

## Biochemical Characteristics and Fatty Acid Compositions of Some Armadillo-derived Mycobacteria and Their Relation to *Mycobacterium gordonae*

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The long-chain components of 75 strains of mycobacteria, cultivated from *Mycobacterium leprae*-infected or non-infected armadillos, and of eight clinical and 15 environmental isolates of *M. gordonae*, were compared. Four major groups could be distinguished based on the presence of 10-methyloctadecanoic (tuberculostearic) and 2-methyl 3-hydroxyeicosanoic acids and secondary alcohols (2-octadecanol and 2-eicosanol). Some heterogeneity was found in strains assigned to *M. gordonae*: the characteristic absence of tuberculostearic acid and secondary alcohols and the presence of the branched C14 and the hydroxylated C20 acids were seen in only 34 of the 49 strains studied. Three strains were identified as *M. malmoense*, one as *M. kansasii*, ten as belonging to the *M. avium*-*M. intracellulare*-*M. scrofulaceum* complex and eight as belonging to new groups of armadillo-derived mycobacteria (ADM 1, ADM 2 and ADM 3) by conventional bacteriological tests and fatty acid compositions, though *M. malmoense* was heterogeneous in its fatty acids composition. Four strains, identified as *M. avium* by conventional tests, differed from this species by their fatty acid compositions. Thirteen strains showed some similarity to *M. simiae* and ten strains differed from all other known mycobacteria.

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### INTRODUCTION

The isolation of several strains of *Mycobacterium* from wild armadillos (F. Portaels & G. P. Walsh, unpublished results) and armadillos experimentally infected with *Mycobacterium leprae* (Portaels *et al.*, 1982, 1985) was recently reported. The majority of the strains have been identified as belonging to the *Mycobacterium avium*-*intracellulare*-*scrofulaceum* (MAIS) complex or as *Mycobacterium gordonae* (Portaels *et al.*, 1985). In addition, unclassified armadillo-derived mycobacteria (ADM) have been found. In a comprehensive study involving several laboratories, five groups of ADM could be distinguished, each different from all other mycobacterial species presently recognized (Portaels *et al.*, 1986).

The potential of fatty acid analysis in systematic studies of mycobacteria has been pointed out by several authors (Minnikin & Goodfellow, 1980; Daffé *et al.*, 1983; Minnikin *et al.*, 1984; Dobson *et al.*, 1985; Larsson *et al.*, 1985a). The objective of the present study was to evaluate the fatty and mycolic acid compositions of several ADM using gas chromatographic (GC) and thin-layer chromatographic (TLC) analyses. For comparison, clinical and environmental isolates of *M. gordonae* were also studied.

### METHODS

*Mycobacteria.* Ninety-eight strains were analysed (Table 1), 75 of which had been isolated from 45 tissues (livers, spleens, lymph nodes) of 35 different armadillos (Portaels *et al.*, 1985). Fifteen strains of *M. gordonae* were isolated from the soil in Zaire and Florida (Portaels, 1978), and eight *M. gordonae* strains were human isolates

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Abbreviations: MAIS, *M. avium*-*intracellulare*-*scrofulaceum* complex; ADM, armadillo-derived mycobacteria.

Table 1. Fatty and mycolic acid composition of *M. gordonae* and some armadillo-derived mycobacteria

The strain numbers refer to the collection of F. Portaels, Institute of Tropical Medicine, Antwerp, Belgium.

GC group*	Mycolic acid patterns†	Source of the strains					Wild armadillos	Clinical samples
		Environment		M. leprae infected armadillos		Wild armadillos		
		Zaire	Florida	Experimentally	Naturally			
Group A <i>M. gordonae</i>	$\alpha$ , methoxy, keto	4324, 4433 4438, 4463 4509	8895, 9105 9150, 9195 9261, 9300 9301, 9375	10182, 10183 10701, 10702 10706, 10712 10716, 10827	10305, 10363 10424, 10540 10674	8760, 8761 8775, 10358 10870, 10965 10988, 10990		
Group B <i>M. gordonae</i>	$\alpha$ , methoxy, keto		10316	10709, 10718	10218, 10288 10299, 10300 10301, 10426 10538, 10539 10542 10302, 10425 10545, 10720 10219, 10296 10365, 10547 10680, 10719 10364, 10541 10809			
' <i>M. avium</i> '	$\alpha$ , $\alpha'$ , keto							
' <i>M. simiae</i> '	$\alpha$ , $\alpha'$ , keto				10066, 10069 10543	10613, 10708 10710, 10734		
<i>M. malmoense</i>	$\alpha$ , $\alpha'$ , keto				9807			
<i>M. kansasii</i>	$\alpha$ , methoxy, keto							
Unclassified	$\alpha$ , methoxy, keto							
Group C <i>M. gordonae</i> MAIS	$\alpha$ , keto, $\omega$ -carboxy $\alpha$ , keto, $\omega$ -carboxy		9191T, 9229		10431	10703, 10728 10729, 10736 11011, 11012 11015, 11065 11068		
ADM 1	$\alpha$ , methoxy, keto, $\omega$ -carboxy				8346, 8634			
ADM 2	$\alpha$ , methoxy, keto, $\omega$ -carboxy				8507	10360G		
ADM 3	keto, $\omega$ -carboxy				8637, 9091	10722, 10723		
Unclassified	$\alpha$ , keto, $\omega$ -carboxy				9251, 10122 10673			
Group D Unclassified	$\alpha$ , $\alpha'$ , keto, $\omega$ -carboxy $\alpha$ , keto, $\omega$ -carboxy				10432	10537 10684		

\* See results for characteristic components of each group.

† Terminology of Minnikin &amp; Goodfellow (1980).

recovered from sputum specimens and skin biopsies (Petroff, 1915). The strains were identified as described by Jenkins *et al.* (1982).

The mycobacteria were cultivated on Löwenstein-Jensen or Ogawa egg yolk medium without malachite green (Portaels *et al.*, 1985). Cells were collected after 1–6 weeks incubation at 37 °C, killed with formalin (1%, v/v) and freeze-dried before being subjected to TLC and GC analysis.

*TLC.* Mycolic acid methyl esters, obtained after heating the cells in an alkaline methanolic solution followed by esterification of the acids using iodomethane (Dobson *et al.*, 1985), were analysed by two-dimensional TLC (Minnikin *et al.*, 1984; Dobson *et al.*, 1985).

*GC.* Freeze-dried cells were heated overnight in methanolic HCl after which the fatty acid methyl esters and long-chain alcohols were extracted (Larsson, 1983). Trifluoroacetylation was used for identification of hydroxylated esters and alcohols. The extracts were introduced and separated on a narrow-bore fused silica capillary column using splitless injection (Larsson & Odham, 1984) as previously reported (Larsson *et al.*, 1985b).

## RESULTS

Of the heterogeneous GC profiles obtained, four major groups (A, B, C and D) of mycobacteria could be distinguished based on the presence of 2-methyltetradecanoic, 10-methyloctadecanoic (tuberculostearic) and 2-methyl 3-hydroxyeicosanoic acids, and secondary alcohols (2-octadecanol and 2-eicosanol). Variations in incubation time and culture medium did not affect these components significantly. A number of compounds eluting after tuberculostearate were occasionally detected; however, most of them were found to originate from small amounts of the culture medium itself present as a contaminant. The results are summarized in Table 1.

Thirty-four strains (13 from armadillos, 13 from the soil and all the eight clinical isolates) were classified as belonging to group A. They were characterized by the presence of the branched-chain C14 and the hydroxylated C20 acid, and the absence (or presence in trace amounts) of tuberculostearic acid and secondary alcohols. These strains were very homogeneous, not only according to the GC profiles but also the mycolate patterns; they all contained  $\alpha$ -, methoxy- and ketomycolates. These strains were identified as *M. gordonae*.

Thirty-seven strains, all isolated from armadillos, belonged to group B. Tuberculostearate, but neither the branched-chain C14 nor the hydroxylated C20 acid, nor any of the secondary alcohols, was detected. Twelve strains produced the typical *M. gordonae* mycolate pattern and were identified as *M. gordonae* by conventional tests. Four strains produced  $\alpha$ -,  $\alpha'$ - and ketomycolates, just as *M. simiae*, but they had the cultural, physiological and biochemical properties of *M. avium*. Thirteen additional strains contained the same mycolates as *M. simiae* but differed from *M. simiae* in that they were non-chromogenic, did not produce niacin and gave less than 45 mm foam in the semi-quantitative catalase test (Table 2). Three strains were identified as *M. malmoense*: Tween 80 was hydrolysed after 1 d, the strains had low catalase activity (less than 45 mm foam) and were sensitive to isoniazid (10  $\mu\text{g ml}^{-1}$ ). One of these strains (10541) was subjected to TLC analysis of its surface lipids (Jenkins, 1980); the unique pattern of spots characteristic for *M. malmoense* was found (P. A. Jenkins, personal communication). The mycolate pattern of two of the strains was identical to that of *M. simiae* ( $\alpha$ -,  $\alpha'$ - and ketomycolates) while one strain (10809) contained  $\alpha$ - and  $\alpha'$ -mycolates, just as *M. chelonae* (Daffé *et al.*, 1983; Minnikin *et al.*, 1984). The three *M. malmoense* strains differed also with respect to their GC patterns: two of them contained an unidentified fatty acid eluting just after C20:0, whereas in the remaining strain (10809) this acid was absent. One strain (9807), which contained 2,4-dimethyl tetradecanoic acid, was identified as *M. kansasii*. Its mycolate pattern was similar to that of *M. gordonae*. Four strains with similar mycolate compositions ( $\alpha$ -, methoxy- and ketomycolates) formed a heterogeneous group according to their cultural, physiological and biochemical properties, and were not considered as belonging to any of the presently recognized mycobacterial species.

Tuberculostearate and secondary alcohols were found in appreciable amounts in the 25 strains (including three *M. gordonae* strains) in group C. Ten of the strains belonged to the MAIS complex and contained  $\alpha$ -, keto- and  $\omega$ -carboxymycolates, this being the characteristic pattern for this complex. Eight strains were divided into homogeneous groups: ADM 1, ADM 2 and

Table 2. *Characteristics of M. simiae and related strains isolated from armadillos*

All the strains tested grew in the presence of thiophene-2-carboxylic acid hydrazide ( $1 \mu\text{g ml}^{-1}$ ) or *p*-nitrobenzoic acid ( $500 \mu\text{g ml}^{-1}$ ), but none grew in the presence of 5% (w/v) NaCl; none of the strains reduced nitrate, produced acid phosphatase or hydrolysed Tween 80.

Character	Percentage of strains positive in each test	
	Results from Wayne <i>et al.</i> (1983)	' <i>M. simiae</i> ' from armadillos
Photochromogenic	90	0
Pigment produced	90	0
Growth in media containing:		
isoniazid ( $10 \mu\text{g ml}^{-1}$ )	70	46
hydroxylamine hydrochloride ( $250 \mu\text{g ml}^{-1}$ )	100	69
Catalase (> 45 mm)	100	0
Urease	100	92
Niacin accumulated	46	0
No. of strains tested	11	13

ADM 3 (Portaels *et al.*, 1986). The ADM 1 and ADM 2 strains contained  $\alpha$ -, methoxy-, keto- and  $\omega$ -carboxymycolates, and the ADM 3 strains keto- and  $\omega$ -carboxymycolates. Four additional group C strains formed a heterogeneous group of mycobacteria which appeared different from all other known mycobacteria: three strains contained  $\alpha$ -, keto- and  $\omega$ -carboxymycolates, and the remaining strain (10537) contained  $\alpha$ -,  $\alpha'$ -, keto- and  $\omega$ -carboxymycolates.

The GC profiles of the two group D strains differed from those of group C by the absence of tuberculostearate. Both group D strains contained  $\alpha$ -, keto- and  $\omega$ -carboxymycolates. One strain (10432) was rapid-growing and the other (10684) slow-growing; both were regarded as 'unclassified' because they did not fit into the established taxonomic schemes of Jenkins *et al.* (1982).

#### DISCUSSION

The present study shows that mycobacteria with very different properties can be isolated from armadillos. Clearly, care must be taken to avoid the presence of such bacteria in preparations of *M. leprae* intended for purposes such as vaccine production.

Long-chain secondary alcohols (2-octadecanol and 2-eicosanol), which are released along with  $\omega$ -carboxymycolates from wax ester mycolates by acid or alkaline hydrolysis, are useful chemical markers in mycobacterial differentiation (Minnikin & Goodfellow, 1980; Daffé *et al.*, 1983; Larsson, 1983; Larsson *et al.*, 1985*a, b*). However, these alcohols (and the  $\omega$ -carboxymycolates), which have so far consistently been detected in strains belonging to the MAIS complex (Larsson, 1983; Daffé *et al.*, 1983; Minnikin *et al.*, 1984), were not found in four of the studied strains tentatively identified by conventional tests as belonging to *M. avium*. Additional studies, such as DNA:DNA homology analysis, are required to confirm their identities; indeed, it has been reported that species which are very similar on the basis of their phenetic characters may show low DNA relatedness among themselves (Portaels *et al.*, 1986).

The heterogeneity found for *M. gordonae* is noteworthy. Absence of tuberculostearic acid and secondary alcohols, and the presence of the branched-chain C14 and the hydroxylated C20 acid, are considered as characteristic features for this species (Tisdall *et al.*, 1979; Julack *et al.*, 1980). These characteristics were found for all of the eight clinical *M. gordonae* isolates, 13 of the 26 strains isolated from armadillos and 13 of the 15 environmental strains. However, 15 *M. gordonae* strains (in groups B and C) produced tuberculostearate in appreciable amounts (about 5% of the total fatty acids) whereas the branched-chain C14 acid was not detected. In addition, three of these strains (belonging to group C) contained the secondary alcohols. None of the *M.*

*gordonae* strains isolated from armadillos experimentally infected with *M. leprae* belonged to group A, and none of the environmental and clinical isolates belonged to group B. A more comprehensive study on *M. gordonae* of different origins should be undertaken in order to relate the different fatty acid compositions to results of genome studies such as DNA:DNA homology analysis. More extensive studies are also necessary for clarifying the taxonomic positions of the two strains classified in group D.

It has been reported that some proposed intermediate MAIS strains may in fact be regarded as belonging to *M. simiae* although photochromogenicity is lacking (Wayne *et al.*, 1983). Even though *M. simiae* might be quite variable in its pigment production (Wayne *et al.*, 1981), our '*M. simiae*' strains all differed from the reference strains by having low catalase activity. Our strains, although isolated from different animals, formed a very homogeneous group of mycobacteria; additional studies are required before it can be suggested that they might constitute a new species.

The *M. malmoense* strains were readily identified by different biochemical tests. The two types of mycolate and fatty acid patterns found may be related to the different lipid patterns reported by Jenkins (1985).

GC analysis of 2-octadecanol and 2-eicosanol is a useful method for detecting certain mycobacterial contaminants in preparations of *M. leprae* (Larsson *et al.*, 1985*b*). However, the armadillo tissues may also be contaminated with mycobacterial species lacking  $\omega$ -carboxymycolates. In view of this, 2-methyltetradecanoic and 2-methyl 3-hydroxyeicosaanoic acids should prove to be useful compounds for revealing the presence of at least certain strains of *M. gordonae*. It seems important to search for additional chemical markers in order to be able to detect all the various cultivable mycobacteria which may be present as contaminants in *M. leprae* preparations.

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