Title:

Mycobacterium arupense sp. nov., a novel moderately growing nonchromogenic bacterium isolated from clinical specimens.

Authors:

Joann L. Cloud[1]

Jay J. Meyer[1,2]

June I. Pounder[1]

Kenneth C. Jost Jr.[3]

Amy Sweeney[4]

Karen C. Carroll[4]

Gail L. Woods[1,2*]

Addresses:

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

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

[3]Texas Department of State Health Services, Austin, TX 78756

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

*New address: Department of Pathology and Laboratory Services, University of Arkansas for Medical Sciences, Little Rock, AR 72205

Corresponding author:

Joann Cloud

cloudjl@aruplab.com

(801) 583-2787 ext. 2439

fax: (801) 584-5901

Running Title:

Mycobacterium arupense sp. nov.

Supplemental Material:

A sequence chromatogram and a sequence alignment showing polymorphisms observed in the ITS1 sequences of *M arupense* strains are shown in 2 supplemental figures in IJSEM Online.

SUMMARY

Several isolates of *Mycobacterium* species related to the *M terrae* complex have been isolated from clinical samples. In the clinical microbiology laboratory, partial 16S rRNA gene sequencing (approximate first 500 base pairs) is often used to identify *Mycobacterium* species rather than full 16S rRNA gene sequencing. Partial 16S rRNA gene sequence analysis revealed 100% identity between 65 clinical isolates and *Mycobacterium* species MCRO 6 (GenBank accession no. X93032). Even after sequencing the nearly full 16S rRNA gene, the closest match to an existing type strain is only 99.6% similar to *M nonchromogenicum* (ATCC 19530^T). Sequencing of the nearly full 16S rRNA gene, the 16S-23S internal transcribed spacer region, and the *hsp*65 gene did not reveal genotypic identity with the type strains of *M nonchromogenicum*, *M terrae*, or *M. triviale*. Although sequence analysis suggests a unique non-established species, mycolic acid analysis by HPLC does not distinguish these clinical isolates from *M. nonchromogenicum*. To further characterize this unique species, phenotypic analysis including growth characteristics, susceptibility testing, and biochemical testing was performed. It is proposed that the strain be recognized as a novel species of Mycobacteria, *Mycobacterium arupense* sp. nov., the type strain of which is ATCC BAA-1242 (=DSM 44942).

INTRODUCTION

The genus *Mycobacterium* consists of 120 validly named species at the time of this document, 31 of which have been described and accepted within the last 5 years (http://www.dsmz.de/species/bacteria.htm). Despite this rapid increase in the number of newly described *Mycobacterium* species, additional *Mycobacterium* species will always need to be formally described (Pauls *et al.*, 2003; Tortoli, 2003; Turenne *et al.*, 2004). Furthermore, many of these un-named species are isolated from clinical specimens and need to be correctly identified for proper patient management.

In the clinical microbiology laboratory, phenotypic and biochemical testing may not accurately identify *Mycobacterium* species as the results of these tests may vary depending on growth conditions and the judgment of the laboratorian. Sequencing the 16S rRNA gene of *Mycobacterium* species in the clinical laboratory has improved the speed and accuracy of identification (Cloud *et al.*, 2002; Turenne *et al.*, 2001; Patel *et al.*, 2000). Sequencing additional gene targets such as the *hsp*65 gene and the 16S-23S internal transcribed spacer region (ITS1) has

increased our ability to describe novel species (Turenne *et al.*, 2004; Tortoli, 2003; Ringuet *et al.*, 1999; Mohamed *et al.*, 2005).

The purpose of this study was to describe a novel species of *Mycobacterium* that appears to be a genotypic match to *Mycobacterium* species MCRO 6 from the GenBank database (accession number X93032). Species MCRO 6 is genotypically related to the *M. terrae* complex which includes *M. terrae*, *M. nonchromogenicum*, and *M. triviale* (Lee *et al.*, 2004). Over a 5-year period, our laboratory has isolated and identified by partial 16S rRNA gene sequencing (positions 24 to 527 of the *Escherichia coli* sequence) a genotypic match to this species from 65 human specimens. Several investigators have reported 'MCRO 6' being isolated from human specimens and leading us to believe the organism is clinically relevant (Torkko *et al.*, 1998; Pauls *et al.*, 2003; Lee *et al.*, 2004). We hereby propose the name *Mycobacterium arupense* for MCRO 6 and present its description.

METHODS

Bacterial strains and growth conditions. Independent data was generated from four separate patient samples (tendon, bronchial wash, sputum, and a finger wound) designated as 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 of MCRO 6 recovered from patient specimens in our lab, all matching perfectly to "MCRO 6" (GenBank accession number X93032). Strain AR08316 was included as a rare genetic variant of this group; being isolated from sputum and growing slower than AR30097^T or AR31431 at room temperature, 30°C, and 37°C. Toward the end of the study, AR30818 was isolated from a finger of a patient showing signs of infection and, therefore, was added for sequence comparison and susceptibility testing. Because interpretation of the endpoint of the susceptibility testing procedure, 4 additional isolates (8 total) were tested for susceptibility patterns to account for variations that may be observed. The strains are described herein as *Mycobacterium arupense* and strain AR30097^T will be referred to as ATCC BAA-1242^T. The following reference strains were also included in the study for comparison: *M. terrae* ATCC 15755^T, *M. nonchromogenicum* ATCC 19530^T, and *M. triviale* ATCC 23292^T (=TMC 1453). All strains had been stored at -70°C in Middlebrook 7H9 broth with 10% DMSO. Growth was obtained after subculture of the isolate to Lowenstein Jensen agar slants.

Phenotypic properties. Culture characteristics and biochemical tests listed in Table 1 were performed on the 3 original isolates of *M. arupense* (AR30097^T, AR31431, and AR08316) as previously described (Vincent *et al.*, 2002). For comparison, phenotypic properties for *M. nonchromogenicum*, *M. terrae*, and *M. triviale* were retrieved from *Bergey's Manual of Systematic Bacteriology* (Wayne & Kubica, 1986).

Susceptibility testing was performed using Sensititre microdilution plates for slowly growing nontuberculous mycobacteria (TREK Diagnostics Systems, Inc.), according to CLSI (formerly NCCLS) guidelines. The following drugs were tested: ciprofloxacin, gatifloxacin, moxifloxacin, linezolid, rifampin, rifabutin, trimethoprim/sulfamethoxazole, ethambutol, clarithromycin, amikacin, and streptomycin. Isolates were tested at a final organism density of approximately 5 x 10^5 organisms per milliliter. Plates were sealed and incubated in plastic bags at 30°C until growth was adequate for interpretation (5-10 days). Minimum inhibitory concentration (MIC) was determined to be the lowest concentration of drug to inhibit the amount of visible growth as observed in the control well. An exception was made for interpretation of trimethoprim/sulfamethoxazole, for which the MIC was determined at 80% inhibition of growth compared to the control well.

HPLC. Mycolic acids were prepared, esterified, and analyzed by fluorescence detection high-performance liquid chromatography (FL-HPLC) as previously described (Brown *et al.*, 1999; Wallace *et al.*, 2002). HPLC reference strains included *M. intracellulare* ATCC 13950^T as well as those described above under the heading "bacterial strains and growth conditions".

16S rRNA gene, *hsp*65 gene, and ITS1 sequencing. DNA was extracted from organisms in pure culture using the PrepMan Ultra reagent (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 determined with Sybr Green™ dye.

Primers used for PCR of their respective targets are listed in table 2. The same primers were used for both PCR and sequencing reactions. PCR was performed in a total volume of 40 µl containing: 0.5 µM each forward and reverse primer, 3 mM $MgCl_2$, and 1X LightCycler® FastStart DNA Master^{PLUS} SYBR Green I prepared according to the manufacturer's instructions (Roche Diagnostics). Annealing temperatures were optimized to be 55°C for 16S rDNA, 55°C for *hsp*65, and 58°C for ITS1. Thermal cycling reactions were initiated with a hold for 10 minutes at

95°C for activation of the polymerase contained in the master mix. Thirty-five cycles of PCR were performed for 16S rDNA and ITS1 sequencing while 50 cycles were performed for *hsp*65. The remaining thermal cycling protocol consisted of denaturation (35 s at 96°C), annealing (20 s at annealing temperature), and extension (30s at 72°C), with a single final extension (5 min at 72°C). The melting protocol consisted of stepping 0.5 degree per second from 75°C to 99°C. Reactions took place in a RotorGene 3000 (Corbett Research). Melting peaks were analyzed with the Rotor-Gene3000 software package version 6.

The PCR products were column-purified using Microcon-100 columns (Amicon). The purified amplicons were sequenced by standard methods using the ABI Prism BigDye Terminator v3.0 Ready Reaction Cycle Sequencing Kit and the ABI Prism 3100 Avant Genetic Analyzer. 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). Alignment methods included CLUSTAL V (neighbor-joining method) and CLUSTAL W (Thompson *et al.*, 1994).

Some strains revealed numerous ambiguous bases within the edited, quality-controlled ITS1 sequences and therefore suggested the presence of multiple *rrn* operons. If more than 2% ambiguities occurred due to this phenomenon, cloning of the ITS1 region was performed by standard protocols using a TA Cloning kit (Invitrogen). PCR and sequencing, was performed on the clones using the M13 primers from the kit to produce unambiguous sequences. All sequences were trimmed at the ITS1 primers before phylogenetic analysis was performed.

Nucleotide sequence accession numbers. The 16S rRNA gene sequence (*E. coli* bases 37 to 1446) of *M. arupense* ATCC BAA-1242^T (= DMS 44942^T) has been deposited in GenBank under accession number DQ157760. The hsp65 gene and 16S-23S internal transcribed spacer (ITS) 1 region has been deposited in GenBank under accession numbers DQ168662 and DQ168663, respectively.

RESULTS AND DISCUSSION

Phenotypic properties

All phenotypic studies were performed on BAA-1242^T, AR31431, and AR08316 with full agreement of results among the 3 strains of *M. arupense*. Organisms of *M. arupense* sp. nov.

stained acid-fast, revealing straight to slightly curved bacilli with moderate beading. Growth was observed from 22°C to 37°C (table 1) with optimal growth at 30°C. There was no growth at 42°C. Growth rate was considered rapid at 30°C with colonies appearing after 5-6 days, while growth was slow at 37°C, as colonies did not appear until after 10-11 days. Results of pyrazinamidase testing for *M. arupense* were negative among the 3 strains whereas Torkko reported that 6 of 7 strains of MCRO 6 were positive (Torkko *et al.*, 1998). Lee did not report results of pyrazinamidase testing among the clinical strains of MCRO 6 (Lee *et al.*, 2004). The remaining phenotypic properties of *M. arupense* revealed similarity with *M. nonchromogenicum* and *M. terrae*.

Susceptibility testing

MIC results were taken from panels incubated at 30°C. All 8 *M. arupense* isolates were susceptible to rifabutin (3 each with an MIC of 0.5 and 0.25 μg/ml and 2 with an MIC of 0.12 μg/ml) and ethambutol (7 with an MIC of 0.5 μg/ml and 1 with an MIC of 1.0 μg/ml). All isolates except one (MIC of 64 μg/ml) were susceptible to clarithromycin (2 each with an MIC of 4, 2, and 1 μg/ml, and 1 with an MIC of 0.5 μg /ml). Susceptibility to amikacin was variable (3 each with an MIC of 16 and 32 μg/ml, 1 with an MIC of 64 μg/ml, and 1 with an MIC of ≥ 128 μg/ml). All isolates were considered resistant to ciprofloxacin (all with an MIC ≥ 32 μg/ml), linezolid (6 with an MIC ≥ 128 μg/ml and 2 with an MIC of 64 μg/ml), rifampin (4 with an MIC ≥ 16 μg/ml and 4 with an MIC of 8 μg/ml), and streptomycin (4 with an MIC ≥ 64 μg/ml and 4 with an MIC of 32 μg/ml). Six of eight isolates were resistant to trimethoprim/sulfamethoxazole (5 with an MIC ≥ 8/152 μg/ml and 1 each with an MIC of 4/76, 2/38, and 1/19 μg/ml). All isolates had an MIC ≥ 16 μg/ml for gatifloxacin and moxifloxacin.

Mycolic acid analysis by FL-HPLC

Three clinical isolates of *M. arupense* were compared to *M. nonchromogenicum* (ATCC 19530^T), *M. terrae* (ATCC 15755^T), *M. triviale* (ATCC 23292^T), and a clinical isolate for *M. triviale* (B02SA22682) for FL-HPLC analysis. The isolates of *M. arupense* 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 the pattern produced by *M. nonchromogenicum*. It was separable from the pattern of the control strain of *M. intracellulare* as well as those of *M. terrae* and *M. triviale*.

Sequencing of the 16S rRNA gene, *hsp*65 gene, and ITS1 region

The 16S rRNA gene was sequenced to near completeness (bases 37-1446 of *Escherichia coli* sequence) for 4 strains of *M. arupense* (ATCC BAA-1242^T, AR31431, AR08316, and AR30818) as well as the reference strains for species of the *M. terrae* complex, ATCC 19530^T (*M. nonchromogenicum*), ATCC 15755^T (*M. terrae*), and ATCC 23292^T (*M. triviale*). Except for 2 ambiguous bases (N) at positions 938 and 939, the nearly complete 16S rRNA gene sequence of ATCC BAA-1242^T, AR31431, AR08316, and AR30818 revealed an identical match with *Mycobacterium* sp. MCRO 6 (GenBank accession X93932). Using type strains of validly accepted species, the closest relative is *M. nonchromogenicum* (99.6% similarity; 5 base mismatches). As shown in table 3 and figure 3, *M. terrae* (98.0% similarity; 27 mismatches) and *M. triviale* (95.9% similarity; 56 mismatches) have a more distant genotypic relationship with *M. arupense*.

The sequence of *M. arupense* has a long helix 18 in the hypervariable region V3 that is commonly seen with slow growing *Mycobacterium* species (Turenne *et al.*, 2004), and occasionally seen with rapid growing *Mycobacterium* species (Menendez *et al.*, 2002). Our observations suggest *M. arupense* is slow growing at 37°C, but rapid growing at 30°C. Therefore, we describe *M. arupense* sp. nov. as moderately growing.

The *hsp*65 gene (401 nt region) of the type strain of *M. arupense* (ATCC BAA-1242^T) revealed a sequence significantly different from strains of the *M. terrae* complex (table 3; figure 4). AR31431 and AR30818 matched *M. arupense* perfectly. The variant strain of *M. arupense*, AR08316, showed only 2 nt mismatches as compared to the most closely related established species, *M. nonchromogenicum*, with 25 nt mismatches.

Some laboratories are using ITS1, rather than 16S rRNA gene, for identification of *Mycobacterium* species because 16S rDNA shows fewer polymorphic sites, which sometimes leads to interspecies homogeneity (Mohamed *et al.*, 2005; Roth *et al.*, 1998). For new 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 (Ji *et al.*, 1994; Roth *et al.*, 1998; Menendez *et al.*, 2002). With ITS1 sequencing, we detected interoperon heterogeneities with some strains of *M. arupense* as well as the reference strain for *M. nonchromogenicum* (supplemental figures A and B). We observed only 5 ambiguous bases for

strain ATCC BAA-1242^T, but the ITS1 sequences for strain AR31431, AR08316, and ATCC 19530^T showed several ambiguous bases, requiring cloning to obtain sequence from a single copy (supplemental figure B).

Even with the number of polymorphisms observed, comparing a single clone of strain AR31431 resulted in only 5 (of 346) nucleotide mismatches (1.4% divergence) with ATCC BAA-1242^T (table 3). Strain AR30818 had no polymorphisms and only 1 nucleotide difference from *M. arupense* (ATCC BAA-1242^T). All strains of *M. arupense* showed relatively low numbers of nt mismatches from the type strain compared to reference strains of the *M. terrae* complex (table 3).

Studies by Roth et al. showed that the lowest ITS1 sequence divergence between any two *Mycobacterium* species was at least 4% (Roth *et al.*, 1998). Although using the sequence of only one clone, the sequence of AR08316 revealed 20 nt mismatches from ATCC BAA-1242^T, or 5.8% (20 of 346 nt). Based on other phenotypic and genotypic properties, we would identify the genetic variant AR08316 as *M. arupense* even though we observed a 5.8% ITS1 sequence divergence from ATCC BAA-1242^T.

We did not obtain all possible clones of each species due to limited resources, but based on our findings there are at least 2 *rrn* operons. Intraspecies heterogeneity has been reported previously among strains of the *M. terrae* complex (Lee *et al.*, 2004). Early reports of intraspecies spacer sequence polymorphisms has been suspected to occur more often in rapidly growing than in slowly growing mycobacteria (Roth *et al.*, 2000; Roth *et al.*, 1998; Turenne *et al.*, 2001). Menendez et al., however, showed it is unlikely that the number of operons or length of helix 18 is related to the growth rate (Menendez *et al.*, 2002).

Clinical relevance

NTM are ubiquitous in the environment, therefore, it would not be uncommon to find NTM in clinical cultures as contaminants. The NTM gain access to the human body through the respiratory tract, the gastrointestinal tract, and direct inoculation into skin and soft tissues (Shinners & H. Yeager, 1999). We have recovered several isolates of *M. arupense*, sp. nov. from clinical cultures of respiratory samples, as well as from various other sources including a tendon, a finger, synovial fluid, pleural fluid, urine, and stool. These isolates have all been identified by partial 16S rRNA gene sequencing as un-named strain MCRO6 (GenBank accession no. X93032).

In our laboratory, most (48 of 65) specimens were from sputum or bronchial wash, 8 originated from sterile sites (lymph node, lung biopsy, pleural fluid, surgical tissues, and urine), 4 were from stool or duodenal contents, and 5 were from unknown sites. In the study by Pauls et al., clinical sources of 7 positive cultures for MCRO 6 (*M arupense*, sp. nov.) included only 3 sputum samples. The remaining 4 samples were suspected to be clinically significant and originated from the sterile body sites of brain (1), lung biopsies (2) and pleural fluid (1) (Pauls *et al.*, 2003). More clinical information from patient charts should be obtained to gain insight into the clinical significance of this organism. Regardless of clinical significance, the organism is being isolated quite commonly in the clinical laboratory and therefore needs to be validly described.

Description of *Mycobacterium arupense* sp. nov.

Mycobacterium arupense is a nonchromogenic acid-fast bacillus growing rapidly (5-7 days) on Lowenstein-Jensen medium at 30°C and, slowly (10-12 days) at 37°C. Isolates do not grow on MacConkey agar lacking crystal violet and are positive for 68°C catalase, 14-day arylsulfatase, and tween-80 hydrolysis; and negative for niacin, nitrate reductase, 3-day arylsulfatase, urease, iron uptake, pyrazinamidase, and tolerance to 5% NaCl. The FL-HPLC mycolic acid pattern is indistinguishable from that produced by *M. nonchromogenicum*. The 16S rRNA gene sequence (GenBank Accession No. DQ157760) reveals a unique species unlike any established species and most similar to the reference strain *M nonchromogenicum* (ATCC 19530^T).

The type strain, ATCC BAA-1242 (=DSM 44942), was representative of 65 human isolates studied in our laboratory over a 5-year period. Several isolates appeared to be clinically significant and were treated with antimicrobials. The isolates were generally susceptible to ethambutol, clarithromycin, and rifabutin while resistant to rifampicin, linezolid, streptomycin, and the quinolones.

ACKNOWLEDGEMENTS

This study was in compliance with human subjects 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.

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

Test	*M. arupense*, sp. nov.	*M. nonchromogenicum*	*M. terrae*	*M. triviale*
Growth at 22°C	+	+	+	+
Growth at 30°C	+	+	+	+
Growth at 37°C	+	+	+	+
Growth at 42°C	-	v	-	-
Pigmentation	N	N	N	N
Niacin accumulation*	-	-	-	-
Nitrate reduction*	-	-	v	+
68°C catalase*	+	+	+	+
3-day arylsulfatase	-	-	-	+
14-day arylsulfatase	+	+	+	+
Tween-80 hydrolysis*	+	+	+	+
Iron uptake	-	-	-	-
Growth on MacConkey without crystal violet	-	-	-	-
Urease activity	-	-	-	-
Tolerance to 5% NaCl*	-	-	-	+
Pyrazinamidase*	-	+	-	-

*Data for reference species (*M. nonchromogenicum*, *M. terrae*, and *M. triviale*) were taken from Wayne and Kubica (1986). v, variable; N, nonchromogenic.

272 Table 2. Primers used for sequence-based studies.

Name	Gene Target	Sequence (5'-3')	Source
16S-27f	16S rRNA	AGAGTTTGATCMTGGCTCAG	Mellmann et al.
16S-519r	16S rRNA	GWATTACCGCGGCKGCTG	Mellmann et al.
16S-359f	16S rRNA	CTCCTACGGGAGGCAGCAGT	Vaneechoutte et al.
16S-971r	16S rRNA	CTCTGCCGGCGTCCTGT	This study
16S-895f	16S rRNA	CGGCGGAGCATGTGGATTA	This study
16S-1482r	16S rRNA	CACCTTCCGGTACGGCTACCT	This study
Tb11	*hsp*65	ACCAACGATGGTGTGTCCAT	Telenti et al.
Tb12	*hsp*65	CTTGTCGAACCGCATACCCT	Telenti et al.
ITS1-1511f	ITS1	AAGTCGTAACAAGGTARCCG	Turenne et al., Harmsen et al.
ITS1-23r	ITS1	TCGCCAAGGCATCCACC	Turenne et al., Harmsen et al.

273

274

Table 3. Sequence analysis describing the number of nucleotide differences from *M. arupense* sp. nov. (ATCC BAA-1242^T) existing with each of the strains in the study.

Strain	16S rRNA	*hsp*65	ITS1
M. arupense (AR30818)	0	0	1
M. arupense (AR31431)	0	0	5*
M. arupense (AR08316)	0	2	20*
M. nonchromogenicum (ATCC 19530^T)	5	25	60
M. terrae (ATCC 15755^T)	27	31	66
M. triviale (ATCC 23292^T)	56	54	144

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

277 Figure 1. FL-HPLC chromatograms for comparison among strains. A, *M. intracellulare* ATCC
278 13950^{T}; B, *M. terrae* ATCC 15755^{T}; C, *M. triviale* ATCC 23292^{T}; D, *M. triviale* clinical isolate
279 B02SA22682; E, *M. nonchromogenicum* ATCC 19530^{T}; F, ATCC BAA-1242^{T}; G, AR08316; H,
280 AR31431.
281
282

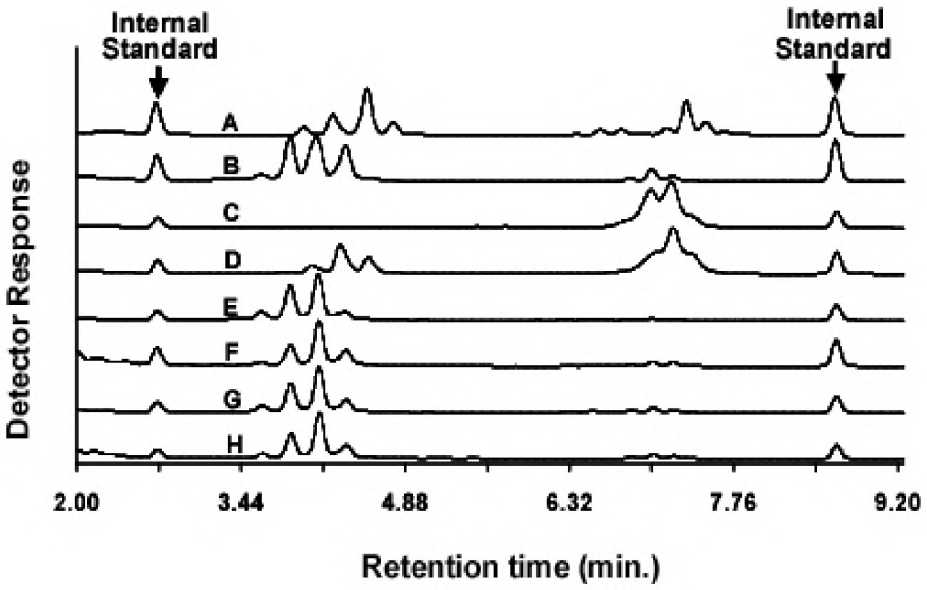


Figure 2. Sequence comparison among *Mycobacterium* species of interest for the 16S rRNA gene hypervariable region B (V3) from *E. coli* positions 428 to 463. Sequences were determined in our lab unless indicated by a GenBank accession number in parentheses.

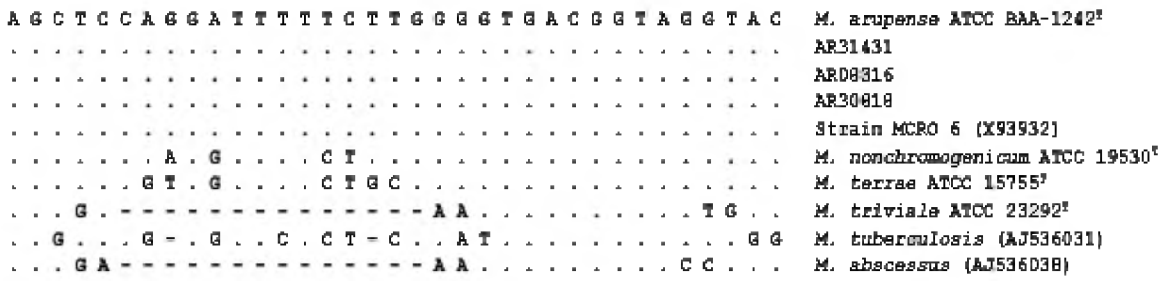


Figure 3. Phylogenetic tree of nearly complete 16S rRNA gene (*E. coli* positions 37 to 1446) sequences showing the genetic relationship among the strains of *M. arupense* and strains of the *M. terrae* complex. The CLUSTAL W method was performed with weightings using the MEGALIGN component of the DNAStar software (Lasergene 5). *M. tuberculosis* and *M. abscessus* were included as out-groups. All sequences were determined in our laboratory except those with GenBank accession numbers in parentheses.

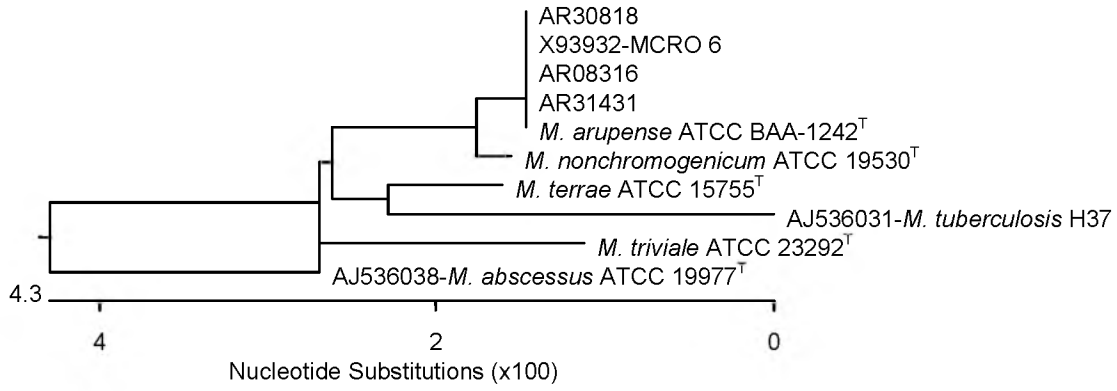
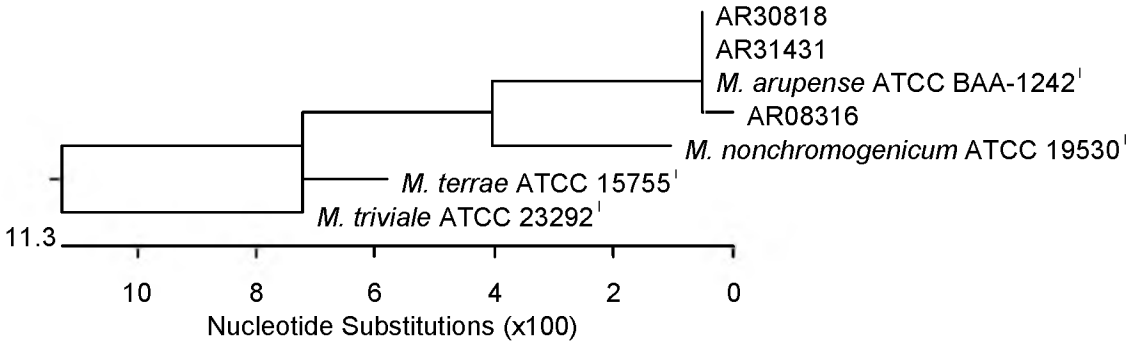


Figure 4. Phylogenetic positions according to 401 nt of the *hsp*65 gene sequence of strains of *M. arupense* in relation to strains of the *M. terrae* complex. The CLUSTAL W method was performed with weightings using the MEGALIGN component of the DNAStar software (Lasergene 5). All sequences were determined in our laboratory.



Supplemental Figure A. Sequence chromatogram showing a region of ambiguities within the 16S-23S internal transcribed spacer region for *M. nonchromogenicum* (ATCC 19530^{T}). The sequence was determined in our laboratory after several repeat analyses on different cultures to rule out a mixed species.

Supplemental Figure B. Alignment of the 16S-23S rRNA spacer (ITS1) sequences of the *M. arupense* strains. The sequence of the type strain, *M. arupense* (ATCC BAA-1424T), shows 5 ambiguous nucleotide positions (K, S, R, Y, and K for positions 100, 108, 313, 314, and 315 respectively).

REFERENCES

Brown, B.A., Springer, B., Steingrube, V.A., Wilson, R.W., Pfyffer, G.E., Garcia, M.J., Menendez, M.C., Rodriguez-Salgado, B., Jost, K.C., Jr., Chiu, S.H., Onyi, G.O., Bottger, E.C. & Wallace, R.J., Jr. (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 Pt 4**, 1493-511.

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**(2), 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**(10), 2829-2840.

Lee, C.K., Gi, H.M., Cho, Y., Kim, Y.K., Lee, K.N., Song, K.-J., Song, J.-W., Park, K.S., Park, E.M., Lee, H. & Bai, G.-H. (2004) The genomic heterogeneity among *Mycobacterium terrae* complex displayed by sequencing of 16S rRNA and *hsp*65 genes. *Microbiol Immunol* **48**(2), 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**(4), 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**(8), 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**(1), 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**(4), 560-566.

Ringuet, H., Akoua-Koffi, C., Honore, S., Varnerot, A., Vincent, V., Berche, P., Gaillard, J.L. & Pierre-Audigier, C. (1999) *hsp*65 sequencing for identification of rapidly growing Mycobacteria. *J Clin. Microbiol.* **37**(3), 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**(1), 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**(3), 1094-1104.

Shinners, D. & H. Yeager, J. (1999) Tuberculosis and non-tuberculosis *Mycobacterium* infections., p. 341. 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. *Nucl. Acids. Res.* **22**, 4673-80.

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**(2), 499-505.

Tortoli, E. (2003) Impact of genotypic studies on Mycobacterial taxonomy: the new Mycobacteria of the 1990s. *Clin. Microbiol. Rev.* **16**(2), 319-354.

Turenne, C.Y., Cook, V.J., Burdz, T.V., Pauls, R.J., Thibert, L., Wolfe, J.N. & Kabani, A. (2004) *Mycobacterium parascrofulaceum* sp. nov., A novel slowly growing, scotochromogenic clinical isolates related to *Mycobacterium simiae*. *Int J Syst Evol Microbiol* **54**(5), 1543-1551.

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**(10), 3637-3648.

Vincent, V., Brown-Elliott, B.A., Jost, K.C. & Jr., R.J.W. (2002) Mycobacterium phenotypic and genotypic identification. In *Manual of Clinical Microbiology*, pp. 563-567. Edited by P.R. Murray, E.J. Baron, J.H. Jorgensen, M.A. Pfaller & R.H. Yolken. Washington D. C.: ASM Press.

Wallace, R.J., Jr., Brown-Elliott, B.A., Hall, L., Roberts, G., Wilson, R.W., Mann, L.B., Crist, C.J., Chiu, S.H., Dunlap, R., Garcia, M.J., Bagwell, J.T. & Jost, K.C., Jr. (2002) Clinical and laboratory features of *Mycobacterium mageritense*. *J.Clin Microbiol* **40**(8), 2930-5.

Wayne, L.G. & Kubica, G.P. (1986) Genus *Mycobacterium*. In *Bergey's Manual of Systematic Bacteriology*, Vol. 2, pp. 1435-1457. Edited by P.H.A. Sneath, N.S. Mair, M.E. Sharp & J.G. Holt. Baltimore: Williams & Wilkins.

Research: Pre-Submission Approval Form

Approval is required **before information is presented outside of ARUP and enters the public domain** to ensure that HIPAA and IRB protocols have been followed. Please ensure that this document is signed and appropriate documents are attached before submitting any information for publication/presentation outside of ARUP.

☑ Attach copy of (please indicate) manuscript, poster, abstract, or other presentation

Presentation/Poster presented at (specify meeting or conference): _____

ARUP Cited: Yes ☐ No ☐ If no, state reason: _____

☐ Global IRB #7275 applies, and PRCS-0020, *Internal Sample Request: De-Identification of Samples* has been followed **OR**

☑ Independent Institutional Review Board (IRB) approval, IRB# 12333
Attach copy of approved IRB protocol.

OR

☐ IRB is not applicable. Please explain: _____

Scientist/Researcher: _____ Date: _____
Signature

Approval Signatures and Dates:

Medical Director: _____ Date: _____

R&D Group Manager or
ARUP Privacy Officer: _____ Date: _____