

Characterization of a novel variant of *Mycobacterium chimaera*

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In this study, nonchromogenic mycobacteria were isolated from pulmonary samples of three patients in the Netherlands. All isolates had identical, unique 16S rRNA gene and 16S–23S ITS sequences, which were closely related to those of *Mycobacterium chimaera* and *Mycobacterium marseillense*. The biochemical features of the isolates differed slightly from those of *M. chimaera*, suggesting that the isolates may represent a possible separate species within the *Mycobacterium avium* complex (MAC). However, the cell-wall mycolic acid pattern, analysed by HPLC, and the partial sequences of the *hsp65* and *rpoB* genes were identical to those of *M. chimaera*. We concluded that the isolates represent a novel variant of *M. chimaera*. The results of this analysis have led us to question the currently used methods of species definition for members of the genus *Mycobacterium*, which are based largely on 16S rRNA or *rpoB* gene sequencing. Definitions based on a single genetic target are likely to be insufficient. Genetic divergence, especially in the MAC, yields strains that cannot be confidently assigned to a specific species based on the analysis of a single genetic target.

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INTRODUCTION

Nontuberculous mycobacteria (NTM) are mostly opportunistic pathogens that are common in the environment (Griffith *et al.*, 2007). The taxonomy of NTM has changed dramatically with the improvements in molecular identification techniques. Within the *Mycobacterium avium* complex (MAC), however, the taxonomy remains unresolved (Tortoli *et al.*, 2004). Currently available commercial identification kits for NTM underestimate the divergence of MAC isolates in particular, as well as NTM in

general. The use of these kits may suffice in the clinical setting, as the treatment of disease caused by MAC isolates is independent of exact speciation results (Griffith *et al.*, 2007), but it hampers understanding of the epidemiology and pathogenesis of disease caused by MAC isolates. Previous authors have suggested the presence of multiple species within the MAC based on the relatedness of internal transcribed spacer (ITS) sequevars classified in *Mycobacterium avium* and *Mycobacterium intracellulare* species compared with more heterogeneous sequevars in other MAC groups (Frothingham & Wilson, 1993; Tortoli *et al.*, 2004). Several novel species have been described as belonging to the MAC in recent years, such as *Mycobacterium colombiense*, *Mycobacterium chimaera*, *Mycobacterium vulneris*, *Mycobacterium arosiense*, *Mycobacterium timonense*, *Mycobacterium marseillense* and *Mycobacterium bouchedurhonense*.

Abbreviations: ITS, internal transcribed spacer; MAC, *Mycobacterium avium* complex; NTM, nontuberculous mycobacteria.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and 16S–23S ITS sequences are FJ794352 and FJ794353, respectively.

Table 1. Baseline characteristics of patients from whom the isolates were collected

Strain	Gender	Age	Material	No. of positive cultures	History
NLA000002037	Female	83	BAL	1/2	Asthma
NLA000200432	Male	70	Sputum	1/5	COPD
NLA000202017	Male	72	Sputum	1/4	COPD

The classification of these species was based on 16S rRNA gene sequence analysis (Tortoli *et al.*, 2004; Murcia *et al.*, 2006; Ben Salah *et al.*, 2009; van Ingen *et al.*, 2009a); however, the *rpoB* gene can also be used for species identification in MAC isolates (Ben Salah *et al.*, 2008).

Aside from the genetic divergence between species grouped in the MAC, the extent of genetic diversity within the species *M. avium* itself was recently determined using an MLST approach (Turenne *et al.*, 2008). Similarly, Park *et al.* (2010) used sequencing of 16S rRNA, ITS and *hsp65* genes to reveal polymorphisms within stains of *M. intracellulare*. Although these two studies revealed significant genetic divergence within species, all the strains still appeared to be distinguishable at the species level.

In this study, we characterized a cluster of three slowly growing, nonchromogenic MAC strains (NLA000002037, NLA000200432, NLA000202017), which proved difficult to identify at the species level by phenotypic and genotypic methods. All three strains were isolated from respiratory samples of three elderly patients who presented with acute exacerbation of chronic pulmonary disease. Clinical and demographical data from this study are summarized in Table 1. The clinical significance seems to be limited as none of the patients met the diagnostic criteria set out by the American Thoracic Society (Griffith *et al.*, 2007).

METHODS

All three isolates were subcultured on Ogawa, Middlebrook 7H10 and Stonebrink solid media, as well as in a mycobacterial growth indicator tube (MGIT) automated liquid culture system. All media were

incubated at 36 °C. Middlebrook 7H10 slants were also incubated at 25 °C, 30 °C and 45 °C.

An AccuProbe MAC probe (Gen-Probe) and INNO-LiPA MYCOBACTERIA v2 line probe assay (Innogenetics) were used for primary identification of the isolates, according to the manufacturer's instructions. To obtain identification at the species level, we sequenced the full 16S rRNA gene and 16S–23S ITS region as well as partial *hsp65* and *rpoB* genes, using previously published approaches (Springer *et al.*, 1996; Roth *et al.*, 1998; Ben Salah *et al.*, 2008; Telenti *et al.*, 1993).

For biochemical and phenotypic identification we investigated colony morphology, growth at temperatures ranging from 25 to 45 °C, niacin accumulation, nitrate reduction, β -glucosidase activity, Tween 80 hydrolysis, 3-day arylsulfatase activity, urease activity, tellurite reduction, 68 °C and semiquantitative catalase activity, growth rate, pigmentation, growth on MacConkey agar, and tolerance to thiophene-2-carboxylic hydrazide (TCH) 5 $\mu\text{g ml}^{-1}$, oleate 250 $\mu\text{g ml}^{-1}$, p-nitrobenzoic acid 500 $\mu\text{g ml}^{-1}$, thiacetazone 10 $\mu\text{g ml}^{-1}$, hydroxylamine 500 $\mu\text{g ml}^{-1}$ and isoniazid 1 $\mu\text{g ml}^{-1}$. All tests were performed on Middlebrook 7H10 agar, following previously published guidelines (Kent & Kubica, 1985).

HPLC was used to investigate the cell-wall mycolic acid composition according to previously reported methods (CDC, 1996). We used the online HPLC *Mycobacterium* library (<http://www.MycobacToscana.it/page4.htm>) for visual comparisons.

We determined MICs of rifampicin, rifabutin, isoniazid, ethambutol, streptomycin, amikacin, clarithromycin, ciprofloxacin, moxifloxacin, cycloserine, prothionamide, clofazimine and linezolid using the Middlebrook 7H10 agar dilution method (van Ingen *et al.*, 2010).

RESULTS

All three isolates gave a positive reaction with the MAC AccuProbe test. The INNO-LiPA MYCOBACTERIA v2

Table 2. Molecular identification results

The top three matches in BLAST searches are shown for 16S rRNA gene and 16S–23S ITS sequences. ITS sequevars and the proportion of matching base pairs are given in parentheses.

Gene (fragment size)	BLAST result
16S rRNA (1440 bp)	99 % <i>M. marseillense</i> 5356591 ^T (1438/1440 bp) 99 % <i>M. chimaera</i> FI-0169 ^T (1436/1440 bp) 99 % <i>M. intracellulare</i> DSM 43223 ^T (1434/1440 bp)
16S–23S ITS (282 bp)	97 % <i>Mycobacterium</i> sp. RiVM9900327 (MAC-P; 277/283 bp) 96 % <i>M. avium</i> complex ATCC 35770 (MAC-D; 272/282 bp) 96 % <i>Mycobacterium</i> sp. RiVM 9600915 (MAC-K; 271/282 bp)
<i>hsp65</i> (402 bp)	100 % <i>M. chimaera</i> FI-0169 ^T (402/402 bp)
<i>rpoB</i> (700 bp)	100 % <i>M. chimaera</i> FI-0169 ^T (700/700 bp)

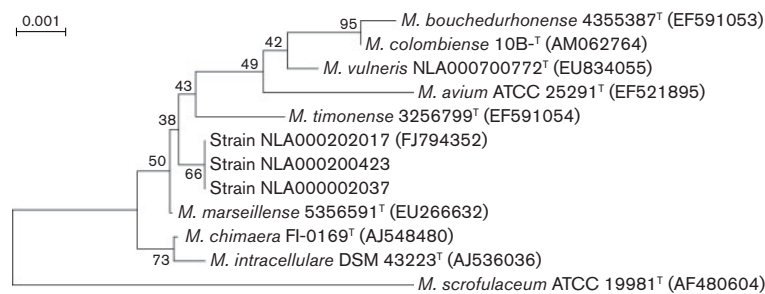


Fig. 1. Phylogenetic relationship between the novel isolates and related species of the genus *Mycobacterium*, based on 16S rDNA gene sequences. The neighbour-joining tree was created and visualized using MEGA 4.0 software (Tamura *et al.*, 2007). Bootstrap values (based on 1000 replications) are indicated at the nodes. Bar, 0.001 substitutions per nucleotide position.

line-probe assay identified the isolates as belonging to the *M. avium*–*M. intracellulare*–*M. scrofulaceum* complex but did not assign them to *M. avium*, *M. chimaera* or *M. intracellulare*, despite the presence of corresponding specific probes.

The sequencing results from this study are listed in Table 2. Sequencing of the full 16S rRNA gene and the 16S–23S ITS identified the bacteria as an *M. chimaera*-like species, with no exact match to sequences in the GenBank/EMBL (NCBI) database. The full 16S rRNA gene sequences of the three isolates were aligned with those of reference strains of the closest related mycobacteria using CLUSTAL_X software (Thompson *et al.*, 1997). The resulting tree topology, inferred by the neighbour-joining method and visualized using the MEGA 4.0 software package (Tamura *et al.*, 2007), was evaluated by bootstrap analysis based on 1000 resamplings (Fig. 1). The low bootstrap values reflect the limitation of 16S rRNA gene sequence-based identifications within MAC. The ITS sequence of the three isolates differed by six base pairs (3%) from the previously described MAC-P sequevar (see Table 2). The partial 65 kDa heat-shock protein (*hsp65*) and *rpoB* gene sequences of the three isolates proved fully identical to those of the *M. chimaera* type strains (Table 2), which does not suggest a separate species status. Multiple-sequence alignment of concatenated 16S rRNA, ITS, *hsp65* and *rpoB* gene sequences of the three isolates and those of reference strains of the closest related mycobacteria by using CLUSTAL_X software

and subsequent visualization using SplitsTree 4 software (Huson & Bryant, 2006) confirmed their phylogenetic position within the MAC as most closely related to *M. chimaera* (Fig. 2).

The HPLC pattern of the isolates comprised two clusters of peaks; the first cluster is largely conserved among MAC members and includes two major and one minor peak; the second emerges later and presents two major and two minor peaks (Fig. 3). This pattern is identical to that of *M. chimaera* and the pattern is distinct from that of the other MAC members, which usually present three clusters of peaks. The relative heights of the peaks in the second and third clusters vary; *M. avium* mostly presents lower peaks in the third cluster and *M. intracellulare* presents lower peaks in the second cluster (Tortoli *et al.*, 2004).

The results of the biochemical identification tests are given in Table 3. The MICs of the drugs in the test panel are given in Table 4; these results are similar to those of other MAC members, including *M. chimaera* (van Ingen *et al.*, 2010).

DISCUSSION

The isolates characterized in this study are phylogenetically related to *M. chimaera*, yet they cannot be assigned to this species due to their lack of the MAC-A ITS sequevar

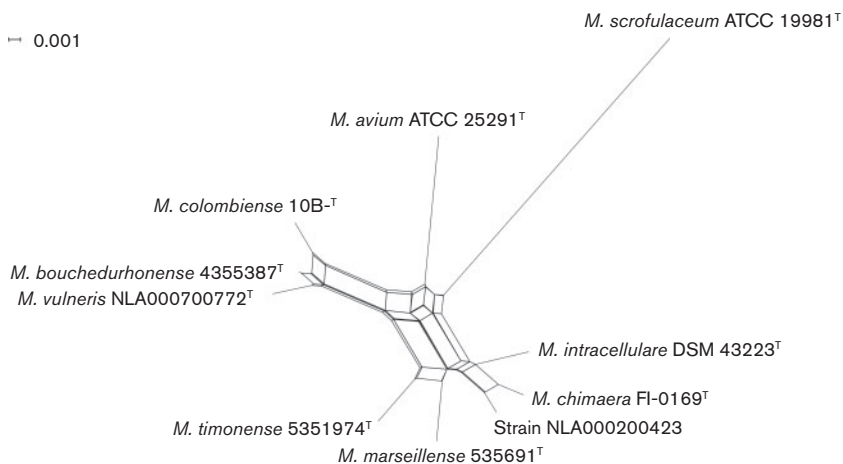


Fig. 2. Phylogenetic relationship between the novel isolates and related species of the genus *Mycobacterium*, based on concatenated 16S rRNA gene, 16S–23S internal transcribed spacer and *hsp65* and *rpoB* gene sequences. No *hsp65* gene sequence is publicly available for *M. timonense*. Bar, 0.001 substitutions per nucleotide position.

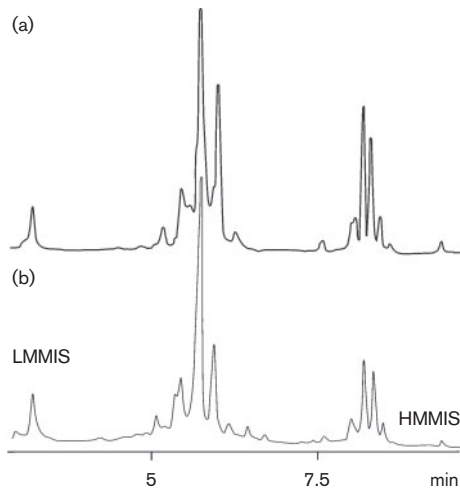


Fig. 3. HPLC results showing cell-wall mycolic acid content. Pattern (a) was obtained from the studied isolates and pattern (b) was obtained from *M. chimaera* FI-0169^T. Two clusters of peaks are apparent for both micro-organisms, which show an identical pattern. LMMIS, Low molecular mass internal standard; HMMIS, high molecular mass internal standard.

(Tortoli *et al.*, 2004). The unique 16S rRNA gene and ITS sequences and slight differences in biochemical properties (Table 3) support their distinct taxonomic position. Designating these isolates as a novel, separate species is difficult owing to the identical *hsp65* and *rpoB* gene sequences as well as the biochemical features, which largely overlap with those of *M. chimaera*. Based on the currently available data, we consider these isolates to represent a novel variant of *M. chimaera*. This does not appear to be an isolated incident pertaining exclusively to this variant and *M. chimaera*. A similar variant was noted, but not extensively typed, in a study that examined the discriminatory power of

the INNO-LiPA MYCOBACTERIA v2 assay and ITS and *hsp65* gene sequencing in a set of MAC isolates (Lebrun *et al.*, 2005). This isolate, too, lacked the MAC-A ITS sequevar but its ITS and *hsp65* gene sequences were different from the isolates in the current study. In addition, *Mycobacterium senegalense* and *Mycobacterium farcinogenes* (Hamid *et al.*, 2002) have identical *hsp65* gene sequences, whereas they have different 16S rRNA gene sequences. The evolutionary background of this phenomenon remains unknown.

There is currently no gold standard for the differentiation of species in the genus *Mycobacterium*. Both 16S rRNA and *rpoB* gene sequences (Adékambi *et al.*, 2006) have been used, although analysis of concatenated sequences of multiple housekeeping genes is considered to be superior to the use of single gene targets (Stackebrandt *et al.*, 2002). Both *hsp65* and *rpoB* gene sequencing are used for the clinical identification of NTM (Ben Salah *et al.*, 2008; Telenti *et al.*, 1993), particularly members of the MAC, to which the isolates from this study belong. For the isolates investigated in this study, however, *hsp65* or *rpoB* gene sequencing alone would have provided a false identification based on the current ITS-based species definition of *M. chimaera* determined here. Our findings support the use of sequencing of multiple housekeeping genes to identify NTM, which has also been recommended for the identification of *Mycobacterium abscessus* group members (Zelazny *et al.*, 2009).

The isolates studied here may have limited clinical relevance, as was shown in a large-scale study of *M. chimaera* isolates in Germany (Schweickert *et al.*, 2008); however, as the clinical relevance of NTM differs from species to species, exact species/subspecies identification can be of clinical importance (van Ingen *et al.*, 2009b). A multiple gene sequencing approach is likely to lead to the recognition of additional species or variants and help to establish their clinical relevance; however, the only way to achieve a new and solid taxonomy for NTM is whole genome

Table 3. Biochemical identification results for the isolated strains and *M. chimaera* FI-0169^T

All studied strains were nonchromogenic slow growers that had positive results in the AccuProbe MAC assay, had negative reactions for urease activity, Tween 80 hydrolysis, β -glucosidase activity, nitrate reduction and niacin accumulation, were tolerant to thiophene-2-carboxylic hydrazide (TCH) 5 $\mu\text{g ml}^{-1}$, thiacetazone 10 $\mu\text{g ml}^{-1}$ and isoniazid 1 $\mu\text{g ml}^{-1}$ and grew at 25 but not 45 °C. +, Positive; -, negative; v, variable.

Test	Strain			
	NLA00002037	NLA000200432	NLA000202017	<i>M. chimaera</i> FI-0169 ^T
68 °C catalase	-	+	+	+
Catalase >45 mm	+	-	-	-
Tellurite reduction	-	-	-	v
3 day Arylsulfatase	-	+	+	-
Colony morphology	Rough	Smooth	Smooth	Smooth
Growth on MacConkey agar	-	+	-	-
<i>p</i> -Nitrobenzoic acid tolerance	+	+	+	v
Hydroxylamine tolerance	+	-	-	v
Oleic acid tolerance	+	+	+	v

Table 4. MICs ($\mu\text{g ml}^{-1}$) for the isolated strains and *M. chimaera* FI-0169^T, determined by the agar dilution method

Antibiotic	Strain			
	NLA000002037	NLA000200432	NLA000202017	<i>M. chimaera</i> FI-0169 ^T
Isoniazid	2	5	5	5
Rifampicin	>5	>5	>5	>5
Ethambutol	10	5	10	10
Streptomycin	>20	>20	>20	>20
Cycloserine	>50	>50	>50	50
Prothionamide	2	2	2	2
Amikacin	>20	>20	>20	>20
Ciprofloxacin	16	>16	>16	>16
Moxifloxacin	>2	>2	>2	>2
Clofazimine	1	2	≤ 0.5	≤ 0.5
Clarithromycin	4	8	8	8
Linezolid	>2	>2	>2	>2
Rifabutin	1	1	0.5	1

sequencing of a large collection of NTM isolates. This will facilitate the establishment of a robust phylogenetic tree and more reliable knowledge of the clinical importance of different NTM on the branches and sub-branches of this tree. Based on this knowledge, decisions can be made on the species designations and rules for the description of additional NTM species.

In summary, we present molecular and biochemical data of three isolates that represent a novel variant of *M. chimaera*. To our knowledge, the existence of such variants has not previously been reported in the genus *Mycobacterium*. Analysis of these isolates has led us to question the currently used methods of species definitions for members of the genus *Mycobacterium*, which are based largely on 16S rRNA or *rpoB* gene sequencing. Definitions based on analysis of a single genetic target are likely to be insufficient. Genetic divergence, especially among MAC members, may result in strains that cannot be confidently assigned to a single species.

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