Tropical Medicine and International Health

VOLUME 2 NO. 2 PP 200–207 FEBRUARY 1997

Characterization of *Mycobacterium avium* complex related mycobacteria isolated from an African environment and patients with AIDS

P.-A. Fonteyne[1], Z. M. Kunze[2], H. De Beenhouwer[1], J. M. Dumonceau[1], D. J. Dawson[3], J. J. McFadden[2] and F. Portaels[1]

[1] *Mycobacteriology Unit, Department of Microbiology, Institute of Tropical Medicine 'Prince Leopold', Antwerpen, Belgium*
[2] *Molecular Microbiology Group, School of Biological Sciences, University of Surrey, Guildford, UK*
[3] *Centre for Public Health Sciences, Queensland Health, Brisbane, Australia*

Summary Thirteen isolates from African AIDS patients and from the environment in Zaire were identified as members of the *Mycobacterium avium* complex by phenotypic tests. RFLP analysis showed that the isolates belong to a genetically homogeneous cluster. The 16S rRNA sequence analysis suggests a close relationship with the P-49 strain (ATCC 35847), a reference strain for the serotype 7 of *M. avium* complex. This work shows the close relationship between certain *M. avium* complex strains responsible for disseminated infection in AIDS patients and *M. avium* complex strains isolated from the environment in Zaire. Further, our findings confirm that atypical mycobacteria may disseminate in AIDS patients in Africa and suggest that infection in these patients probably originates in their environment.

keywords AIDS, MAC, *Mycobacterium intracellulare*, 16S rRNA, Africa

correspondence P.-A. Fonteyne, Mycobacteriology Unit, Department of Microbiology, Institute of Tropical Medicine 'Prince Leopold', Nationalestraat 15, 2000 Antwerpen, Belgium. e-mail: fonteyne@itg.be

Introduction

The importance of *Mycobacterium avium* complex (MAC) disseminated infection in patients with AIDS has been thoroughly documented during the last decade (for review, see Inderlied *et al.* 1993). These infections primarily develop in industrialized countries. Very few data are available about MAC infections in developing countries. Cases have been reported but no extensive work has been done to characterize the isolates (Barreto *et al.* 1993; Idigbe *et al.* 1994; Koivula *et al.* 1996). For epidemiological purposes, however, and for a better understanding of the pathogenesis of disease due to MAC, it is important to characterize the strains grouped in MAC at species and subspecies level.

The MAC includes two species, *M. avium* and *M. intracellulare*, as well as a cluster of related taxa. *Mycobacterium paratuberculosis* and *M. lepraemurium* are considered as subspecies of *M. avium* (Shinnick & Good 1994). Most clinical MAC isolates can be assigned to either *M. avium* or *M. intracellulare* by biochemical assays or hybridization with commercially available DNA probes. However, some MAC from clinical and environmental sources cannot be identified as *M. avium* or *M. intracellulare*. Serotyping has been very useful in order to constitute a collection of reference strains representative of

Table 1 RFLP-type-H-MAC isolates

ITM strain no.	Specimen	Origin	Date of primary culture
11074	sputum	patient 1	13/12/84
925	blood	patient 2	21/01/86
926	sputum	patient 2	11/03/86
1329	sputum	patient 3	07/08/86
1331	faeces	patient 3	11/08/86
1788	BAL*	patient 4	03/02/87
1794	sputum	patient 4	11/02/87
1812	sputum	patient 4	11/02/87
1818	faeces	patient 4	04/02/87
2069	sputum	patient 5	29/09/87
4541	water	lower Zaire	12/05/72
4543	algae	lower Zaire	07/10/72
5280	fish bowel	lower Zaire	17/05/72

* Broncho-alveolar lavage.

the MAC diversity. But the main value of serotyping lies in subtyping rather than identification. Sequence data analysis seems to be the most definitive technique to characterize MAC related strains (Böddinghaus *et al.* 1990; Kirschner & Böttger 1992; Soini *et al.* 1994; Frothingham & Wilson 1993).

Between 1986 and 1991, MAC identified by phenotypic procedures at the Institute of Tropical Medicine, Antwerp (ITM), were isolated from patients originating from Western and Central Africa who are being treated for AIDS in Belgium. In a previous study using DNA probes identifying restriction fragment length polymorphisms (RFLP) (Hampson *et al.* 1989; Kunze *et al.* 1990), it was shown that most of these 'African' strains constitute a genetically homogenous group (RFLP type H) distinct from MAC from human, animal, and environmental sources in industrialized countries (USA, Europe and Australia). Some MAC related environmental strains isolated between 1970 and 1974 from various locations in Zaire had also been characterized by the same RFLP H type pattern (Portaels 1978a; Portaels *et al.* 1991). The pMB22 probe used for the RFLP analysis, being non-specific for MAC, has prompted the decision to further identify the phenotypic and genotypic characteristics of RFLP H type strains. The aim of this study was to specify the relationship between RFLP type H strains and other MAC, and to determine features which can be used to differentiate them.

Materials and methods

Strains

Clinical strains were isolated from 5 different patients (Table 1), according to previously described methods (Jenkins *et al.* 1982). All patients had lived in Africa for more than 5 years; for all of them, the first positive isolation occurred within 7 months of their arrival in Belgium. The environmental strains (Table 1) were isolated and first identified in 1972 (Portaels 1978). The cultures were kept freeze-dried and regrown on Löwenstein–Jensen (LJ) for the present study.

Identification

Cultural, biochemical and physiological properties were determined as described previously (Vincent-Lévy-Frébault & Portaels 1992). The arylsulphatase assay was performed according to the method of Tarshis modified by Tomioka *et al.* (1990).

Mycolic acid pattern

Mycolic acid patterns were determined as follows. The mycobacteria were cultivated for 3 weeks at 37°C on LJ. The mycolic acids were released from 5–10 mg of bacteria by alkaline hydrolysis followed by esterification with iodomethane (Dobson *et al.* 1985). Methyl esters of the mycolic acids were

separated by one-dimensional thin-layer chromatography on two silica gel plates and revealed by spraying the plates with a 5% (wt/vol) solution of phosphomolybdic acid in ethanol. The patterns were interpreted according to Daffé *et al.* (1983).

Serotyping

Isolates were screened with antisera specific for serovars of *M. avium*, *M. intracellulare*, and *M. scrofulaceum* according to the method of Reznikov and Leggo (1972).

GEN PROBE testing

GEN PROBE testing (GEN-PROBE Inc., San Diego, CA) was performed according to the manufacturer's instructions. The three different chemiluminescent labelled AccuProbe for *M. avium* complex, *M. avium* and *M. intracellulare* were used.

Sequencing of 16S rRNA gene

A total of 1398 base pairs of the 16S rRNA gene (corresponding to positions 65–1521) on the *Escherichia coli* sequence (Brosius *et al.* 1978) were sequenced for the clinical isolates 926 and 1818. Partial sequencing of the 16S rRNA gene was performed for the environmental strains 4543 and 5280 (from positions 65–255 and 1350–1521). Starting from bacterial lysates, two overlapping fragments of the 16S rRNA gene were amplified and sequenced. The primers used for amplification and sequencing reactions are described below:

- G64R, (44-64), *GAGAATTC*CGTGCTTAACAC ATGCAAGTCG.
- M82, (54-82), CATGCAAGTCGAACGGAAAGG.
- r290B, (305-290), GGCCGGACACCCTCTC.
- RogS, (518-536), CAGCAGCCGCGGTAATAC.
- 585R, (565-585), *CTGAATTC*TGGGCGTAAAG AGCTCGTAGG.
- rM592B, (609-592), TTTCACGAACAACGCGAC.
- 1005R, (986-1005), GGAATTCTGGGTTTGAC ATGCACAGGA.
- r1333, (1350-1333), GATTACTAGCGACTC CGA.
- s1366, (1348-1366), ATCGCAGATCAGCAA CGC.
- r1475R, (1491-1475), *ACAAGCTT*CGTCCCA ATCGCCGATC.
- rRogB, (1542-1522), AAGGAGGTGATCCAGC CGCA.

The nucleotide positions indicate the target sites of the primers in 16S rRNA as represented in the sequence of *E. coli* (Brosius *et al.* 1978); primers RogS and rRogB are described in Böddinghaus *et al.* (1990).

The amplification was performed in a total volume of 50 ml containing 20 pmol of each primer, 1 unit of Ampli Taq DNA polymerase (Roche Molecular Systems, Branchburg, New Jersey, USA), 200 mM of each deoxynucleotide triphosphate, 1.5 mM $MgCl_2$, 0.1% Triton X100, 10 mM Tris/HCl pH 8.4 (20°C). The mixture was overlaid with mineral oil and placed in a thermal cycler (Hybaid Ltd, Teddington, Middlesex, UK). After the initial denaturation step (5 min at 94°C) the mixture was submitted to 30 amplification cycles (45 s 94°C, 1 min at the appropriate annealing temperature, 45 s at 72°C). The last cycle was followed by a final extension step (7 min at 72°C).

Forty ml of the PCR product were used for sequencing. The strands were separated with streptavidin-coated magnetic beads (Dynabeads M-280 streptavidin, Dynal SA, Oslo, Norway) and a magnet (Dynal MPC-6, Dynal SA) according to the manufacturer's instructions. Each DNA strand was then sequenced with the appropriate primer with the T7 DNA sequencing kit (Pharmacia Biotech Benelux, Roosendaal, NL) with α-^{33}P dATP. Three ml of each solution was loaded onto a 6% polyacrylamide gel. After 3 or 6 hours electrophoresis, the gels were fixed in 10% acetic acid, 10% methanol, and dried. The gel was exposed to X-ray film for 12 hours.

Sequence analysis

The 16S rRNA sequences were compared with the sequences for 19 reference strains belonging to different serovars of *M. avium* and *M. intracellulare* published by Böddinghaus *et al.* (1990), the sequences for the strains *M. intracellulare* ATCC 15985 (EMBL access codes X52925 and M59276)

Table 2 Phenotypic characteristics of type H strains isolated from African environment and patients with AIDS

Characteristic	Type H	*M. avium**	*M. intracellulare*[1]	*M. simiae*-like[2]
Scotochromogenicity	−	−	−	−
Photochromogenicity	−	−	−	−
Growth in presence of				
Isoniazid (10 μg/ml)	+	M	M	M
Hydroxylamine (250 μg/ml)	+	F	M	+
p-Nitrobenzoate (500 μg/ml)	+	+	+	+
Thiophene-2-carboxylic hydrazide (2 μg/ml)	+	+	+	+
Enzymatic properties				
Urease	+	−	−	+
Tween hydrolysis (10 days)	−	−	−	−
Acid phosphatase	−	−	−	−
Catalase (semi-quantitative)	−	−	F	M
Nitrate reduction	−	−	−	−
Niacin production	−	−	−	−
Mycolate types				
α-Mycolates	+	+	+	+
α′-Mycolates	−	−	−	+
Ketomycolates	+	+	+	+
ω-Carboxymycolates	+	+	+	−

+>85% positive; − <15% positive; M, 50–85% positive; F, 15–49% positive.
[1] Data from Vincent Lévy-Frébault and Portaels (1992).
[2] Data from Portaels *et al.* (1987).

and *M. avium* DSM 43216 (EMBL access codes X52918 and M59264), published by Rogall *et al.* (1990a,b), the sequences for two *M. avium* clinical isolates (EMBL access codes M29572 and M29573) published by Stahl *et al.* (1990) and the sequences for 5 *M. intracellulare* strains (EMBL access codes M61682, M61683, M61684, M61685, M61686) and 7 *M. avium* strains (EMBL access codes M61673, M61667, M61668, M61669, M61670, M61671, M61672) from Urbance *et al.* (unpublished).

Results

Phenotypic characterization

All type H strains presented the same phenotypic characteristics, given in Table 2. According to classic identification schemes (Jenkins *et al.* 1982) the strains described in this study are slow growing acid-fast bacilli with an identification pattern compatible with *M. avium*, *M. intracellulare* or some *M. simiae*-like organisms producing a low catalase activity (Portaels *et al.* 1987) (positive urease). However, their mycolic acid profiles (α, keto and ω-carboxy-mycolates) clearly differentiate them from *M. simiae* (α, α′ and keto-mycolates) (Vincent-Lévy-Frébault & Portaels 1992).

Two additional phenotypic tests considered useful for the differentiation between *M. avium* and *M. intracellulare*, i.e. growth at 45°C and the level of arylsulphatase activity (Tomioka *et al.* 1990) were used. The arylsulphatase test gives the OD value higher than 0.2, considered as indicative of *M. intracellulare* for 5 isolates out of 11. The incapacity to grow at 45°C, indicative of *M. intracellulare*, is observed for all tested strains.

Most isolates tested gave multiple or non-typeable seroagglutination patterns. All typeable clinical isolates showed non-specific agglutination with at least the antiserum for *M. avium* ser. 2. Multiple isolates from the same patient gave identical reaction patterns.

GEN PROBE testing

No hybridization was detected in a test performed with the *M. avium* and *M. intracellulare* probes.

However, all isolates scored positive with the probe for MAC.

16S rRNA, DNA sequence analysis

The 16S rRNA gene sequence of isolates 926, 1818, 4543 and 5280 were identical. The highest percentage of homology was obtained with the sequence published by Böddinghaus *et al.* (1990) for the serovar 7, reference strain Runyon P-49 (ATCC 35847) (MIs7, Figure 1), the only difference being a single A to G substitution in the region where sequences are available for all MAC strains (Position B, Figure 1).

Discussion

The initial phenotype identification pattern of the RFLP type H strains is compatible with MAC or *M. simiae*-like organisms isolated from armadillos (Portaels *et al.* 1987). The strains are positive for urease activity. However, they produce α, keto and ω-carboxy mycolates, which clearly distinguishes these strains from *M. simiae* (α, α′ and keto-mycolates). The absence of growth at 45°C is more in keeping with the species *M. intracellulare* than *M. avium*.

Most RFLP H type isolates showed non-specific reaction with various antisera for MAC serovars and do not belong to described serovars. The patterns of reaction by isolates from the same patient agree, confirming that these 10 isolates represent 5 patient strains.

However, these results confirm that serotyping should not be seen as a means of identifying MAC strains, but rather as a basis for subtyping them, once the species *M. avium* or *M. intracellulare* has been established. The same general comment can be made for the 2 other tests that discriminate between *M. avium* and *M. intracellulare* (arylsulphatase and 45°C). The highly conserved RFLP pattern indicates the close genetic relationship of the H type strains and strongly suggests that in spite of the variability of the arylsulphatase activity, these strains are a homogeneous group.

The strains react with the newer version of Accu-Probe *Mycobacterium avium* complex. The sequence of this commercial probe is not known. The GEN-PROBE company has introduced this new version of the MAC probe in order to detect clinical isolates which were not recognized by the *M. avium* and *M. intracellulare* Accu-Probe (Bull & Shanson 1992).

The previously published 16S rRNA sequences of MAC strains can be classified to 8 different types representative for the MAC heterogeneity, 5 for *M. intracellulare* (MI in Figure 1) 3 for *M. avium* (MA in Figure 1).

In the hypervariable region comprising helix 10, the RFLP H type sequences is closer to the *M. intracellulare* than to the *M. avium* 16S rRNA sequences (Figure 1 positions B, C and D). Nevertheless, the RFLP H type sequence at positions A, E and F (Figure 1) indicates patterns which are present in all *M. avium* sequences and in none of the sequences available for *M. intracellulare* MIs7 excepted (Figure 1). These observations underline the importance of considering different segments of the 16S rRNA sequence for comparative sequencing for taxonomic purpose.

The sequences of the H strains are very similar to the sequence published for ATCC 35847 (Runyon P-49, serovar 7) and to a lesser extent ATCC 35770 (Melnick, serovar 18) (Böddinghaus *et al.* 1990), and the *M. intracellulare* sequences M61682, M61683, M61684 (Urbance *et al.*, unpublished) (MIs7, MIs18 and MIRRDJ in Figure 1). The Runyon P-49 and Melnick strains do not hybridize either with the *M. avium* or *M. intracellulare* Accuprobe (Wayne *et al.* 1993).

Wayne *et al.* (1993) suggest that the Runyon P-49 strain should be considered as a *M. scrofulaceum*. The comparison of the 16S rRNA sequence determined for the Runyon P-49 strain (Böddinghaus *et al.* 1990) with that obtained for *M. scrofulaceum* (Rogall *et al.* 1990b) invalidates this hypothesis.

Clinical evidence (which is not discussed in the present paper) as well as the multiplicity of isolations, especially from patients 2 and 4, shows that the RFLP H type strains are able to disseminate in patients at the last stage of AIDS. The phenetic and genetic similarity of the RFLP H type strains from African AIDS patients and those isolated from the environment in Zaire strongly suggests that the source of MAC infection in these patients originates from their environment. These RFLP H type strains also appear to be able to colonize an HIV positive patient for a long time. Indeed, some patients

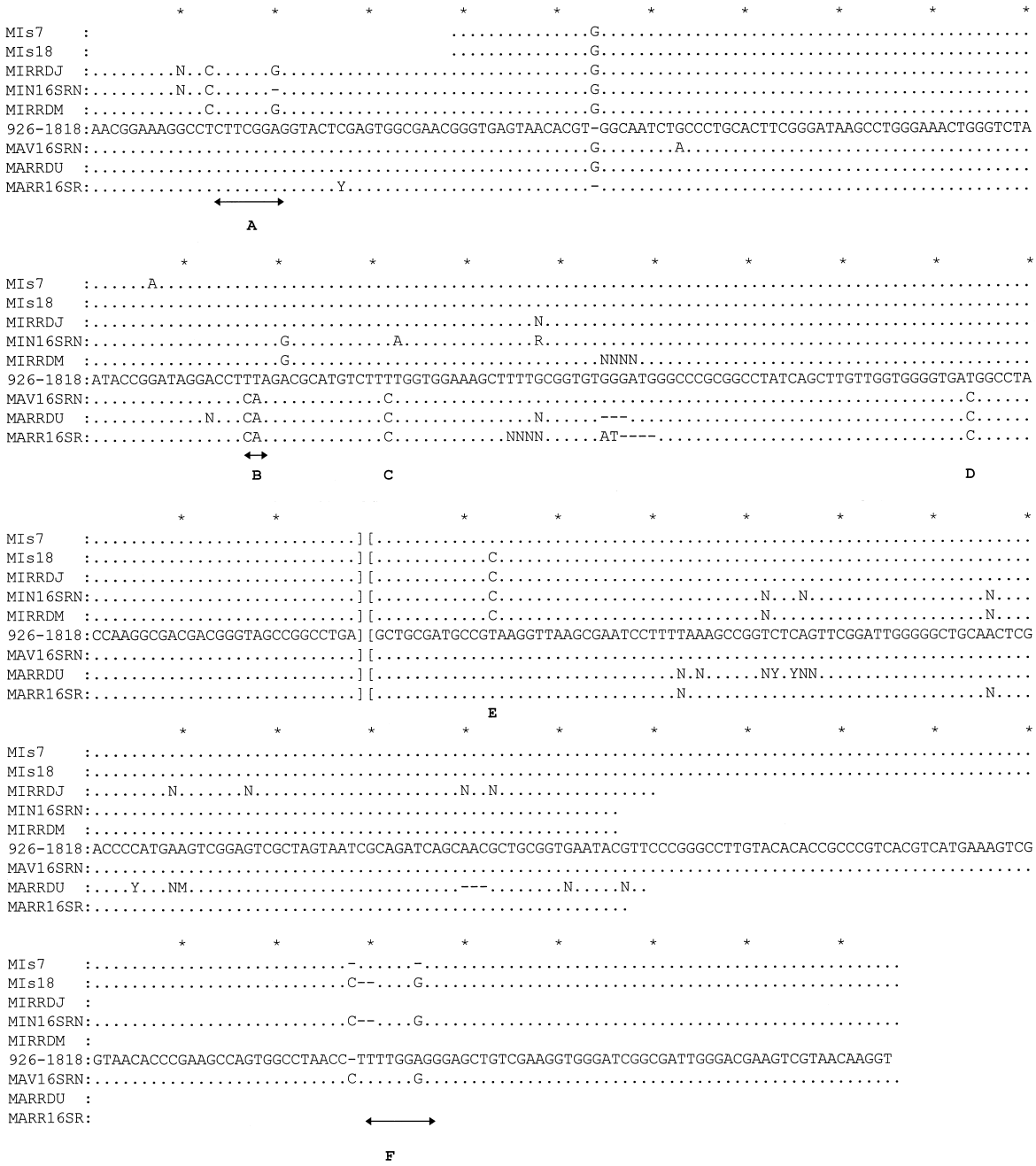

Figure 1 The sequences presented here correspond to positions 65 to 302, and 1255 to 1475 on the sequence of the non-coding strand of 16S rRNA gene in *E. coli* (Bro 78). These have been published and/or introduced in the EMBL data bank by different authors. The bibliographic references are given in the text. The accessing numbers are following: MIRRDJ (EMBL, AC: M61682), MIRRDM (EMBL, AC: M61685), MARRDU (EMBL, AC: M61667), MARR16SR (EMBL, AC: M29573), MIN16SR (EMBL, AC: X52927), and MAV16SRN (EMBL, AC: X52918). s7 and s18 have been published by Böddinghaus *et al.* (1990).

developed a mycobacterial disease only a few months after their arrival in Belgium (Portaels 1995).

This ability has been frequently described as an important prerequisite for an opportunistic pathogen. Two recent prospective studies have demonstrated that HIV-positive patients with colonization of respiratory or gastrointestinal tract by MAC had a greater risk of MAC dissemination than those without colonization (Havlik *et al.* 1993; Chin *et al.* 1994).

MAC disseminated infection has been recently reported in Kenya, using GEN-PROBE culture confirmation tests for MAC and/or *M. avium* (Gilks *et al.* 1995). The authors did not specify which of these two probes was positive for the 3 isolates. More recently, Koivula *et al.* (1996) reported on the isolation of MAC Accu-Probe positive strains from patients in Guinea-Bissau. It would be interesting to compare these strains with the type H strains.

Even if tuberculosis is by far the most important mycobacterial infection in Africa, the possibility of an atypical mycobacteriosis has to be taken into consideration when dealing with African patients with AIDS. Our paper emphasizes the clinical and epidemiological importance of a correct and detailed phenotypic and genetic analysis of clinical and environmental isolates. It also establishes that atypical mycobacteria may disseminate in AIDS patients in Africa, and strongly suggests that the source of MAC infection in these patients originates from their environment.

Acknowledgements

This work was supported by grants from the European Commission TS2-0080-UK, the Belgian Funds for Medical Scientific Research (grant 3.4601.90), and the Damien Foundation.

We thank P. Schel and P. de Rijk for their excellent technical assistance.

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