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Chemotypes of *Mycobacterium malmoense* Based on Glycolipid Profiles

MARJA-LEENA KATILA,[1]* ELJAS BRANDER,[2] ERIK JANTZEN,[3] RAIJA HUTTUNEN,[2] AND LAURA LINKOSALO[1]

Department of Clinical Microbiology, Kuopio University Central Hospital, SF-70210 Kuopio,[1] and Tuberculosis Laboratory, National Public Health Institute, SF-00250 Helsinki,[2] Finland, and National Institute of Public Health, N-0462 Oslo 4, Norway[3]

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Thin-layer chromatographic analysis of 72 Finnish clinical mycobacterial isolates presumptively identified as *Mycobacterium malmoense* revealed four major glycolipid profiles with two minor variations. An additional glycolipid profile was found in three British *M. malmoense*-like strains. No clear distinction between the strains could be made by means of gas chromatography of cellular fatty acids. The two *M. malmoense*-specific constituents, 2-methyleicosanoate and 2,4,6-trimethyltetracosanoate, were detected in all strains. The frequency of chemotypes other than that of the type strain was 8% among the Finnish isolates. This variation should be recognized when confirmative identification of mycobacteria is based on thin-layer chromatography of glycolipid extracts.

Mycobacterium malmoense is an environmental pathogen of increasing clinical importance in northern and northwestern Europe. It causes a variety of clinical manifestations, e.g., pulmonary disease, cervical adenitis (5, 9, 16), localized infections (15), and even disseminated infections (4). The correct identification of this species is particularly important since colonization of the respiratory tract by *M. malmoense* is uncommon and a positive isolation is most likely clinically significant (1, 11). In our retrospective analysis of the clinical data of 63 consecutive Finnish adult patients positive for *M. malmoense*, 50 (81%) had a relevant clinical infection (11).

Presumptive identification of *M. malmoense* relies on biochemical test profiles (16), but these features only give a tentative identity. Glycolipid analysis by thin-layer chromatography (TLC) (8, 16) and cellular fatty acid analysis by gas chromatography (GLC) (7, 17) have been found useful for confirmatory identification of this species. However, information on *M. malmoense* glycolipids is limited (13). Strains resembling *M. malmoense* but not meeting the diagnostic criteria for the species sensu stricto in glycolipid content are known to exist (8, 13).

In 1971 to 1988, 72 mycobacterial strains were isolated and presumptively identified as *M. malmoense* in Finland. TLC analyses revealed that 66 of these strains had the glycolipid profile of the *M. malmoense* type strain. The remaining six strains had distinct but different glycolipid profiles. For further description of these strains, comparative analyses were performed by TLC and GLC.

MATERIALS AND METHODS

Twenty-seven mycobacterial isolates, one per patient, from Finnish patients with pulmonary mycobacteriosis (n = 26) or cervical adenitis (n = 1) were selected from among the 72 isolates presumptively identified as *M. malmoense*. This collection included all isolates with atypical glycolipid profiles (n = 6) as well as a randomly selected group of strains with typical glycolipids (n = 21). The biochemical features of these isolates are listed in Table 1. Five British *M. malmoense* (12514) or *M. malmoense*-like (13754, 462, 18698, and 6328) strains (gifts from P. A. Jenkins, Mycobacterium Reference Unit, Public Health Laboratory Service, Cardiff, United Kingdom) were also included in the TLC and GLC analyses.

The strains, deposited in Middlebrook 7H9 broth at −70°C, were checked for purity on Middlebrook 7H11 agar before further processing. The strains were grown for 14 to 28 days on Löwenstein-Jensen or Middlebrook 7H11 agar medium for TLC and on Middlebrook 7H11 agar for GLC. Harvested cells were killed by heating the cells to 70°C for 30 min and drying them. A minimum of 20 and 0.5 mg (dry weight) of cells was grown for TLC and GLC, respectively.

TLC. The dried cells were extracted by the method of Marks et al. (12), in an ethanol-ether-water solution (17:17:6) overnight at room temperature. Samples (20 μl) of the extract were spotted on silica-gel TLC plates (Merck Art 5567) and run in one dimension in propanol-butanol-water-ammonium solvent (57:20:20:3). Runs were also performed by using the solvent system propanol-water-ammonium (150:64:9) for further separation of glycolipid bands. The spots were visualized with orcinol-sulfuric acid spraying and heating before photography. Selected strains were also analyzed for alkali stability of glycolipids (2).

GLC. Formation and extraction of fatty acid methyl esters was performed as described earlier (7). A Perkin-Elmer Sigma 300 gas chromatograph (Norwalk, Conn.) equipped with a capillary column and with split/splitless inlet was used in the splitless mode. A fused silica column (25 m by 0.2 mm) coated with cross-linked SE-30 methyl silicone (SGE, Ringwood, Victoria, Australia) was used. The temperature of both the injector and detector was 325°C. The column was operated at 120 to 280°C, increasing the temperature by 8 degrees/min with a carrier gas (He) flow rate of 1.2 ml/min. Peak areas and retention times were recorded by a Perkin-Elmer LC1-100 integrator.

Identification of the eluted substances was confirmed by use of authentic standards and additional GLC-mass spectometry analyses with Finnigan 700 Ion-trap mass spectrometric detector (San Jose, Calif.). No standards were available

* Corresponding author.

TABLE 1. Biochemical profiles of the Finnish *M. malmoense* isolates with typical and atypical TLC glycolipid patterns

Characteristic	No. of isolates with typical t-1 pattern (n = 21) showing indicated result[a]:			Reaction of isolates showing atypical pattern					
				F-2	F-3			F-4	
	+	±	−	6829	2038	18377	14216	25772	21640
Pigment	0	0	21	−	−	−	−	−	−
Growth at:									
25°C	21	0	0	+	+	+	+	+	+
37°C	21	0	0	+	+	+	+	+	+
45°C	0	0	21	−	−	−	−	−	−
Arylsulfatase									
3 days	0	1	20	−	−	−	−	−	−
14 days	9	12	0	−	±	±	±	+	+
Catalase (mm)	13 (4–13)	7 (1–3)	1 (<1)	± (1)	± (3)	+ (7)	+ (6)	+ (12)	± (3)
Niacin production	0	0	21	−	−	−	−	−	−
Nicotin-amidase	2	18	1	±	±	±	−	+	+
Nitrate reduction	0	7	14	−	−	−	−	±	−
Pyratzin-amidase	3	17	1	±	±	±	±	+	+
Tween 80 hydrolysis	8	9	4	±	+	±	±	+	+
Urease	1	9	11	±	±	−	−	−	+

[a] +, Strong positive; ±, weak positive; −, negative.

for the 2-methyl- and 2,4,6-trimethyl-branched acid methyl esters, and their identity was tentatively determined by their retention properties, supported by the characteristic mass spectometry fragments with m/z of 88, 101, and 120 as previously described (6, 7, 14).

RESULTS

The glycolipid pattern regarded as typical of *M. malmoense* (t-1) consists of two yellow-greenish bands with R_f values between 0.12 and 0.20 (Fig. 1, lanes 1 and 2). This simple pattern was detected in 21 of the 27 Finnish strains and in one British strain (12514). Chromatograms of the remaining strains deviated significantly from this pattern (Fig. 1). In six Finnish strains and four British strains, four major patterns of various complexity (F-2, B-2, F-3, and F-4) were seen, with two minor variations characterized by an additional weak band h with a R_f value of 0.42 (Fig. 2). The yellow-greenish color of the glycolipids of the type strain was shared by all glycolipid bands. Alkali lability, a feature of the two glycolipid spots of *M. malmoense* (13), was also demonstrated.

By GLC, it was demonstrated that all strains, with both typical and atypical glycolipid profiles, contained the two fatty acids 2-methyleicosanoate and 2,4,6-trimethyltetracosanoate, regarded as unique for *M. malmoense* (17), along with tuberculostearic acid and other fatty acids typical of the genus *Mycobacterium*. No clear distinction between the strains could be made by means of fatty acid profiles (Table 2).

DISCUSSION

The present evidence reveals that *M. malmoense* resembles *M. avium* complex and some other mycobacterial species by intraspecies heterogeneity in glycolipid composition (2). Of the 72 consecutive Finnish isolates of *M. malmoense*, six (8%) had TLC profiles different from the typical two-banded pattern t-1. Atypical patterns were also found in four of the British strains analyzed. This indicates that *M. malmoense* can be separated into at least five glycolipid variants (chemotypes). The two glycolipids previously shown to be characteristic of *M. malmoense* are known to be antigenic in nature similar to the glycolipids of other atypical mycobacteria (2, 13). Hence the additional glycolipids seen in the atypical patterns indicate that the number of specific *M. malmoense* antigens is considerably higher.

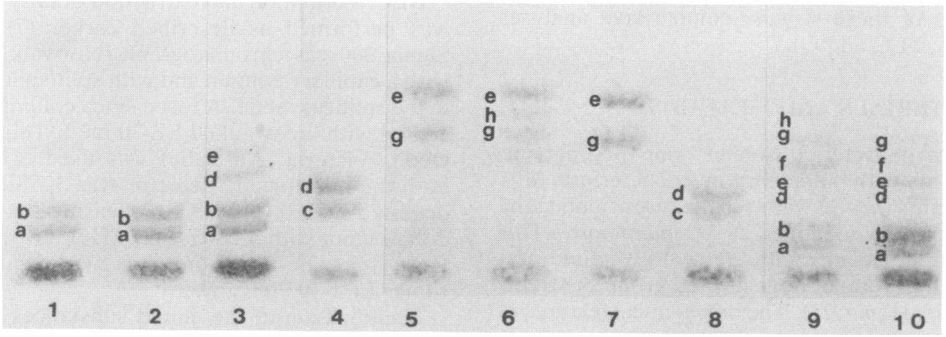

FIG. 1. TLC glycolipid profiles of some *M. malmoense* strains. Lanes: 1 and 2, banding pattern t-1; 3, F-2; 4 and 8, B-2; 5, B-3; 6, F-3 (identical to B-3, but with an additional faint band h); 7, F-3 (without band h); 9, F-4; 10, F-4 (without band h). The R_f values of the individual bands are given in Fig. 2.

TABLE 2. Fatty acid markers detected by gas chromatography in *Mycobacterium malmoense* isolates[a]

TLC pattern	No. of strains	% Fatty acids[b], mean ± SD (range)			
		10-Me18:0	2-Me20:0	2,4,6-triMe24:0	26:0
t-1	21	18.5 ± 4.6 (8.3–25.4)	3.8 ± 1.1 (1.3–5.8)	3.2 ± 0.9 (1.2–5.7)	3.6 ± 2.5 (0.6–11.3)
F-2	1	13.2 ± 2.6 (10.5–15.8)	3.2 ± 0.9 (2.3–4.1)	3.1 ± 0.7 (2.4–3.7)	5.0 ± 2.4 (2.6–7.3)
B-2	3	17.7 ± 2.1 (16.1–20.6)	2.5 ± 1.1 (1.0–3.5)	1.8 ± 0.9 (0.5–2.5)	1.6 ± 0.3 (1.3–2.0)
F-3	3	19.6 ± 3.1 (17.6–24.8)	4.2 ± 0.7 (3.4–5.2)	3.7 ± 0.6 (3.0–4.4)	2.7 ± 1.3 (1.6–4.9)
B-3	1	24.0	3.5	2.8	1.6
F-4	2	16.4 ± 3.3 (10.9–20.4)	5.1 ± 1.7 (3.1–7.5)	4.1 ± 0.9 (2.8–5.0)	4.7 ± 3.1 (1.1–9.3)

[a] Repeated analyses of chemotypes other than t-1 are included in the calculations.
[b] 10-Me18:0, Tuberculostearate; 2-Me20:0, 2-methyleicosanoate; 2,4,6-triMe24:0, 2,4,6-trimethyltetracosanoate; 26:0, hexacosanoate.

Patient isolates containing more than one serotype of the *M. avium* complex have recently been described (3). We checked our atypical Finnish primary isolates for this possibility and observed a similar phenomenon. Two isolates (2038 and 18377) were originally demonstrated to comprise both the typical t-1 and F-3 patterns.

M. malmoense may be underestimated as the causative agent of clinical infections because of the recognized difficulties in its primary cultivation and identification (8, 10). No biochemically distinct characteristics allow for its precise differentiation from other pathogenic or nonpathogenic mycobacteria. The ability to hydrolyze Tween 80 which is regarded as one of the key tests in the preliminary identification of *M. malmoense* (8) is neither unique for the species nor always positive (Table 1). At the present stage therefore, identification has to be confirmed by lipid analysis. GLC detects the two *M. malmoense*-specific constituents, 2-methyleicosanoate and 2,4,6-trimethyltetracosanoate (7, 17), and the instrumentally less demanding TLC technique provides specific glycolipid patterns. The latter technique also has the advantage of distinguishing among glycolipid chemotypes, which is not possible with GLC. Although chemotyping per se is unnecessary in a diagnostic routine, the heterogeneity in glycolipid patterns among *M. malmoense* strains should be known to avoid confusion in certain cases.

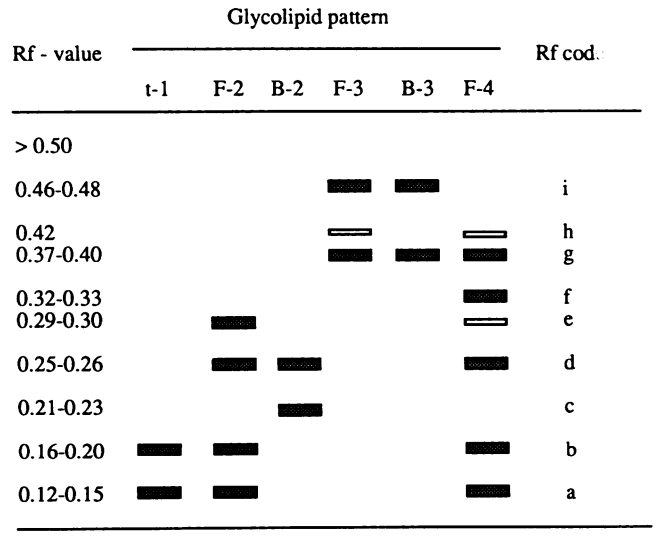

FIG. 2. Schematic presentation of the glycolipid patterns detected in the *M. malmoense* strains. Patterns F-3 and F-4 were either with or without band h (R_f of 0.42). Hence the F-3 and B-3 patterns were regarded as belonging to a single chemotype and the F-4 pattern with and without band h was regarded as another chemotype.

The chemotypes examined did not differ in drug susceptibility tests from the general pattern of *M. malmoense*. The patients infected with the unusual chemotypes had progressing clinical manifestations typical of mycobacteriosis and were without other known causative agents. Therefore these atypical chemotypes apparently are of comparable pathogenicity to *M. malmoense* with typical glycolipids.

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