International Journal of Systematic and Evolutionary Microbiology (2006), **56**, 2049–2054 **DOI** 10.1099/ijs.0.64190-0

Mycobacterium colombiense sp. nov., a novel member of the *Mycobacterium avium* complex and description of MAC-X as a new ITS genetic variant

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Forty-five mycobacterial strains isolated from 23 Colombian HIV-positive patients were identified as members of the *Mycobacterium avium* complex (MAC) and were characterized using different molecular approaches. Seven of the isolates showed characteristic features that allowed them to be differentiated from other members of the complex. The isolates had a novel 16S–23S rRNA internal transcribed spacer (ITS 1) gene sequence which is described as a new sequevar, MAC-X. All of the seven novel isolates gave a positive result with the MAC-specific AccuProbe (Gen-Probe), but tested negative for *Mycobacterium avium* and *Mycobacterium intracellulare* species-specific probes (64 and 100 % of the isolates, respectively). The novel isolates could be differentiated phenotypically from other members of the MAC on the basis of the production of urease and by a consistent mycolic acid pattern. The novel isolates shared some characteristics with *M. avium*, such as the avium variant I (av-I) pattern of the *hsp*65 gene as determined by PCR restriction analysis and a positive PCR result for the *mig* (macrophage-induced) gene. However, the novel isolates showed a unique 16S rRNA gene sequence. DNA–DNA relatedness values, from 24 to 44 %, confirmed the distinction of the novel isolates from other members of the MAC at the genetic level and their status as members of a separate species. The novel isolates are proposed as representatives of a novel species, *Mycobacterium colombiense* sp. nov., that is closely related to *M. avium* within the MAC. The type strain is 10B^T (=CIP 108962^T=CECT 3035^T).

Members of the *Mycobacterium avium* complex (MAC) are currently identified on the basis of positive results with the commercial MAC-specific probe, AccuProbe (Gen-Probe). Originally, two species were identified within the MAC, namely *Mycobacterium avium* and *Mycobacterium intracellulare*. However, recent studies have drawn attention to the wide diversity of isolates that can be detected in the complex (Mijs *et al.*, 2002; Smole *et al.*, 2002; Lebrun *et al.*, 2005). Analysis of the 16S–23S rRNA internal transcribed spacer (ITS 1) sequence, a molecular approach frequently used for the identification of these bacteria, has revealed the existence of more than 30 different sequevars (Tortoli *et al.*, 2004). Only a few of these sequevars belong to members of *M. avium* (seven sequevars) or *M. intracellulare* (four sequevars). Recently, members of the MAC represented by sequevar MAC-A were described as the novel species *Mycobacterium chimaera*. This novel species has similar characteristics to *M. intracellulare* (Tortoli *et al.*, 2004).

During a study to characterize MAC isolates from HIV-positive Colombian patients, a group of distinct strains was identified. These isolates showed several features that allowed them to be differentiated from other members of the MAC and recognized as a separate novel species.

Abbreviations: ITS 1, 16S–23S rRNA internal transcribed spacer; MAC, *Mycobacterium avium* complex; PRA, PCR restriction analysis; RAPD, randomly amplified polymorphic DNA.

The GenBank/EMBL/DDBJ accession number for the sequence of the 16S rRNA gene and ITS 1 regions of strain 10B is AM062764 and the accession number for the partial *hsp*65 gene sequence of the same strain is AM062765.

An alignment of the *hsp*65 gene sequences examined in this study and the RAPD patterns for the *M. colombiense* strains are available as supplementary material in IJSEM Online.

Seven bacterial strains were isolated from four HIV-positive patients in 1995 (four strains) and 2000 (three strains). One strain was isolated from sputum and the remaining six were isolated from blood samples. Patients were hospitalized in three hospitals in Bogota, Colombia (Table 1). All patients died as a consequence of their underlying immunocompromised clinical status.

The seven bacterial isolates tested positive with the commercial MAC-specific probe and tested negative with the *M. intracellulare* species-specific probe. Four of the isolates also gave a negative result when tested with the *M. avium* species-specific probe (AccuProbe; Gen-Probe) (Table 1). The isolates were also investigated by using the INNO LiPA probe assay (Innogenetics) and were identified as belonging to the *Mycobacterium avium–intracellulare–scrofulaceum* (MAIS) group, but different from *M. avium*, *M. intracellulare* and *Mycobacterium scrofulaceum*.

Phenotypic identification

Samples were isolated using a biphasic medium. Ogawa Kudoh agar supplemented with iron citrate was used as the solid phase and Sauton Tween-albumin modified medium was used as the liquid phase. The novel mycobacterial isolates were initially considered to be non-pigmented and slow-growing, but some colonies developed some pigmentation with age. The isolates were unable to grow on MacConkey agar, Sauton agar supplemented with picric acid or Löwenstein–Jensen agar supplemented with NaCl (5 %). A set of biochemical tests was performed (Kent & Kubica, 1985) and some variability in results was found when the seven isolates were compared. All isolates were positive for urease activity which was the main biochemical characteristic that enabled the novel strains to be distinguished from other members of the complex (Table 1).

HPLC analysis was performed by employing a standard method (CDC, 1996) on a System Gold instrument (Beckman) equipped with an XL Ultrasphere column (Beckman). The chromatographic patterns were visually compared with the HPLC library (available at http://www.mycobactoscana.it/page4.htm) and, in particular, with the profiles of species belonging to the MAC. Analysis of mycolic acids revealed the three-clustered pattern typical of strains belonging to the species *M. avium*, *M. intracellulare* and *M. chimaera*. In the novel isolates, however, the peaks of the first and second clusters consistently appeared in increasing peak height, which differed from the bell-shaped appearance that is characteristic of other members of the MAC (Fig. 1).

These results, together with the results of the commercial probe tests, did not enable the novel isolates to be identified any further than their recognition as members of the MAC.

Table 1. Comparison of characteristics of *Mycobacterium colombiense* sp. nov. and other members of the MAC

All isolates were obtained from HIV-positive patients. Patient A came from Hospital Simon Bolivar, patients B and C were from Hospital San Juan de Dios and patient 57B was from the Instituto de Seguro Social. All of these institutions are located in Bogota, Colombia. All strains were able to grow at 37 °C and gave a positive result in the MAC AccuProbe test. All strains were negative in tests for niacin production, acid phosphatase activity and Tween hydrolysis. INNO LiPA, Line probe assay; MAIS, *Mycobacterium avium–intracellulare–scrofulaceum*; +, positive; −, negative; +/−, intermediate; UN, unknown.

Characteristic	*Mycobacterium colombiense* sp. nov. strains							*M. avium*	*M. intracellulare*	*M. chimaera*
	6B	7B	9B	10B^T	16B	19B	57B			
Patient	A	A	B	B	C	B	D			
Source	Blood	Blood	Blood	Blood	Blood	Blood	Sputum			
INNO LiPA	MAIS	MAIS	MAIS	MAIS	MAIS	MAIS	MAIS	*M. avium*	*M. intracellulare* i	*M. intracellulare* ii
AccuProbe test:										
M. avium	+/−	−	−	−	+	+	−	+	−	−
M. intracellulare	−	−	−	−	−	−	−	−	+	+
Catalase activity:										
Room temperature	+	+	+	+	+	+	+	UN	UN	UN
68 °C	+	+	+	+	−	−	+	−	−	+
Nitrate reduction	−	−	−	−	−	+	−	−	−	−
Urease	+	+	+	+	+	+	+	−	−	−
Aryl sulphatase activity:										
3 days	−	−	+	+/−	+/−	+/−	+	−	−	−
14 days	−	−	+	+	+	+/−	+	−	+/−	UN
Growth temperature:										
Room temperature	+	+	+	+	+	+	+	+/−	+	+
45 °C	−	−	−	−	−	−	+	−	+/−	−

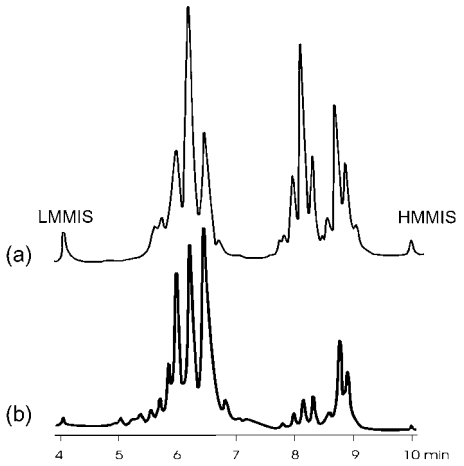

Fig. 1. HPLC profile of *Mycobacterium avium* (a) compared with *Mycobacterium colombiense* sp. nov. (b). LMMIS, Low molecular mass internal standard; HMMIS, high molecular mass internal standard.

Genotypic characterization

Several molecular methods were used to characterize the novel strains. Bacterial DNA was purified from liquid cultures as described previously (van Soolingen *et al.*, 1993).

PCR restriction analysis (PRA) and sequencing of the 65 kDa heat-shock protein gene. PRA of the *hsp*65 gene (Telenti *et al.*, 1993) is a frequently used method for the identification of mycobacteria because it is easy and rapid. Many PRA patterns are already available for recognized members of the genus (Wong *et al.*, 2003; Leao *et al.*, 2005). All of the novel isolates displayed the *M. avium* variant I (av-I) *hsp*65 gene restriction pattern (Smole *et al.*, 2002) after digestion with *Hae*III and *Bst*EII restriction enzymes. The amplified products were sequenced for a more complete characterization (model 3730 automated sequencer; Applied Biosystems). A BLAST comparison (at the NCBI database) of the sequence showed up to five single mutations when compared with the av-I *hsp*65 gene reference sequence (GenBank accession number AF126031). In contrast, the sequence displayed 100 % similarity with that of the strain recently deposited as MAIS-28 by Lebrun *et al.* (2005). An alignment of the *hsp*65 sequences examined is available as Supplementary Fig. 1 in IJSEM Online.

PCR detection of selected targets. Several PCR amplifications were performed to detect other molecular targets previously described as being characteristic of *M. avium* such as insertion sequences IS*1245* (Guerrero *et al.*, 1995) and IS*1311* (Roiz *et al.*, 1995) and the macrophage-induced gene (*mig*; Beggs *et al.*, 2000). Two isolates, 9B and 10B^T, tested negative for both insertion sequences while the other novel strains were weakly positive for one or both IS elements (data not shown). Possible cross-hybridization with an IS*1245*/*1311*-related insertion sequence in these isolates could not be discounted (Keller *et al.*, 2002).

The *mig* gene has been identified as encoding an acyl-CoA synthetase, a central enzyme in the metabolic pathway of bacteria (Morsczeck *et al.*, 2001). All of the novel isolates showed positive results for this target by PCR (data not shown) and confirmation was obtained by sequencing of the amplified products. The resulting sequences revealed the amplification of a gene encoding an acyl-CoA synthetase that was most closely related to that of *M. avium* (Beggs *et al.*, 2000; GenBank accession number U43598) with 85·5 % identity at the amino acid level (data not shown).

Gene sequencing. The 16S rRNA gene (Kirschner *et al.*, 1993) and the 16S–23S ITS 1 spacer (Novi *et al.*, 2000) were amplified and sequenced by using an automated sequencing system (model 3730; Applied Biosystems). The nucleotide sequences were compared with those in GenBank. The complete ITS 1 and 16S rRNA gene sequences were aligned with other known related mycobacterial sequences using CLUSTAL X version 1.81 software. Two separate phylogenetic trees were constructed for the ITS 1 and 16S rRNA gene sequences, using *Mycobacterium tuberculosis* and *Mycobacterium fortuitum*, respectively, as outgroups. Tree topologies were generated by using the neighbour-joining method with the Kimura two-parameter model and bootstrap analysis with 1000 replications (Figs 2 and 3).

The novel isolates displayed a new ITS 1 sequence, named MAC-X, that differed from all other previously described sequences within the complex and that was more closely related to MAC-R (Fig. 2).

A BLAST analysis of the ITS 1 sequence showed 100 % nucleotide similarity with the MAC strain MAIS-28 (Lebrun *et al.*, 2005). Strain MAIS-28 also shared some other genomic characteristics with the novel isolates such as the *M. avium* PRA pattern and the MAIS result by the INNO LiPA probe assay (Lebrun *et al.*, 2005).

The 16S rRNA gene sequence is considered the gold standard molecular methodology for the identification of mycobacteria (Tortoli, 2003; Clarridge, 2004; Devulder *et al.*, 2005). The sequence of the entire 16S rRNA gene was determined for all of the novel isolates. All of the isolates possessed identical 16S rRNA gene sequences. Comparisons of this sequence with other 16S rRNA gene sequences in GenBank indicated that it was more closely related to the sequence of *M. avium* (Fig. 3). The 16S rRNA gene sequence of the novel isolates was found to be identical to another previously submitted sequence (*Mycobacterium* sp. HSC 1852; GenBank accession number AY184225). This mycobacterial strain, isolated from a human lymphadenitis, differs from the novel isolates as it gives a negative result with the MAC AccuProbe (Pauls *et al.*, 2003) (Table 1).

Strains with similar ITS 1 and 16S rRNA gene sequences have been isolated from blood samples taken from patients

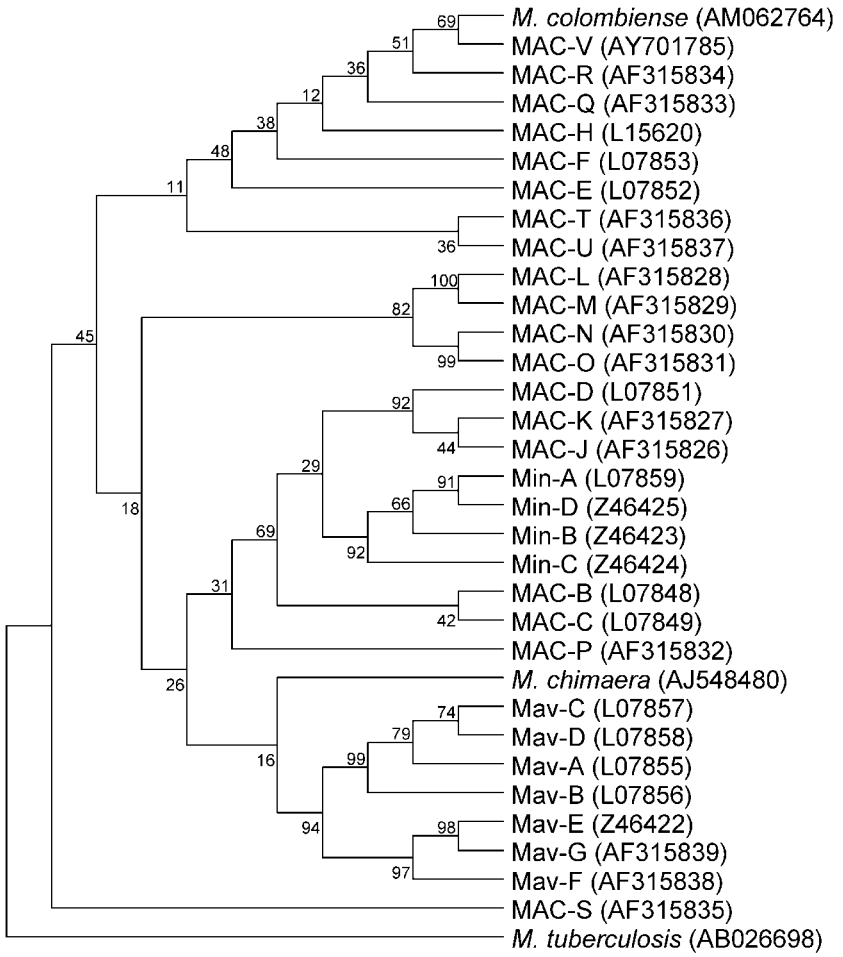

Fig. 2. Phylogenetic tree based on 16S–23S ITS 1 gene sequences showing the relationships between the sequevar of the novel species (MAC-X) and other sequevars of the MAC. Each sequence is identified by the sequevar name. GenBank accession numbers are shown in parentheses. MAC, *Mycobacterium avium* complex; Mav, *M. avium*; Min, *M. intracellulare*.

in the USA and Thailand (Turenne *et al.*, 2006). This could indicate a putative wider distribution of the novel isolates. Further studies are required to ascertain whether strains MAIS-28 (Lebrun *et al.*, 2005), HSC 1852 (Pauls *et al.*, 2003) and other isolates (Turenne *et al.*, 2006) are also members of our proposed novel species.

DNA–DNA relatedness

Global genomic relatedness, as determined by DNA–DNA hybridization, still remains the reference methodology to confirm bacterial distinction from other closely related species (Stackebrandt *et al.*, 2002; Zeigler, 2003). Experiments were performed to analyse the total genomic relationships of the novel isolates with other members of the MAC. DNA–DNA hybridization experiments were performed on membrane filters by using a dot-blot-based procedure as described previously (Jimenez *et al.*, 2004). Genomic DNA from strain 10B^T (considered as the type-strain of the proposed novel species) was used as the reference DNA. The results are summarized in Table 2. Levels of DNA–DNA hybridization between the novel isolates and strain 10B^T were greater than 75 %, indicating that they are all members of the same single species. The level of DNA–DNA hybridization between strain 10B^T and other members of the MAC varied from around 40 % with *M. avium* strains to lower values with *M. intracellulare* (24 %) and *M. chimaera* (28 %). DNA–DNA hybridization with *M. fortuitum* ATCC 6841^T, a rapidly growing mycobacterium, was as low as 12 %. These data indicate that our isolates can be distinguished as a separate novel species within the *M. avium* complex, being most closely related to *M. avium*.

Finally, the DNA–DNA relatedness value between strain 10B^T and strain 57B was at the limit for species differentiation (74 %, Table 2). This result could indicate some internal variability within this novel species.

Genetic typing

The internal variability of the isolates under study was investigated by molecular typing methods. Some of the isolates showed weakly positive bands by PCR for the two insertion sequences IS*1245* and IS*1311*. None of the isolates showed RFLP patterns under standard conditions and with both IS*1245* (van Soolingen *et al.*, 1998) and IS*1311* (Roiz *et al.*, 1995) as probes. We were therefore unable to type the isolates by this procedure. To differentiate the strains, randomly amplified polymorphic DNA (RAPD) analysis was performed according to a previously described protocol

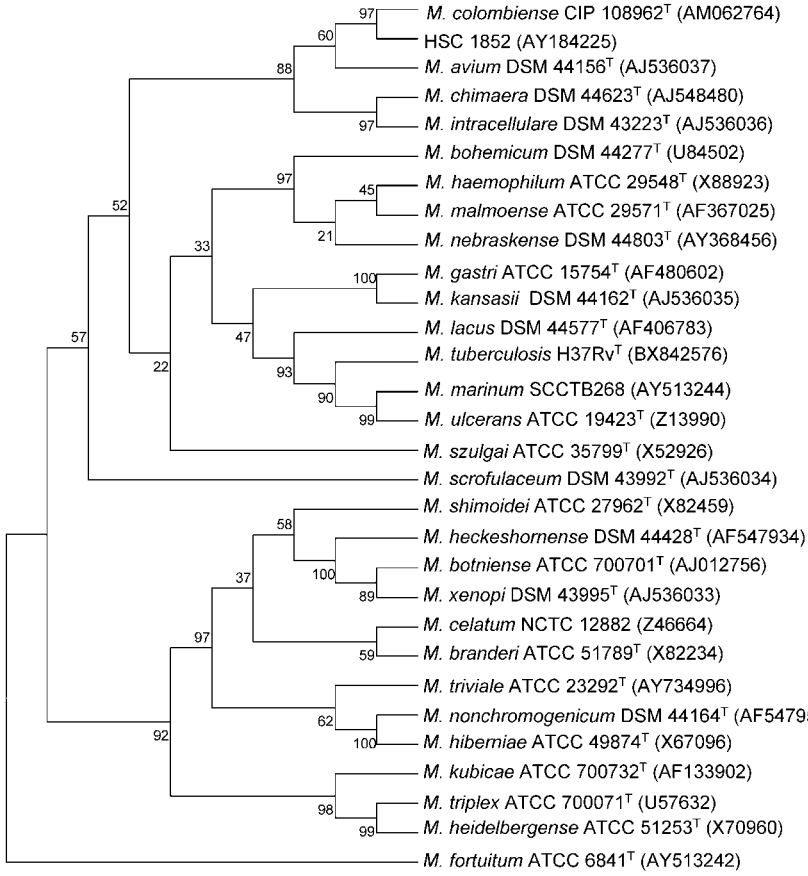

Fig. 3. Phylogenetic tree based on 16S rRNA gene sequences showing the relationships of *Mycobacterium colombiense* sp. nov. and other selected slow-growing mycobacteria. The topology was calculated using bootstrap values derived from 1000 replicates (see methods). *M. fortuitum* ATCC 6841^T was included as an outgroup. GenBank accession numbers are shown in parentheses.

(Zhang *et al.*, 1997) and oligonucleotides INS2 and M13 (Welsh & McClelland, 1990; Linton *et al.*, 1994) were used as primers. Both oligonucleotides revealed similar patterns' distribution. Up to four different patterns were identified using INS2 as a primer (see Supplementary Fig. S2 in IJSEM Online). As expected, some correlation emerged between the patterns obtained and patient/hospital origin of the strains.

Table 2. DNA–DNA hybridization between *M. colombiense* sp. nov. strain 10B^T and other members of the MAC

Mean values ± standard deviation from two separate experiments.

Strains	DNA–DNA relatedness (%)
M. colombiense	
10B^T	100
6B	97±9
7B	118±12
9B	81±12
16B	82±13
19B	107±25
57B	74±6
M. avium ATCC 25291^T	44±6
M. intracellulare (clinical isolate)	24±5
M. chimaera FI 01069^T	28±4
M. fortuitum ATCC 6841^T	12±2·5
Calf thymus DNA	4·2±0·5

Description of *Mycobacterium colombiense* sp. nov.

Mycobacterium colombiense (co.lom.bi′en.se. N.L. neut. adj. *colombiense* pertaining to Colombia, the South American country where the strains were first isolated).

Cells are acid-fast non-motile rods. Visible growth with non-pigmented rough colonies appears in 3 weeks. Growth occurs at 20 and 37 °C. Grows on Löwenstein-Jensen, Ogawa Kudoh and Sauton agars, but does not grow on MacConkey agar. Negative in tests for niacin production, acid phosphatase activity and Tween hydrolysis. Positive for catalase activity at room temperature (approx. 20 °C). Can be differentiated from the other three species within the MAC by the ability to produce urease. The HPLC pattern, although similar to that of *M. avium*, *M. intracellulare* and *M. chimaera*, allows the strains to be identified on the basis of the relative height of the peaks. Results of DNA analysis, such as the unique ITS 1 sequence (MAC-X) and the unique 16S rRNA gene sequence, allow the strains to be differentiated from other members of the MAC. The *hsp*65 gene PRA pattern is identical to that of *M. avium* variant I (av-I). DNA–DNA relatedness clearly differentiates *M. colombiense* as a separate species within the MAC.

The type-strain, isolate $10B^T$ (=CIP 108962^T=CECT 3035^T), was isolated from the blood of an HIV-positive patient in Colombia, South America.

Acknowledgements

This work has been partially supported by INCO-DEV EU grants ICA4-CT-2001-10087 and ICA4-CT-2002-10063. The authors are grateful to C. Turenne for her helpful revision, interesting comments and for allowing the use of still unpublished data.

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