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

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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 19530T. 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 AR30097T (= ATCC BAA-1242T = DSM 44942T).

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
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 3-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 AR30097T, AR31431, AR08316 and AR08318, respectively. Strain AR30097T 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 AR30097T, AR31431 and AR08318 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 15755T, M. nonchromogenicum ATCC 19530T and M. triviale ATCC 23292T (= TMC 1453T). 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 AR30097T, 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 AR30097T, 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 AR30097T were taken from panels incubated at 30°C. All eight isolates were susceptible to rifampicin (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

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

<table>
<thead>
<tr>
<th>Test</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth at 42°C</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>–</td>
<td>–</td>
<td>+*</td>
<td>+*</td>
</tr>
<tr>
<td>Arylsulfatase (3 days)</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tolerance of 5% NaCl</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Pyrazinamidase</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*Data taken from Wayne & Kubica (1986).
isolates were considered to be resistant to ciprofloxacin (all with an MIC $\geq 32$ mg ml$^{-1}$), linezolid (six with an MIC $\geq 128$ mg ml$^{-1}$ and two with an MIC of 64 mg ml$^{-1}$), rifampicin (four with an MIC $\geq 16$ mg ml$^{-1}$ and four with an MIC of 8 mg ml$^{-1}$) and streptomycin (four with an MIC $\geq 64$ mg ml$^{-1}$ and four with an MIC of 32 mg ml$^{-1}$). Six of the eight isolates were resistant to trimethoprim/sulfamethoxazole (five with an MIC $\geq 8/152$ mg ml$^{-1}$ and one each with an MIC of 4/76, 2/38 and 1/19 mg ml$^{-1}$). All isolates had an MIC $\geq 16$ mg 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$^\text{T}$ as well as the type strains of *M. terrae*, *M. nonchromogenicum* and *M. triviale*.

Results of mycolic acid analysis for AR30097$^\text{T}$, AR31431 and AR08316 were compared with those of *M. nonchromogenicum* ATCC 19530$^\text{T}$, *M. terrae* ATCC 15755$^\text{T}$, *M. triviale* ATCC 23292$^\text{T}$ and a clinical isolate of *M. triviale*, B02SA22682. Isolates AR30097$^\text{T}$, 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 $-20^\circ$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 Rotor-Gene 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 x LightCycler FastStart DNA MasterPLUS SYBR Green I prepared according to the manufacturer’s instructions (Roche Diagnostics). Melting peaks were analysed with the RotorGene3000 software package (version 6).

The nucelotide 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 SEQUAN 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$^\text{T}$, AR31431, AR08316 and AR30818) as well as for the type strains of species of the *M. terrae* complex (*M. nonchromogenicum* ATCC 19530$^\text{T}$, *M. terrae* ATCC 15755$^\text{T}$, *M. triviale* ATCC 23292$^\text{T}$). 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$^\text{T}$, 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$^\text{T}$.

The sequence of AR30097$^\text{T}$ 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$^\text{T}$ 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$^\text{T}$ 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$^\text{T}$ perfectly. The variant strain, AR08316, showed only 2 nt mismatches.
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 19530T (Supplementary Figs S2 and S3 in IJSEM Online).

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 AR30097T (Table 2). Strain AR30818 had no polymorphisms and only 1 nt difference from strain AR30097T. Strains AR31431 and AR08316 showed relatively small numbers of nucleotide mismatches with AR30097T 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 AR30097T, 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 AR30097T.

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. AR30097T and other mycobacterial isolates

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

*Multiple *rrn* operon copies existed; only one clone was analysed.
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 AR30097T 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 AR30097T 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 AR30097T, but has been reported to occur with M. nonchromogenicum (Torkko et al., 1998). Pyrazinamidase production was negative for AR30097T but positive for M. nonchromogenicum, although results for this test may show inconsistencies between laboratories. At 30°C, M. nonchromogenicum grows slowly whereas AR30097T grows rapidly. Although culture characteristics, phenotypic properties and mycolic acid patterns are similar between AR30097T and M. nonchromogenicum ATCC 19530T, distinct gene sequence differences have been found. Using 16S rRNA gene sequence data, 5 nt differences were consistently found between several isolates similar to AR30097T and M. nonchromogenicum ATCC 19530T, Sequence data for the hsp65 gene showed 25 nt differences between AR30097T and M. nonchromogenicum ATCC 19530T. When using the less-conserved genetic target ITS1 for sequence comparison, 60 nt differences were found. The taxon represented by AR30097T 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 19530T and other members of the M. terrae complex, strain AR30097T 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 arsulfatase and hydrolysis of Tween 80; negative for niacin, nitrate reductase, 3-day arsulfatase, 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 19530T.

The type strain, AR30097T (=ATCC BAA-1242T = DSM 44942T), was isolated from a human tendon and is representative of 65 human isolates studied in our laboratory over a 5-year period.

Acknowledgements

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References


