, whereas Torkko *et al.* (1998) reported that six of seven strains of MCRO 6 were positive for pyrazinamidase. A separate report did not reveal results of pyrazinamidase testing among clinical strains of MCRO 6 (Lee *et al.*, 2004). Other phenotypic properties of AR30097^T,

Table 1. Comparison of biochemical characteristics of clinical strains of *Mycobacterium arupense* sp. nov. and closely related species of the *M. terrae* complex

Taxa: 1, *M. arupense*; 2, *M. nonchromogenicum*; 3, *M. terrae*; 4, *M. triviale*. +, Positive; –, negative; v, variable. Growth was observed for all strains at 22, 30 and 37 °C. All strains were non-pigmented, negative for iron uptake and urease activity, did not grow on MacConkey agar without crystal violet and showed a positive reaction for arylsulfatase at 14 days. Strains of *M. arupense* were negative for niacin accumulation and positive for 68 °C catalase and hydrolysis of Tween 80; according to Wayne & Kubica (1986), the reference taxa share these properties.

Test	1	2	3	4
Growth at 42 °C	–	v	–	–
Nitrate reduction	–	–*	v*	+*
Arylsulfatase (3 days)	–	–	–	+
Tolerance of 5% NaCl	–	–*	–*	+*
Pyrazinamidase	–	+*	–*	–*

*Data taken from Wayne & Kubica (1986).

AR31431 and AR08316 were identical to those for *M. nonchromogenicum* and *M. terrae*.

Susceptibility testing was performed using Sensititre microdilution plates for slowly growing non-tuberculous mycobacteria (NTM) (TREK Diagnostic Systems, Inc.), according to guidelines of the Clinical and Laboratory Standards Institute (formerly NCCLS). The following antibiotics were tested: ciprofloxacin, gatifloxacin, moxifloxacin, linezolid, rifampicin, rifabutin, trimethoprim/sulfamethoxazole, ethambutol, clarithromycin, amikacin and streptomycin. Isolates were tested at a final density of approximately 5×10^5 organisms ml^{–1}. Plates were sealed and incubated in plastic bags at 30 °C until cells mass was adequate for testing (5–10 days). The minimum inhibitory concentration (MIC) was determined to be the lowest concentration of drug that was able to inhibit the amount of visible growth as observed in a control well. An exception was made for the interpretation of trimethoprim/sulfamethoxazole, for which the MIC was determined at 80% inhibition of growth compared with the control well.

The results of susceptibility testing for eight isolates resembling AR30097^T were taken from panels incubated at 30 °C. All eight isolates were susceptible to rifabutin (three with an MIC of 0.5 µg ml^{–1}, three with an MIC of 0.25 µg ml^{–1} and two with an MIC of 0.12 µg ml^{–1}) and ethambutol (seven with an MIC of 0.5 µg ml^{–1} and one with an MIC of 1.0 µg ml^{–1}). All isolates except one (with an MIC of 64 µg ml^{–1}) were susceptible to clarithromycin (two each with an MIC of 4, 2 and 1 µg ml^{–1}, and one with an MIC of 0.5 µg ml^{–1}). Susceptibility to amikacin was variable (three each with an MIC of 16 and 32 µg ml^{–1}, one with an MIC of 64 µg ml^{–1} and one with an MIC of ≥ 128 µg ml^{–1}). All

isolates were considered to be resistant to ciprofloxacin (all with an MIC ≥ 32 $\mu\text{g ml}^{-1}$), linezolid (six with an MIC ≥ 128 $\mu\text{g ml}^{-1}$ and two with an MIC of 64 $\mu\text{g ml}^{-1}$), rifampicin (four with an MIC ≥ 16 $\mu\text{g ml}^{-1}$ and four with an MIC of 8 $\mu\text{g ml}^{-1}$) and streptomycin (four with an MIC ≥ 64 $\mu\text{g ml}^{-1}$ and four with an MIC of 32 $\mu\text{g ml}^{-1}$). Six of the eight isolates were resistant to trimethoprim/sulfamethoxazole (five with an MIC $\geq 8/152$ $\mu\text{g ml}^{-1}$ and one each with an MIC of 4/76, 2/38 and 1/19 $\mu\text{g ml}^{-1}$). All isolates had an MIC ≥ 16 $\mu\text{g ml}^{-1}$ for gatifloxacin and moxifloxacin.

Mycolic acids were prepared, esterified and analysed by fluorescence detection HPLC (FL-HPLC) as described by Brown *et al.* (1999) and Wallace *et al.* (2002). Reference strains for HPLC included *Mycobacterium intracellulare* ATCC 13950^T as well as the type strains of *M. terrae*, *M. nonchromogenicum* and *M. triviale*.

Results of mycolic acid analysis for AR30097^T, AR31431 and AR08316 were compared with those of *M. nonchromogenicum* ATCC 19530^T, *M. terrae* ATCC 15755^T, *M. triviale* ATCC 23292^T and a clinical isolate of *M. triviale*, B02SA22682. Isolates AR30097^T, AR31431 and AR08316 yielded mycolic acid chromatograms that were typical of *Mycobacterium* species. All produced a pattern of two closely clustered sets of peaks (Fig. 1). This pattern was indistinguishable from that produced by *M. nonchromogenicum*, but differed from that of the control strain of *M. intracellulare* as well as those of *M. terrae* and *M. triviale*.

DNA was extracted from organisms in pure culture using PrepMan Ultrareagent (Applied BioSystems) and frozen at -20°C until analysis (1–3 days). Sequencing of real-time PCR amplicons was performed after attainment of appropriate melting temperatures as determined with SYBR Green dye. The primers used for PCR and their respective targets are given in Supplementary Table S1 available in IJSEM

Online. The same primers were used for both PCR and sequencing reactions. PCR was performed using a RotorGene 3000 (Corbett Research). PCR volumes were 40 μl and consisted of DNA template, 0.5 μM each forward and reverse primer, 3 mM MgCl_2 and $1 \times$ LightCycler FastStart DNA Master^{PLUS} SYBR Green I prepared according to the manufacturer's instructions (Roche Diagnostics). Melting peaks were analysed with the RotorGene3000 software package (version 6).

The nucleotide sequences of both forward and reverse DNA strands were determined. If there were more than 2 % base differences between the strands, sequencing was repeated. Sequence editing, alignments and phylogenetic analyses were performed using the SEQMAN and MEGALIGN components of DNASTAR (Lasergene 5). Sequences were aligned using CLUSTAL W (Thompson *et al.*, 1994) and phylogenetic analysis was performed and trees were generated by the neighbour-joining method with Kimura's two-parameter distance correction model (Kimura, 1980).

The nearly complete 16S rRNA gene was sequenced (bases 28–1497 of the *E. coli* sequence) for four strains (AR30097^T, AR31431, AR08316 and AR30818) as well as for the type strains of species of the *M. terrae* complex (*M. nonchromogenicum* ATCC 19530^T, *M. terrae* ATCC 15755^T, *M. triviale* ATCC 23292^T). For phylogenetic analysis, 14 sequences of various *Mycobacterium* species were retrieved from GenBank and compared with the above sequences. The ends of the 16S rRNA gene sequences were trimmed so each would match closely in length. Except for two ambiguous bases (N) at positions 938 and 939, Fig. 2 shows that the nearly complete 16S rRNA gene sequences of AR30097^T, AR31431, AR08316 and AR30818 match identically with that of *Mycobacterium* sp. MCRO 6. Using the type strains of recognized *Mycobacterium* species, the closest relative is *M. nonchromogenicum* (99.6 % similarity; five base mismatches). As shown in Table 2 and Fig. 2, *M. terrae* (98.0 % sequence similarity; 27 mismatches) and *M. triviale* (95.9 % sequence similarity; 56 mismatches) have a more distant genotypic relationship with AR30097^T.

The sequence of AR30097^T has a long helix 18 in the hypervariable region V3 (see Supplementary Fig. S1 in IJSEM Online). This is commonly seen with slowly growing *Mycobacterium* species, with the exception of *Mycobacterium simiae*; at least 14 genetically related species consistently show a short helix 18 (Turenne *et al.*, 2004). Occasionally, a long helix 18 has been seen with rapidly growing *Mycobacterium* species (Menendez *et al.*, 2002). Our phenotypic observations suggest that AR30097^T grows slowly at 37°C but rapidly at 30°C . Therefore, we describe this strain as having an intermediate growth rate.

The *hsp65* gene (401 nt region) of strain AR30097^T revealed a sequence significantly different from those of strains of the *M. terrae* complex (Table 2 and Fig. 3). Strains AR31431 and AR30818 matched strain AR30097^T perfectly. The variant strain, AR08316, showed only 2 nt mismatches

Fig. 1. FL-HPLC chromatograms for comparison among mycobacterial strains. Traces: A, *M. intracellulare* ATCC 13950^T; B, *M. terrae* ATCC 15755^T; C, *M. triviale* ATCC 23292^T; D, *M. triviale* clinical isolate B02SA22682; E, *M. nonchromogenicum* ATCC 19530^T; F, AR30097^T; G, AR08316; H, AR31431.

Fig. 2. Phylogenetic tree of nearly complete sequences of the 16S rRNA gene (*E. coli* positions 38–1384) using the neighbour-joining method with Kimura’s two-parameter distance correction model. End gaps were removed from the sequences prior to analysis. The tree shows the genetic relationship between strains AR30097^T, AR30818, AR08316 and AR31431 and other *Mycobacterium* species. *Nocardia asteroides* ATCC 19247^T was included as an outgroup. GenBank accession numbers are given in parentheses.

compared with the most closely related recognized species, *M. nonchromogenicum*, with 25 nt mismatches.

Some laboratories are using ITS1, rather than the 16S rRNA gene, for identification of *Mycobacterium* species because the latter shows fewer polymorphic sites, which sometimes leads to interspecies homogeneity (Mohamed *et al.*, 2005; Roth *et al.*, 1998). For novel species descriptions, ITS1 data should be included along with that of the 16S rRNA gene. ITS1 shows many polymorphic sites between species, but also shows intraspecies heterogeneity due to interoperon heterogeneities among species from which multiple *rrn* operons exist (see Supplementary Fig. S2 in IJSEM Online) (Ji *et al.*, 1994; Roth *et al.*, 1998; Menendez *et al.*, 2002). With

ITS1 sequencing, we detected interoperon heterogeneities with some strains, including *M. nonchromogenicum* ATCC 19530^T (Supplementary Figs S2 and S3 in IJSEM Online). We observed only five ambiguous bases for strain AR30097^T, but the ITS1 sequences for AR31431, AR08316 and *M. nonchromogenicum* ATCC 19530^T showed several ambiguous bases, requiring cloning to obtain sequence from a single copy.

Even with the number of polymorphisms observed, comparing a single clone of strain AR31431 resulted in only 5 nt (of 346 nt) mismatches (1·4 % sequence divergence) with AR30097^T (Table 2). Strain AR30818 had no polymorphisms and only 1 nt difference from strain AR30097^T. Strains AR31431 and AR08316 showed relatively small numbers of nucleotide mismatches with AR30097^T compared with reference strains of the *M. terrae* complex (Table 2).

Roth *et al.* (1998) showed that the lowest ITS1 sequence divergence between any two *Mycobacterium* species was at least 4 %. Although using the sequence of only one clone, the sequence of AR08316 revealed 20 nt mismatches from AR30097^T, or 5·8 % sequence divergence (20 of 346 nt). Based on other phenotypic and genotypic properties, however, we conclude that the genetic variant AR08316 represents the same species as strain AR30097^T.

We did not obtain all possible clones of each species owing to limited resources, but based on our findings there are at least two *rrn* operons. Intraspecies heterogeneity has been reported previously among strains of the *M. terrae* complex (Lee *et al.*, 2004). Intraspecies spacer sequence polymorphisms have been suspected to occur more often in rapidly

Table 2. Numbers of nucleotide differences between *M. arupense* sp. nov. AR30097^T and other mycobacterial isolates

Strain	Sequence		
	16S rRNA gene	<i>hsp65</i>	ITS1
<i>M. arupense</i> AR30818	0	0	1
<i>M. arupense</i> AR31431	0	0	5*
<i>M. arupense</i> AR08316	0	2	20*
<i>M. nonchromogenicum</i> ATCC 19530 ^T	5	25	60
<i>M. terrae</i> ATCC 15755 ^T	27	31	66
<i>M. triviale</i> ATCC 23292 ^T	56	54	144
Nucleotides compared	1347	401	346

*Multiple *rrn* operon copies existed; only one clone was analysed.

Fig. 3. Phylogenetic tree based on sequence analysis of the *hsp65* gene using the neighbour-joining method with Kimura's two-parameter distance correction model. The tree shows the genetic relationship between strains AR30097^T, AR30818, AR08316 and AR31431 and other *Mycobacterium* species. See legend to Fig. 2 for other details.

growing than in slowly growing mycobacteria (Roth *et al.*, 1998, 2000; Turenne *et al.*, 2001). Menendez *et al.* (2002), however, showed that it was unlikely that either the number of operons or the length of helix 18 was related to growth rate.

NTM are ubiquitous in the environment, and therefore it would not be uncommon to find NTM in clinical cultures as contaminants. NTM gain access to the human body through the respiratory tract, the gastrointestinal tract and direct inoculation into skin and soft tissues (Shinners & Yeager, 1999). We have recovered several isolates similar to AR30097^T from clinical cultures of respiratory samples, as well as from various other sources. Among these isolates, many appeared to be clinically significant and the patients were treated with antimicrobial drugs. These isolates have all been identified based on partial 16S rRNA gene sequencing as representing *Mycobacterium* sp. MCRO 6.

In our laboratory, most (48 of 65) specimens were from sputum or bronchial wash; eight specimens originated from sterile sites (lymph node, lung biopsy, pleural fluid, surgical tissues and urine), four were from stool or duodenal contents and five were from unknown sites. In the study by Pauls *et al.* (2003), clinical sources of seven cultures identified as representing MCRO 6 included only three sputum samples. The remaining four samples were suspected to be clinically significant and to have originated from sterile body sites: brain tissue (one), lung biopsies (two) and pleural fluid (one). More clinical information will need to be obtained to gain insight into the clinical significance of this organism.

Strains related to AR30097^T have been isolated quite commonly in the clinical laboratory, and they clearly represent a species different from its closest relative, *M. nonchromogenicum*. Growth at 42 °C does not occur with AR30097^T, but has been reported to occur with *M. nonchromogenicum*

(Torkko *et al.*, 1998). Pyrazinamidase production was negative for AR30097^T but positive for *M. nonchromogenicum*, although results for this test may show inconsistencies between laboratories. At 30 °C, *M. nonchromogenicum* grows slowly whereas AR30097^T grows rapidly. Although culture characteristics, phenotypic properties and mycolic acid patterns are similar between AR30097^T and *M. nonchromogenicum* ATCC 19530^T, distinct gene sequence differences have been found. Using 16S rRNA gene sequence data, 5 nt differences were consistently found between several isolates similar to AR30097^T and *M. nonchromogenicum* ATCC 19530^T. Sequence data for the *hsp65* gene showed 25 nt differences between AR30097^T and *M. nonchromogenicum* ATCC 19530^T. When using the less-conserved genetic target ITS1 for sequence comparison, 60 nt differences were found. The taxon represented by AR30097^T is particularly important because it has been isolated from clinical specimens for several years and has the potential to reduce confusion surrounding the taxonomy of the *M. terrae* complex. Because of its phenotypic and genotypic differences with *M. nonchromogenicum* ATCC 19530^T and other members of the *M. terrae* complex, strain AR30097^T is considered to be the type strain of a novel species, for which the name *Mycobacterium arupense* sp. nov. is proposed.

Description of *Mycobacterium arupense* sp. nov.

Mycobacterium arupense (a.rup.en'se. N.L. neut. adj. *arupense* pertaining to the ARUP Institute for Clinical and Experimental Pathology, where the type strain was characterized).

Cells are non-chromogenic, acid-fast bacilli. The organism grows rapidly (5–7 days) on Lowenstein–Jensen medium at 30 °C and slowly (10–12 days) at 37 °C; no growth occurs at 42 °C. Isolates do not grow on MacConkey agar lacking

crystal violet. Positive for 68 °C catalase, 14-day arylsulfatase and hydrolysis of Tween 80; negative for niacin, nitrate reductase, 3-day arylsulfatase, urease, iron uptake, pyrazinamidase and tolerance of 5 % NaCl. The FL-HPLC mycolic acid pattern is indistinguishable from that produced by *M. nonchromogenicum*. Isolates are generally susceptible to ethambutol, clarithromycin and rifabutin, but resistant to rifampicin, linezolid, streptomycin and the quinolones. The 16S rRNA gene reveals a unique sequence unlike that of any established species and most similar to that of *M. nonchromogenicum* ATCC 19530^T.

The type strain, AR30097^T (=ATCC BAA-1242^T=DSM 44942^T), was isolated from a human tendon and is representative of 65 human isolates studied in our laboratory over a 5-year period.

Acknowledgements

This study was undertaken in compliance with human subject research regulations and was approved by the University of Utah Institutional Review Board. This work was supported by the ARUP Institute for Clinical and Experimental Pathology.

References

- Brown, B. A., Springer, B., Steingrube, V. A. & 10 other authors (1999). *Mycobacterium wolinskyi* sp. nov. and *Mycobacterium goodii* sp. nov., two new rapidly growing species related to *Mycobacterium smegmatis* and associated with human wound infections: a cooperative study from the International Working Group on mycobacterial taxonomy. *Int J Syst Bacteriol* **49**, 1493–1511.
- Cloud, J. L., Neal, H., Rosenberry, R., Turenne, C. Y., Jama, M., Hillyard, D. R. & Carroll, K. C. (2002). Identification of *Mycobacterium* spp. by using a commercial 16S ribosomal DNA sequencing kit and additional sequencing libraries. *J Clin Microbiol* **40**, 400–406.
- Ji, Y., Colston, M. & Cox, R. (1994). The ribosomal RNA (*rrn*) operons of fast-growing mycobacteria: primary and secondary structures and their relation to *rrn* operons of pathogenic slow-growers. *Microbiology* **140**, 2829–2840.
- 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.
- Lee, C. K., Gi, H. M., Cho, Y. & 8 other authors (2004). The genomic heterogeneity among *Mycobacterium terrae* complex displayed by sequencing of 16S rRNA and *hsp65* genes. *Microbiol Immunol* **48**, 83–90.
- Menendez, M. C., Garcia, M. J., Navarro, M. C., Gonzalez-y-Merchand, J. A., Rivera-Gutierrez, S., Garcia-Sanchez, L. & Cox, R. A. (2002). Characterization of an rRNA operon (*rrnB*) of *Mycobacterium fortuitum* and other mycobacterial species: implications for the classification of mycobacteria. *J Bacteriol* **184**, 1078–1088.
- Mohamed, A. M., Kuyper, D. J., Iwen, P. C., Ali, H. H., Bastola, D. R. & Hinrichs, S. H. (2005). Computational approach involving use of the internal transcribed spacer 1 region for identification of *Mycobacterium* species. *J Clin Microbiol* **43**, 3811–3817.
- Patel, J. B., Leonard, D. G. B., Pan, X., Musser, J. M., Berman, R. E. & Nachamkin, I. (2000). Sequence-based identification of *Mycobacterium* species using the MicroSeq 500 16S rDNA bacterial identification system. *J Clin Microbiol* **38**, 246–251.
- Pauls, R. J., Turenne, C. Y., Wolfe, N. & Kabani, A. (2003). A high proportion of novel mycobacteria species identified by 16S rDNA analysis among slowly growing accuprobe-negative strains in a clinical setting. *Am J Clin Pathol* **120**, 560–566.
- Ringuet, H., Akoua-Koffi, C., Honore, S., Varnerot, A., Vincent, V., Berche, P., Gaillard, J. L. & Pierre-Audigier, C. (1999). *hsp65* sequencing for identification of rapidly growing mycobacteria. *J Clin Microbiol* **37**, 852–857.
- 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.
- Roth, A., Reischl, U., Streubel, A., Naumann, L., Kroppenstedt, R. M., Habicht, M., Fischer, M. & Mauch, H. (2000). Novel diagnostic algorithm for identification of mycobacteria using genus-specific amplification of the 16S–23S rRNA gene spacer and restriction endonucleases. *J Clin Microbiol* **38**, 1094–1104.
- Shinners, D. & Yeager, H., Jr (1999). Nontuberculous mycobacterial infection: clinical syndromes and diagnosis: overview. In *Tuberculosis and Non-Tuberculosis Mycobacterial Infections*, 4th edn, pp. 341–350. Edited by D. Schlossberg. Philadelphia: W. B. Saunders.
- 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.
- Torkko, P., Suutari, M., Suomalainen, S., Paulin, L., Larsson, L. & Katila, M.-L. (1998). Separation among species of *Mycobacterium terrae* complex by lipid analyses: comparison with biochemical tests and 16S rRNA sequencing. *J Clin Microbiol* **36**, 499–505.
- Tortoli, E. (2003). Impact of genotypic studies on mycobacterial taxonomy: the new mycobacteria of the 1990s. *Clin Microbiol Rev* **16**, 319–354.
- Turenne, C. Y., Tschetter, L., Wolfe, J. & Kabani, A. (2001). Necessity of quality-controlled 16S rRNA gene sequence databases: identifying nontuberculous *Mycobacterium* species. *J Clin Microbiol* **39**, 3637–3648.
- Turenne, C. Y., Cook, V. J., Burdz, T. V., Pauls, R. J., Thibert, L., Wolfe, J. N. & Kabani, A. (2004). *Mycobacterium parascrofulaceum* sp. nov., novel slowly growing, scotochromogenic clinical isolates related to *Mycobacterium simiae*. *Int J Syst Evol Microbiol* **54**, 1543–1551.
- Vincent, V., Brown-Elliott, B. A., Jost, K. C., Jr & Wallace, R. J., Jr (2003). *Mycobacterium*: phenotypic and genotypic identification. In *Manual of Clinical Microbiology*, 8th edn, vol. 1, pp. 563–567. Edited by P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller & R. H. Tenover. Washington, DC: American Society for Microbiology.
- Wallace, R. J., Jr, Brown-Elliott, B. A., Hall, L. & 9 other authors (2002). Clinical and laboratory features of *Mycobacterium mageritense*. *J Clin Microbiol* **40**, 2930–2935.
- Wayne, L. G. & Kubica, G. P. (1986). Genus *Mycobacterium* Lehmann and Neumann 1896, 363^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 2, pp. 1436–1457. Edited by P. H. A. Sneath, N. S. Mair, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins.