Gas-chromatographic lipid profiles in identification of currently known slowly growing environmental mycobacteria

Pirjo Torkko,^{1,2} Marja-Leena Katila² and Merja Kontro¹†

Correspondence

Pirjo Torkko

pirjo.torkko@ktl.fi

¹Laboratory of Environmental Microbiology, National Public Health Institute, PO Box 95, FIN-70701 Kuopio, Finland

²Department of Clinical Microbiology, Kuopio University Hospital, PO Box 1777, FIN-70211 Kuopio, Finland

Cellular fatty acid analysis by GLC is widely used in the species identification of mycobacteria. Combining mycolic acid cleavage products with shorter cellular fatty acids increases the informative value of the analysis. A key has been created to aid in the identification of all currently known slowly growing environmental species. In this scheme, the species are classified into six categories, each characterized by a combination of fatty markers shared by those species. Within each category, individual species may be distinguished by the presence or absence of specific marker substances, such as methyl-branched fatty acids or secondary alcohols. This study also describes earlier unpublished GLC profiles of 14 rare, slowly growing, environmental mycobacteria, *Mycobacterium asiaticum*, *Mycobacterium botniense*, *Mycobacterium branderi*, *Mycobacterium conspicuum*, *Mycobacterium cookii*, *Mycobacterium doricum*, *Mycobacterium heckeshornense*, *Mycobacterium heidelbergense*, *Mycobacterium hiberniae*, *Mycobacterium kubicae*, *Mycobacterium lentiflavum*, *Mycobacterium scrofulaceum*, *Mycobacterium triplex* and *Mycobacterium tusciae*. Though no single identification technique alone, even sequencing of an entire single gene such as 16S rRNA, can identify all mycobacterial species accurately, GLC has proven to be both reliable and reproducible in the identification of slowly growing mycobacteria. In cases of earlier unknown species, it generates useful information that allows their further classification and may lead to the description of novel species.

Received 1 November 2002

Accepted 18 December 2002

INTRODUCTION

Environmental mycobacteria are an emerging problem as causative agents of diseases, especially among immunocompromised hosts. Many mycobacterial species previously regarded as non-pathogenic to man are today classified as inducers of mycobacterioses, this occurring predominantly in AIDS patients (Carbonara *et al.*, 2000; Luque *et al.*, 1998; Mayo *et al.*, 1998). The precise identification of species is of great importance for appropriate patient management. Environmental microbial flora, also rich in mycobacteria, represent a reservoir of previously unknown species (Torkko *et al.*, 2002). In the 1980s, the genus *Mycobacterium* amounted to about 40 documented species. Today, the number has increased to over 90, and novel species, both saprophytic and potential pathogens, are continually being

introduced (Tortoli *et al.*, 2001b; Willumsen *et al.*, 2001; Wilson *et al.*, 2001; Torkko *et al.*, 2002). The taxonomic classification of a new bacterial species is based on the genetic and biochemical characteristics of a group of identical or highly similar unclassifiable isolates. The chemical analysis of their lipid composition is regarded as essential for categorization of a new species (Vincent Lévy-Frébault & Portaels, 1992). Both fatty acid analysis by GLC and mycolic acid analysis by HPLC are commonly applied techniques (Luquin *et al.*, 1991; Butler & Guthertz, 2001).

In the routine identification of isolates recovered from both clinical and environmental sources, GLC analysis has proven to be highly applicable for the identification of the increasing number of validly described mycobacterial species. It has also been found to be a helpful tool in classifying isolates that do not meet the identification criteria of currently accepted species (Brander *et al.*, 1992; Koukila-Kähkölä *et al.*, 1995; Torkko *et al.*, 2000, 2001, 2002). If the GLC analysis is extended to cover both mycolic acid cleavage products

†Present address: Department of Ecological and Environmental Sciences, University of Helsinki, Niemenkatu 73, FIN-15140 Lahti, Finland.

Abbreviation: MACP, mycolic acid cleavage product.

(MACPs) and cellular fatty acids, the method is increasingly reliable for species identification (Chou *et al.*, 1998; Müller *et al.*, 1998; Torkko *et al.*, 1998).

In the present work, the main focus was on GLC identification of recently described or rare slowly growing species and their differentiation from the well-established species. The identification scheme devised relied on visual assessment of the fatty acid profile. Three rapid biochemical tests, which were found to be useful for final confirmation of certain species, were incorporated into the scheme.

METHODS

Bacterial strains. Strains used in this study were mostly type strains, originating from several type culture collections (Table 1). They were stored in Middlebrook 7H9 broth (Difco) at -80°C and subcultured for GLC analyses on Middlebrook 7H11 agar supplemented with OADC enrichment (Difco) at 36°C . Haemin and mycobactin J were respectively added for growth of *Mycobacterium haemophilum* and *Mycobacterium genavense*. *Mycobacterium cookii*, *M. haemophilum* and *Mycobacterium marinum* were incubated at 30°C and *Mycobacterium botniense*, *Mycobacterium heckeshornense* and *Mycobacterium xenopi* at 42°C . For this study, cells were harvested after 3–5 weeks incubation to ensure the production of all fatty acids.

GLC method. In the direct transesterification procedure, the bound fatty acids were cleaved from cells by mild acid hydrolysis and derivatized to the corresponding methyl esters to convert them into volatile compounds suitable for GLC. The method was described previously in detail (Jantzen *et al.*, 1989; Torkko *et al.*, 1998) and was used with minor modifications. In practice, one loopful of bacteria was harvested into acidic methanol and incubated for 16 h at 85°C . Fatty acid methyl esters were extracted with *n*-hexane. The analyses were performed with a Perkin-Elmer AutoSystem gas-liquid chromatograph, equipped with a fused-silica capillary column coated with methylpolysiloxane (NB-30, $25\text{ m} \times 0.32\text{ mm} \times 0.25\text{ }\mu\text{m}$; HNU-Nordion). TurboChrom Workstation software (version 6.1.2.0.1; Perkin-Elmer) was used in the operation of sampling, analysis and integration of the chromatographic data. The carrier gas was helium at a pressure of 8 lb in^{-2} (55.2 kPa). The injector and flame-ionization detector temperatures were 325°C and the oven temperature was programmed from 125°C (hold for 1 min) to 280°C , rising by $10^{\circ}\text{C min}^{-1}$ and holding at 280°C for 7.5 min. Sample size was $1\text{ }\mu\text{l}$ in the splitless injection; the split was opened after 1 min. Individual fatty acids were identified by comparing their retention times with those of a standard mixture of straight-chain saturated fatty acid methyl esters between C_{14} and C_{26} . Identification of methyl-branched fatty acids and alcohols was based on the appropriate retention time relationships observed in previously published profiles of known mycobacterial species. The identity of fatty marker substances was additionally determined by mass spectrometry (MS) using a Hewlett Packard G1800A GCD System chromatograph equipped with an electron ionization detector, an HP-5 ($30\text{ m} \times 0.25\text{ mm} \times 0.25\text{ }\mu\text{m}$) column and an HP 7673 automatic sampler, as described previously in detail (Torkko *et al.*, 1998).

Biochemical methods. Tests for Tween 80 hydrolysis and semi-quantitative catalase were performed as described previously (Vincent Lévy-Frébault & Portals, 1992; Torkko *et al.*, 1998, 2000). Urease activity was determined using commercial discs (Rosco), following the manufacturer's recommendations but using overnight incubation at 36°C .

Table 1. Mycobacterial reference strains representing the species included in the study

Species	Strain
<i>Mycobacterium asiaticum</i>	ATCC 25274
<i>Mycobacterium asiaticum</i>	ATCC 25276 ^T
<i>Mycobacterium avium</i>	ATCC 15769
<i>Mycobacterium bohemicum</i>	DSM 44277 ^T
<i>Mycobacterium botniense</i>	ATCC 700701 ^T
<i>Mycobacterium branderi</i>	ATCC 51789 ^T
<i>Mycobacterium celatum</i> type 1	ATCC 51131 ^T
<i>Mycobacterium celatum</i> type 2	ATCC 51130
<i>Mycobacterium celatum</i> type 3	NCTC 12882
<i>Mycobacterium conspicuum</i>	DSM 44136 ^T
<i>Mycobacterium cookii</i>	ATCC 49103 ^T
<i>Mycobacterium doricum</i>	DSM 44339 ^T
<i>Mycobacterium gastri</i>	ATCC 15754 ^T
<i>Mycobacterium genavense</i>	ATCC 51234 ^T
<i>Mycobacterium gordonae</i>	ATCC 14470 ^T
<i>Mycobacterium haemophilum</i>	ATCC 29548 ^T
<i>Mycobacterium heckeshornense</i>	DSM 44428 ^T
<i>Mycobacterium heidelbergense</i>	ATCC 51253 ^T
<i>Mycobacterium hiberniae</i>	ATCC 49874 ^T
<i>Mycobacterium interjectum</i>	ATCC 51457 ^T
<i>Mycobacterium intermedium</i>	ATCC 51848 ^T
<i>Mycobacterium intracellulare</i>	ATCC 13950 ^T
<i>Mycobacterium kansasii</i>	ATCC 12478 ^T
<i>Mycobacterium kubicae</i>	CIP 106428 ^T
<i>Mycobacterium lentiflavum</i>	ATCC 51985 ^T
<i>Mycobacterium malmoense</i>	ATCC 29571 ^T
<i>Mycobacterium marinum</i>	ATCC 927 ^T
<i>Mycobacterium nonchromogenicum</i>	ATCC 19530 ^T
<i>Mycobacterium palustre</i>	DSM 44572 ^T
<i>Mycobacterium scrofulaceum</i>	ATCC 19981 ^T
<i>Mycobacterium shimoidei</i>	ATCC 27962 ^T
<i>Mycobacterium simiae</i>	ATCC 25275 ^T
<i>Mycobacterium szulgai</i>	ATCC 35799 ^T
<i>Mycobacterium terrae</i>	ATCC 15755 ^T
<i>Mycobacterium triplex</i>	ATCC 700071 ^T
<i>Mycobacterium triviale</i>	ATCC 23292 ^T
<i>Mycobacterium tusciae</i>	DSM 44338 ^T
<i>Mycobacterium xenopi</i>	ATCC 19250 ^T

RESULTS AND DISCUSSION

The cellular fatty acids present in all of the mycobacterial species examined were hexadecanoic (16:0), tetradecanoic (14:0), hexadecenoic (16:1), octadecanoic (18:0) and octadecenoic (18:1) acids, as determined previously by Luquin *et al.* (1991). MACPs ranging from eicosanoic acid (20:0) to hexacosanoic acid (26:0) were detected in varying combinations and secondary alcohols and methyl-branched fatty acids were recovered in some of the species. On the basis of fatty acid, MACP and alcohol composition, the GLC profiles of slowly growing mycobacteria could be divided into six categories that formed the basis for our identification scheme. As shown in the key (Fig. 1), each main path was

Fig. 1. A fatty acid/alcohol marker-based key for the identification of slowly growing environmental mycobacteria.

characterized by a combination of fatty markers regarded as being typical for that category (Table 2). Each category included several species, some with and others without species-specific lipid markers. Whenever more than one species had an approximately similar basic profile without any distinguishing species-specific markers, one to three auxiliary tests were used to confirm the identification. Only tests that were quick to perform, gave reproducible results and had a high discriminatory power in the separation of the species with similar combinations of lipid content were used (Table 2).

Category 'Avium'

The category 'Avium' encompasses ten species: *Mycobacterium avium*, *Mycobacterium bohemicum*, *M. cookii*, *Mycobacterium hiberniae*, *Mycobacterium intracellulare*, *Mycobacterium nonchromogenicum*, *Mycobacterium scrofulaceum*, *Mycobacterium shimoidei*, *Mycobacterium terrae* and *Mycobacterium tusciae*. The major MACP, tetracosanoic acid (24:0), is accompanied by two secondary alcohols, 2-octadecanol (2-OH-18:0 alc) and 2-eicosanol (2-OH-20:0 alc) (Table 2; Fig. 2). Hexacosanoic acid (26:0) is not detected or it is present in only minute amounts. Some of the species in the 'Avium' category have specific lipid markers, allowing definite species identification (Table 2). The species lacking specific markers, *M. avium* complex, *M. terrae*, *M. scrofulaceum*, *M. cookii* and *M. scrofulaceum*, can be separated with the help of biochemical tests. For example, *M. terrae* is differentiated by a positive Tween 80 hydrolysis test result. In those isolates negative for Tween 80 hydrolysis, tests for urease and semi-quantitative catalase can separate *M. avium* complex from *M. scrofulaceum* and *M. cookii* (Table 2) but molecular probes are necessary for the separation of the two clinically important species among the *M. avium* complex, *M. avium* and *M. intracellulare* (Viljanen *et al.*, 1993; Tortoli *et al.*, 2001a). Our earlier results have highlighted a weakness in the use of AccuProbe for the *M. avium* complex (Gen-Probe). A novel species, *Mycobacterium*

palustre, can hybridize with the *M. avium* complex AccuProbe (Torkko *et al.*, 2002) and it could therefore be misidentified as *M. avium* complex if the identification is reliant on this probe test alone. However, in contrast to *M. avium* complex, *M. palustre* can hydrolyse Tween 80. This simple test should always be applied in the verification of yellow isolates hybridizing with *M. avium*-complex AccuProbe. The GLC scheme applied easily separates *M. palustre*, which has a species-specific GLC profile (see Category 'Palustre'). The identification of *M. scrofulaceum* is confirmed by positive results in urease and semi-quantitative catalase tests. Rough colony morphology, intense yellow pigment and the inability to grow at 36 °C can separate *M. cookii* from *M. scrofulaceum* and also from yellow-pigmented strains of *M. avium* complex.

Category 'Triviale'

The category 'Triviale' is composed of ten species, *Mycobacterium asiaticum*, *Mycobacterium doricum*, *Mycobacterium gastri*, *Mycobacterium gordonae*, *M. haemophilum*, *Mycobacterium kansasii*, *Mycobacterium kubicae*, *M. marinum*, *Mycobacterium szulgai* and *Mycobacterium triviale*. In the basic profile represented by *M. triviale*, the main MACP is 24:0 and no secondary alcohols are present. Species other than *M. triviale* contain combinations of species-specific markers that enable their correct identification (Table 2). *M. asiaticum* was placed in the 'Triviale' category on the basis of the GLC profile of the type strain (ATCC 25276^T). In a visual inspection, the GLC profile of another reference strain of *M. asiaticum* appeared to be identical. Unexpectedly, GLC-MS analysis indicated that the two peaks following 18:0 (Fig. 3) were not identical in the two strains. They were 2-methyloctadecanoic (2-Me-18:0) and nonadecanoic acid (19:0) in the *M. asiaticum* type strain (ATCC 25276^T), compared with tuberculostearic acid (10-Me-18:0) and 2-OH-20:0 alc in *M. asiaticum* ATCC 25274. The amounts of both 19:0 and 2-OH-20:0 alc were small. In 16S rDNA sequencing, *M. asiaticum* ATCC 25274 had a one base

Table 2. Fatty acid and MACP markers, pigment production and selected biochemical tests as a basis for species identification of slowly growing environmental *Mycobacterium* species, with special emphasis on recently classified species

For each GLC category, the typical lipid markers are given in parentheses. Results for Tween 80 hydrolysis, urease and semi-quantitative catalase (>45 mm foam) are scored as positive, negative or variable (v).

Species	Species-specific markers*	Pigment†	Tween	Urease	Catalase	Reference(s) describing the profile
Category 'Avium' (24:0, 2-OH-18:0 alc, 2-OH-20:0 alc)						
<i>M. avium</i>	None	None‡	—	—	—	Chou <i>et al.</i> (1996), Garcia-Barceló <i>et al.</i> (1993), Jantzen <i>et al.</i> (1989), Larsson <i>et al.</i> (1985), Luquin <i>et al.</i> (1991)
<i>M. bohemicum</i>	2-Me-20:0, 2,4-diMe-22:0, 2,4,6-triMe-22:0, 2,4,6-triMe-24:0	Yellow ^{sc}	v	+	+	Torkko <i>et al.</i> (2001)
<i>M. cookii</i>	None	Yellow ^{sc}	—	—	+	This study (Fig. 2)
<i>M. hiberniae</i>	(8,15-diMe-22:1 alc), 8,17-diMe-24:1 alc	Red ^{sc}	—	—	+	This study (Fig. 2)
<i>M. intracellulare</i>	None	None	—	—	—	Chou <i>et al.</i> (1996), Luquin <i>et al.</i> (1991)
<i>M. nonchromogenicum</i>	8,15-diMe-22:1 alc, 8,17-diMe-24:1 alc	None	+	—	+	Torkko <i>et al.</i> (1998)
<i>M. scrofulaceum</i>	None	Yellow ^{sc}	—	+	+	This study (Fig. 2)
<i>M. shimoides</i>	2-OH-22:0 alc	None	+	—	—	Koukila-Kähkölä <i>et al.</i> (2000)
<i>M. terrae</i>	(8,17-diMe-24:1 alc)	None	+	—	+	Torkko <i>et al.</i> (1998)
<i>M. tusciae</i>	22:0>24:0	Yellow ^{sc}	+	+	—	This study (Fig. 2)
Category 'Triviale' (24:0)						
<i>M. asiaticum</i>	2-Me-18:0§, 2,4-diMe-20:0	Yellow ^{ph}	+	—	+	This study (Fig. 3)
<i>M. doricum</i>	2-OH-18:0 alc, (2-OH-20:0 alc)	Yellow ^{sc}	—	+	—	This study (Fig. 3)
<i>M. gastri</i>	2,4-diMe-14:0	None	+	+	—	Larsson (1983)
<i>M. gordonae</i>	2-Me-14:0, lack of TBSA	Yellow ^{sc}	+	—	+	Luquin <i>et al.</i> (1991); Chou <i>et al.</i> (1996)
<i>M. haemophilum</i>	22:0>24:0	None	—	—	—	Portaels <i>et al.</i> (1993)
<i>M. kansasii</i>	2-Me-14:0, 2,4-diMe-14:0	Yellow ^{ph}	+	+	+	Chou <i>et al.</i> (1996), Garcia-Barceló <i>et al.</i> (1993), Jantzen <i>et al.</i> (1989), Luquin <i>et al.</i> (1991)
<i>M. kubicae</i>	2-Me-14:0	Yellow ^{sc}	+	—	+	This study (Fig. 3)
<i>M. marinum</i>	10-Me-16:0, 2,4-diMe-16:0, (26:0)	Yellow ^{ph}	+	+	—	Chou <i>et al.</i> (1996), Jantzen <i>et al.</i> (1989), Luquin <i>et al.</i> (1991)
<i>M. szulgai</i>	2-Me-14:0, 10-Me-16:0, 2,4-diMe-20:0, 2,4,6-triMe-22:0	Yellow ^{ph,sc}	+	+	+	Chou <i>et al.</i> (1996), Luquin <i>et al.</i> (1991), Müller <i>et al.</i> (1998)
<i>M. triviale</i>	None	None	+	v	+	Luquin <i>et al.</i> (1993)
Category 'Xenopi' (26:0>24:0, 2-OH-20:0 alc)						
<i>M. botniense</i>	2-OH-22:0 alc, 2,4,6,x-tetraMe-20:0, 2,4,6,x,x-pentaMe-22:0	Yellow ^{sc}	—	—	—	This study (Fig. 4)

<i>M. branderi</i>	None	None	—	—	—	This study (Fig. 4)
<i>M. celatum</i>	None	None [‡]	—	—	—	Butler <i>et al.</i> (1993)
<i>M. heckeshornense</i>	2-OH-22:0 alc>2-OH-20:0 alc	Yellow ^{sc}	—	—	—	This study (Fig. 4)
<i>M. xenopi</i>	(12:0), 2-OH-22:0 alc>2-OH-20:0 alc	Yellow ^{sc}	—	—	—	Alugupalli & Larsson (1992), Butler <i>et al.</i> (1993), Chou <i>et al.</i> (1996), Jantzen <i>et al.</i> (1989), Larsson <i>et al.</i> (1989), Luquin <i>et al.</i> (1991)
Category 'Simiae' (26:0>24:0)						
<i>M. genavense</i>	None	None	—	+	+	Chou <i>et al.</i> (1998)
<i>M. heidelbergense</i>	2-Me-20:0, (2,4-diMe-22:0), 2,4,6-triMe-24:0	None	+	+	—	This study (Fig. 5)
<i>M. lentiflavum</i>	None	Yellow ^{sc}	—	—	—	This study (Fig. 5)
<i>M. malmoense</i>	2-Me-20:0, (2,4-diMe-22:0), 2,4,6-triMe-24:0	None	+	v	+	Chou <i>et al.</i> (1996), Jantzen <i>et al.</i> (1989), Luquin <i>et al.</i> (1991), Valero-Guillén <i>et al.</i> (1988)
<i>M. simiae</i>	None	Yellow ^{ph}	—	+	+	Chou <i>et al.</i> (1998), Garcia-Barceló <i>et al.</i> (1993)
<i>M. triplex</i>	None	None	—	+	+	This study (Fig. 5)
Category 'Interjectum' (24:0, 26:0, 2-OH-18:0 alc, 2-OH-20:0 alc)						
<i>M. conspicuum</i>	2-Me-12:0, 2-Me-14:0	None	+	—	—	This study (Fig. 6)
<i>M. interjectum</i>	2-Me-10:0, 2-Me-20:0, 2,4-diMe-22:0, 2,4,6-triMe-24:0	Yellow ^{sc}	+	+	+	Torkko <i>et al.</i> (2002)
Category 'Palustre' (24:0, 26:0)						
<i>M. intermedium</i>	12-Me-12:0, 2-Me-14:0, 2,4-diMe-20:0, 2,4,6-triMe-22:0	Yellow ^{ph}	+	v	+	Torkko <i>et al.</i> (2002)
<i>M. palustre</i>	2-Me-10:0, 2-Me-19:0, 2-Me-20:0, 2,9-diMe-20:0, 14-phe-14:0, 2-Me-16-phe-16:0	Yellow ^{sc,ph¶}	+	+	—	Torkko <i>et al.</i> (2002)

*Me, Methyl; diMe, dimethyl; triMe, trimethyl; tetraMe, tetramethyl; pentaMe, pentamethyl; phe, phenyl; TBSA, tuberculostearic acid (10-methyloctadecanoic acid). Substances in parentheses may be detected in minor amounts.

†sc, Scotochromogenic; ph, photochromogenic.

‡Older cultures may develop yellowish pigment (Wayne *et al.*, 1993; Butler *et al.*, 1993).

§See text.

||Photochromogenic at 25 °C, scotochromogenic at 37 °C.

¶Scotochromogenic at 36 °C, 85 % of isolates photochromogenic at 42 °C.

Fig. 2. GLC profiles of *M. cookii*, *M. scrofulaceum*, *M. tusciae* and *M. hiberniae* in the category 'Avium'. FID, Flame-ionization detector. See Table 2 for explanation of other abbreviations.

difference from the type strain (Turenne *et al.*, 2001), at position 1020 of the 16S rDNA. *M. doricum*, also included in this category, contains only trace amounts of 2-OH-20:0 alc (Tortoli *et al.*, 2001b).

Category 'Xenopi'

The species placed in the category 'Xenopi' have a GLC pattern characterized by the presence of 2-OH-20:0 alc and a large amount of 26:0 (26:0 > 24:0). This category consists of *M. botniense*, *Mycobacterium branderi*, *Mycobacterium celatum*, *M. heckeshornense* and *M. xenopi*. The GLC profiles of *M. branderi* and *M. celatum* are characterized by their large

Fig. 3. GLC profiles of *M. asiaticum*, *M. doricum* and *M. kubicae* in the category 'Triviale'.

amounts of 2-OH-20:0 alc. In *M. branderi*, the amount of 26:0 is always greater than that of 24:0, whereas the three subtypes of *M. celatum* (types 1, 2 and 3) differ in their relative amounts of 24:0 and 26:0. Though *M. branderi* and *M. celatum* are indistinguishable by both GLC and biochemical tests (Koukila-Kähkölä *et al.*, 1995), *M. branderi* is always non-pigmented whereas, in our experience, *M. celatum* always produces variable amounts of yellow pigment. A recent report has indicated that the tellurite tolerance test could be used to differentiate between these two species (Wolfe *et al.*, 2000). The GLC profiles of *M. botniense*, *M. heckeshornense* and *M. xenopi* contain large amounts of 2-docosanol (2-OH-22:0 alc). Of these species, *M. botniense* is separated by the presence of two specific markers (Table 2; Fig. 4), one of which co-elutes with 2-OH-22:0 alc (Torkko *et al.*, 2000). Differentiation of *M. heckeshornense* from *M. xenopi* is problematic (Roth *et al.*, 2000, 2001; Richter *et al.*, 2001) and, to our knowledge, reliable separation between the two can only be attained by 16S rDNA sequencing at present.

Category 'Simiae'

The category 'Simiae' consists of *M. genavense*, *Mycobacterium heidelbergense*, *Mycobacterium lentiflavum*, *Mycobacter-*

Fig. 4. GLC profiles of *M. botniense*, *M. branderi* and *M. heckeshornense* in the category 'Xenopi'.

Fig. 5. GLC profiles of *M. heidelbergense*, *M. lentiflavum* and *M. triplex* in the category 'Simiae'.

ium malmoense, *Mycobacterium simiae* and *Mycobacterium triplex*. The major MACP is 26:0, and its amount is always greater than that of 24:0. The basic profile of this category is similar to that of *Mycobacterium tuberculosis* with the exception that the 14:0 peak is always substantial in this category, whereas it is a minor peak in *M. tuberculosis*. *M. malmoense* and *M. heidelbergense* have the same species-specific fatty markers. However, in the profile of *M. malmoense*, the amount of 2,4,6-trimethyltetracosanoic acid (2,4,6-triMe-24:0) is always greater than that of 24:0. This ratio is opposite to the profiles of the two isolates of *M. heidelbergense* available for our analyses so far. However, more isolates of *M. heidelbergense* should be analysed to confirm this observation. Fastidious growth and the requirement for mycobactin *J* separate *M. genavense* from the other species in this group (Böttger *et al.*, 1993). Three species, *M. lentiflavum*, *M. simiae* and *M. triplex*, have highly similar profiles (Fig. 5) (Chou *et al.*, 1998; Garcia-Barceló *et al.*, 1993). *M. triplex*, with its variants (Suomalainen *et al.*, 2001), does not produce pigment. The scotochromogenic *M. lentiflavum* is negative in most biochemical tests (Springer *et al.*, 1996). It grows as pinpoint colonies, in contrast to the photochromogenic *M. simiae*, growth of which is more abundant. According to

Chou *et al.* (1998), *M. simiae* can be differentiated from *M. genavense* and *M. tuberculosis* by the presence of *cis*-11-hexadecenoic acid (*cis*-11-16:1) and the lack of *cis*-10-hexadecenoic acid (*cis*-10-16:1) in the GLC pattern.

Categories 'Interjectum' and 'Palustre'

The remaining two categories, 'Interjectum' and 'Palustre', are characterized by the presence of several methyl-branched fatty markers and both 24:0 and 26:0 as MACPs. The differentiating feature of the category 'Interjectum' is the presence of the secondary alcohols 2-OH-18:0 alc and 2-OH-20:0 alc. *Mycobacterium interjectum* and *Mycobacterium conspicuum* differ in their specific fatty markers (Table 2; Fig. 6) and are therefore easily identifiable by GLC. In contrast, the category 'Palustre' lacks secondary alcohols. The two species in this category, *M. palustre* and *Mycobacterium intermedium*, have unique combinations of methyl-branched fatty acids (Torkko *et al.*, 2002).

Conclusions

GLC and HPLC are widely used in the routine identification of mycobacteria, in spite of the increasing number of genetic

Fig. 6. GLC profile of *M. conspicuum* in the category 'Interjectum'.

methods designed for the identification of mycobacteria. In our experience, GLC is reliable and reproducible in the identification of both pathogenic and environmental slowly growing mycobacteria. In our identification scheme, we have added one to three biochemical tests to confirm the initial identification result.

The criteria for identification of a species should never be based on a single isolate, regardless of the method used. This also applies to GLC analysis. More than one isolate has to be analysed to ensure the significance of the marker substances that are being regarded as typical of this species. This principle has also been used as the basis of the scheme developed, though, for the present article, the GLC profiles of the type strains were selected. In this study, our major emphasis was placed on rare species whose fatty profiles have remained unavailable in the literature. Though the number of isolates of some species has remained small to date, more than one isolate has been included in our analyses.

Our experience has also verified the usefulness of GLC in the grouping of mycobacteria belonging to previously undescribed species (Koukila-Kähkölä *et al.*, 1995; Torkko *et al.*, 2000, 2002). This grouping can be used as a basis for an extended identification scheme, which may eventually lead to the description of a novel species. In our research studies and in the routine identification of clinical and veterinary isolates, all but the most recent species have been represented. Nonetheless, among mycobacteria recovered from a variety of environmental sources (Iivanainen *et al.*, 1993; Katila *et al.*, 1995), the majority of isolates represent species other than those fully characterized today. This leads us to assume that species identification will become an even more complicated procedure in the future.

According to our current experience, no single identification technique alone, including sequencing of a single gene such as 16S rRNA, allows infallible identification of all established mycobacterial species. Therefore, a rational battery of techniques should be available in all laboratories that perform mycobacterial identification.

REFERENCES

- Alugupalli, S. & Larsson, L. (1992). Secondary fatty alcohols of *Mycobacterium xenopi*. *J Gen Microbiol* **138**, 2499–2502.
- Böttger, E. C., Hirschel, B. & Coyle, M. B. (1993). *Mycobacterium genavense* sp. nov. *Int J Syst Bacteriol* **43**, 841–843.
- Brander, E., Jantzen, E., Huttunen, R., Julkunen, A. & Katila, M.-L. (1992). Characterization of a distinct group of slowly growing mycobacteria by biochemical tests and lipid analyses. *J Clin Microbiol* **30**, 1972–1975.
- Butler, W. R. & Guthertz, L. S. (2001). Mycolic acid analysis by high-performance liquid chromatography for identification of *Mycobacterium* species. *Clin Microbiol Rev* **14**, 704–726.
- Butler, W. R., O'Connor, S. P., Yakrus, M. A. & 8 other authors (1993). *Mycobacterium celatum* sp. nov. *Int J Syst Bacteriol* **43**, 539–548.
- Carbonara, S., Tortoli, E., Costa, D. & 7 other authors (2000). Disseminated *Mycobacterium terrae* infection in a patient with advanced human immunodeficiency virus disease. *Clin Infect Dis* **30**, 831–835.
- Chou, S., Chedore, P., Haddad, A., Paul, N. R. & Kasatiya, S. (1996). Direct identification of *Mycobacterium* species in Bactec 7H12B medium by gas-liquid chromatography. *J Clin Microbiol* **34**, 1317–1320.
- Chou, S., Chedore, P. & Kasatiya, S. (1998). Use of gas chromatographic fatty acid and mycolic acid cleavage product determination to differentiate among *Mycobacterium genavense*, *Mycobacterium fortuitum*, *Mycobacterium simiae*, and *Mycobacterium tuberculosis*. *J Clin Microbiol* **36**, 577–579.
- Garcia-Barceló, M., Luquin, M., Belda, F. & Ausina, V. (1993). Gas chromatographic whole-cell fatty acid analysis as an aid for the identification of mixed mycobacterial cultures. *J Chromatogr* **617**, 299–303.
- Iivanainen, E. K., Martikainen, P. J., Väänänen, P. K. & Katila, M.-L. (1993). Environmental factors affecting the occurrence of mycobacteria in brook waters. *Appl Environ Microbiol* **59**, 398–404.
- Jantzen, E., Tangen, T. & Eng, J. (1989). Gas chromatography of mycobacterial fatty acids and alcohols: diagnostic applications. *APMIS* **97**, 1037–1045.
- Katila, M.-L., Iivanainen, E., Torkko, P., Kauppinen, J., Martikainen, P. & Väänänen, P. (1995). Isolation of potentially pathogenic mycobacteria in the Finnish environment. *Scand J Infect Dis Suppl* **98**, 9–11.
- Koukila-Kähkölä, P., Springer, B., Böttger, E. C., Paulin, L., Jantzen, E. & Katila, M.-L. (1995). *Mycobacterium branderi* sp. nov., a new potential human pathogen. *Int J Syst Bacteriol* **45**, 549–553.
- Koukila-Kähkölä, P., Paulin, L., Brander, E., Jantzen, E., Eho-Remes, M. & Katila, M.-L. (2000). Characterisation of a new isolate of *Mycobacterium shimoidei* from Finland. *J Med Microbiol* **49**, 937–940.
- Larsson, L. (1983). Acidic methanolysis v. alkaline saponification in gas chromatographic characterization of mycobacteria: differentiation between *Mycobacterium avium*–intracellular and *Mycobacterium gastri*. *Acta Pathol Microbiol Immunol Scand Sect B* **91**, 235–239.
- Larsson, L., Jantzen, E. & Johnsson, J. (1985). Gas chromatographic fatty acid profiles for characterisation of mycobacteria: an interlaboratory methodological evaluation. *Eur J Clin Microbiol* **4**, 483–487.
- Larsson, L., Jiménez, J., Valero-Guillén, P., Martín-Luengo, F. & Kubin, M. (1989). Establishment of 2-docosanol as a cellular marker compound in the identification of *Mycobacterium xenopi*. *J Clin Microbiol* **27**, 2388–2390.
- Luque, A. E., Kaminski, D., Reichman, R. & Hardy, D. (1998). *Mycobacterium szulgai* osteomyelitis in an AIDS patient. *Scand J Infect Dis* **30**, 88–91.
- Luquin, M., Ausina, V., López Calahorra, F., Belda, F., García Barceló, M., Celma, C. & Prats, G. (1991). Evaluation of practical chromatographic procedures for identification of clinical isolates of mycobacteria. *J Clin Microbiol* **29**, 120–130.
- Luquin, M., Ausina, V., Vincent-Lévy-Frébault, V., Lanéeille, M. A.,

- Belda, F., García-Barceló, M., Prats, G. & Daffé, M. (1993). *Mycobacterium brumae* sp. nov., a rapidly growing, nonphotochromogenic mycobacterium. *Int J Syst Bacteriol* **43**, 405–413.
- Mayo, J., Collazos, J. & Martínez, E. (1998). *Mycobacterium nonchromogenicum* bacteremia in an AIDS patient. *Emerg Infect Dis* **4**, 124–125.
- Müller, K.-D., Schmid, E. N. & Kroppenstedt, R. M. (1998). Improved identification of mycobacteria by using the Microbial Identification System in combination with additional trimethylsulfonium hydroxide pyrolysis. *J Clin Microbiol* **36**, 2477–2480.
- Portaels, F., Dawson, D. J., Larsson, L. & Rigouts, L. (1993). Biochemical properties and fatty acid composition of *Mycobacterium haemophilum*: study of 16 isolates from Australian patients. *J Clin Microbiol* **31**, 26–30.
- Richter, E., Niemann, S., Ruesch-Gerdes, S. & Harmsen, D. (2001). Description of *Mycobacterium heckeshornense* sp. nov. *J Clin Microbiol* **39**, 3023.
- Roth, A., Reischl, U., Schönfeld, N., Naumann, L., Emler, S., Fischer, M., Mauch, H., Loddenkemper, R. & Kroppenstedt, R. M. (2000). *Mycobacterium heckeshornense* sp. nov., a new pathogenic slowly growing *Mycobacterium* sp. causing cavitary lung disease in an immunocompetent patient. *J Clin Microbiol* **38**, 4102–4107.
- Roth, A., Mauch, H., Schönfeld, N., Loddenkemper, R., Reischl, U., Naumann, L. & Kroppenstedt, R. M. (2001). Description of *Mycobacterium heckeshornense* sp. nov. – Authors' reply. *J Clin Microbiol* **39**, 3023–3024.
- Springer, B., Wu, W.-K., Bodmer, T. & 10 other authors (1996). Isolation and characterization of a unique group of slowly growing mycobacteria: description of *Mycobacterium lentiflavum* sp. nov. *J Clin Microbiol* **34**, 1100–1107.
- Suomalainen, S., Koukila-Kähkölä, P., Brander, E., Katila, M.-L., Piilonen, A., Paulin, L. & Mattson, K. (2001). Pulmonary infection caused by an unusual, slowly growing nontuberculous mycobacterium. *J Clin Microbiol* **39**, 2668–2671.
- Torkko, P., Suutari, M., Suomalainen, S., Paulin, L., Larsson, L. & Katila, M.-L. (1998). Separation among species of *Mycobacterium terrae* complex by lipid analyses: comparison with biochemical tests and 16S rRNA sequencing. *J Clin Microbiol* **36**, 499–505.
- Torkko, P., Suomalainen, S., Iivanainen, E., Suutari, M., Tortoli, E., Paulin, L. & Katila, M.-L. (2000). *Mycobacterium xenopi* and related organisms isolated from stream waters in Finland and description of *Mycobacterium botniense* sp. nov. *Int J Syst Evol Microbiol* **50**, 283–289.
- Torkko, P., Suomalainen, S., Iivanainen, E. & 7 other authors (2001). Characterization of *Mycobacterium bohemicum* isolated from human, veterinary, and environmental sources. *J Clin Microbiol* **39**, 207–211.
- Torkko, P., Suomalainen, S., Iivanainen, E., Tortoli, E., Suutari, M., Seppänen, J., Paulin, L. & Katila, M.-L. (2002). *Mycobacterium palustre* sp. nov., a potentially pathogenic, slowly growing mycobacterium isolated from clinical and veterinary specimens and from Finnish stream waters. *Int J Syst Evol Microbiol* **52**, 1519–1525.
- Tortoli, E., Nanetti, A., Piersimoni, C. & 11 other authors (2001a). Performance assessment of new multiplex probe assay for identification of mycobacteria. *J Clin Microbiol* **39**, 1079–1084.
- Tortoli, E., Piersimoni, C., Kroppenstedt, R. M., Montoya-Burgos, J. I., Reischl, U., Giacometti, A. & Emler, S. (2001b). *Mycobacterium doricum* sp. nov. *Int J Syst Evol Microbiol* **51**, 2007–2012.
- Turenne, C. Y., Tschetter, L., Wolfe, J. & Kabani, A. (2001). Necessity of quality-controlled 16S rRNA gene sequence databases: identifying nontuberculous *Mycobacterium* species. *J Clin Microbiol* **39**, 3637–3648.
- Valero-Guillén, P., Martín-Luengo, F., Larsson, L., Jimenez, J., Juhlin, I. & Portaels, F. (1988). Fatty and mycolic acids of *Mycobacterium malmoeense*. *J Clin Microbiol* **26**, 153–154.
- Viljanen, M. K., Olkkonen, L. & Katila, M.-L. (1993). Conventional identification characteristics, mycolate and fatty acid composition, and clinical significance of MAIX AccuProbe-positive isolates of *Mycobacterium avium* complex. *J Clin Microbiol* **31**, 1376–1378.
- Vincent Lévy-Frébault, V. & Portaels, F. (1992). Proposed minimal standards for the genus *Mycobacterium* and for description of new slowly growing *Mycobacterium* species. *Int J Syst Bacteriol* **42**, 315–323.
- Wayne, L. G., Good, R. C., Tsang, A. & 13 other authors (1993). Serovar determination and molecular taxonomic correlation in *Mycobacterium avium*, *Mycobacterium intracellulare*, and *Mycobacterium scrofulaceum*: a cooperative study of the International Working Group on Mycobacterial Taxonomy. *Int J Syst Bacteriol* **43**, 482–489.
- Willumsen, P., Karlson, U., Stackebrandt, E. & Kroppenstedt, R. M. (2001). *Mycobacterium frederiksborgense* sp. nov., a novel polycyclic aromatic hydrocarbon-degrading *Mycobacterium* species. *Int J Syst Evol Microbiol* **51**, 1715–1722.
- Wilson, R. W., Steingrube, V. A., Böttger, E. C. & 11 other authors (2001). *Mycobacterium immunogenum* sp. nov., a novel species related to *Mycobacterium abscessus* and associated with clinical disease, pseudo-outbreaks and contaminated metalworking fluids: an international cooperative study on mycobacterial taxonomy. *Int J Syst Evol Microbiol* **51**, 1751–1764.
- Wolfe, J., Turenne, C., Alfa, M., Harding, G., Thibert, L. & Kabani, A. (2000). *Mycobacterium branderi* from both a hand infection and a case of pulmonary disease. *J Clin Microbiol* **38**, 3896–3899.