ELSEVIER

Journal of Chromatography B, 689 (1997) 341–347

JOURNAL OF CHROMATOGRAPHY B

Easy differentiation of *Mycobacterium mucogenicum* from other species of the *Mycobacterium fortuitum* complex by thin-layer and gas chromatography of fatty esters and alcohols

Manuel Muñoz[a], Esther Julián[a], Merçé Garcia-Barceló[b], Vicente Ausina[b], Marina Luquin[a,*]

[a]*Departamento de Genética y Microbiología, Unidad de Microbiología, Facultad de Ciencias, Universidad Autónoma de Barcelona, 08193 Bellaterra, Barcelona, Spain*

[b]*Servicio de Microbiología, Hospital Universitario Germans Trias i Pujol, 08916 Badalona, Barcelona, Spain*

Received 20 March 1996; revised 11 July 1996; accepted 11 July 1996

Abstract

The mycolate pattern of a recently recognized mycobacterial pathogen, *Mycobacterium mucogenicum* (formerly *Mycobacterium chelonae*-like organism), was established for the first time. The reference strains, together with 31 environmental and clinical isolates belonging to this species, were examined for their mycolate composition by thin-layer chromatography. All strains tested exhibited the same mycolate profile. Mycolates were identified as belonging to the type without additional oxygenated chemical groups (mycolate I) and the type with a dicarboxylic group (mycolate VI); the identification of the latter was reinforced by the presence of 2-octadecanol, as seen by gas–liquid capillary chromatography. This mycolate profile permits the clear differentiation of *M. mucogenicum* from other related species, as members of the *Mycobacterium fortuitum* complex. This fact is especially important because strains of *M. mucogenicum* are very difficult to differentiate from other species of the *M. fortuitum* complex by means of conventional biochemical tests. Moreover, the characteristic mycolate profile exhibited by the strains of *M. mucogenicum* supports the recent proposal which considers them as members of a new species.

Keywords: *Mycobacterium mucogenicum*; *Mycobacterium fortuitum* complex; Mycolates; Mycolic acid methyl ester; Fatty acid methyl esters; Alcohols;

1. Introduction

Mycobacterium mucogenicum is a group of rapidly growing non-pigmented mycobacteria common in potable water, more specifically, tap water. They were implicated in nosocomial outbreaks of peritonitis which occurred in 1976 and 1978 in Washington State, involving the use of hospital-based automated chronic peritoneal dialysis machines [1]. *M. mucogenicum* was later identified as a cause of nosocomial infections associated with haemodialysis in Louisiana [2] and California [3]. In all cases *M. mucogenicum* was recovered from tap water used in the dialysis process. A collection of 87 sporadic clinical isolates of these organisms was recently characterized [4]. The majority of these isolates

*Corresponding author.

PII S0378-4347(96)00330-1

(62%) were respiratory isolates and had no clinical significance, except for 2 strains isolated from patients with AIDS. Among 33 non-respiratory isolates, 20 were clinically significant. Clinical diseases included postraumatic wound infections and catheter related sepsis, infections which are widely recognized as being caused by the other groups of rapidly growing pathogenic mycobacteria [5].

Biochemical analysis of the 87 *M. mucogenicum* isolates revealed that they were fairly homogeneous and had typical characteristics of members of the *M. fortuitum* complex. Members of this complex are defined as non-pigmented mycobacteria that grow within 7 days, show a positive 3-day arylsulfatase reaction, and grow on MacConkey agar without crystal violet at 28°C [6]. Most *M. mucogenicum* strains meet these inclusion criteria. As shown by Wallace et al. [4], routine biochemical features failed to clearly differentiate *M. mucogenicum* from *Mycobacterium chelonae* and *Mycobacterium abscessus* (two species of the *M. fortuitum* complex), and only two special tests (utilization of mannitol as the sole carbon source and susceptibility to cephalothin) helped to separate *M. mucogenicum* from *M. chelonae* and *M. abscessus*. The interest in clearly differentiating *M. mucogenicum* from the other two is due to the fact that *M. mucogenicum* strains show a characteristic susceptibility pattern, that is, they are more drug susceptible than *M. chelonae* and *M. abscessus* [7]. Mycolic acids are major lipid constituents in the cell wall of mycobacteria and related taxa. They are α-branched, β-hydroxy fatty acids which are characterized in mycobacteria by very long chains (up to 90 atoms long) and by the presence of various functional groups in the longest (mero) chain [8,9].

Silicic acid thin-layer chromatography (TLC) is a simple and efficient method to separate mycolic acid methyl esters in accordance with the presence and nature of oxygenated chemical functions in their long chain. Depending on the nature of these functional groups, different chromatographic behaviours are observed when preparations are subjected to TLC. Seven types of mycolates have been recognized in this way; these mycolates are type I and II mycolates (long and short mycolates with no additional oxygenated functions), type III (methoxymycolates), type IV (ketomycolates), type V (epoxymycolates), type VI (dicarboxymycolates) and type VII (ω-1 methoxymycolates) [8–10]. Mycolate TLC patterns for the various mycobacterial species have already been published either in catalogues or in previous studies [8,9,11–13]. The data obtained from these studies demonstrated that determination of mycolate patterns is useful in the differentiation of mycobacterial species, and that mycolate composition is a stable taxonomic characteristic, since the content in mycolates (except for minor components) is common to all strains of a species. Only vaccine strains of *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) produce a mycolate pattern that is devoid of type III mycolates, and is thus distinct from authenticated profiles containing type I, III and IV mycolates found in all *M. bovis* strains and other tubercle bacilli [9]. One consequence of these studies is the recommendation that the TLC pattern of mycolates be included in the description of new *Mycobacterium* species [14].

In this study, for the first time, TLC analysis was applied to strains of *M. mucogenicum*, to try to find an easy and reproducible method to differentiate this group of microorganisms from other related species.

Additional information, such as the presence of secondary alcohols related to dicarboxymycolates (type VI), and the composition in non-hydroxylated fatty acids, was obtained by gas–liquid capillary chromatography (GLC).

2. Experimental

2.1. Mycobacterial strains

Clinical and environmental isolates of *M. mucogenicum* (31 strains) were characterized by cultural, biochemical and physiological standard procedures [6,15,16]. In addition to these, three *M. mucogenicum* reference strains, ATCC 49649, ATCC 49650^T and ATCC 49651, were tested.

Also, for identification purposes, partial sequencing of the 16S rRNA gene was performed in four *M. mucogenicum* isolates. The 16S rRNA gene was amplified by using two primers whose oligonucleotide sequences were as follows: 5′ GAGAGTTTGATCCTGGCTCAG 3′ corresponding to the *E. coli* 16S rRNA gene from positions 9 to 30,

used in combination with 5′ TTTCAC-GAACAACGCGACAA 3′ corresponding to *E. coli* 16S rRNA gene from positions 609 to 590. These primers allowed the amplification of partial 16S rRNA gene that yielded a DNA fragment containing the species-specific character for mycobacteria [17,18].

The nucleic acid sequence obtained was compared with more than 30 mycobacterial 16S rRNA gene sequences, including *M. mucogenicum* ATCC 49650^T, deposited in the Gene Bank and EMBL data base. The Gene Bank nucleotide accession number for a selected isolate (strain CR-51) is U19366.

Reference strains, *M. fortuitum* ATCC 6841^T and *M. chelonae* NCTC 946^T, were included for thin-layer and gas chromatography comparative purposes. *Mycobacterium terrae* ATCC 15755^T and *Mycobacterium xenopi* ATCC 19250^T, two slow-growing mycobacteria which are distantly related to *M. mucogenicum* were used exclusively to provide a pattern for identification on TLC of dicarboxymycolate (mycolate VI). Species of mycobacteria closely related to *M. mucogenicum*, and displaying dicarboxymycolates do not, in fact, exist [8,11,13]. We therefore chose *M. terrae* and *M. xenopi* for TLC comparative purposes. However, other species which do possess dicarboxymycolates but which are equally distant taxonomically from *M. mucogenicum*, could have been chosen for this purpose [8,11,13]. For isolation of lipid components, all strains were grown on plates of Sauton agar and incubated at 37°C. The incubation period was two weeks for *M. mucogenicum*, *M. fortuitum* and *M. chelonae*, and six weeks for *M. terrae* and *M. xenopi*.

2.2. Extraction of lipids

A spadeful of bacteria (10 mg wet weight) was scraped from the surface of Sauton agar plates and treated with 2 ml of a methanol–benzene solution (8:2, v/v) containing 5% (w/v) potassium hydroxide in a screw-cap test tube (14×120 mm) fitted with a Teflon-lined cap. The mixture was heated in a covered bath at 80°C for 8 h. After cooling at room temperature, the samples were acidified by the addition of 20% (v/v) sulfuric acid, and the lipids were extracted into diethyl ether. The ether extracts were washed with water until neutral, dried over anhydrous sodium sulfate, filtered and concentrated by evaporation to dryness.

2.3. Preparation of methyl esters

Lipids were methylated with freshly prepared diazomethane (diazomethane is toxic and has to be handled under a hood). Diazomethane was prepared from a commercially available compound, N-Nitroso-N-methylurea (Sigma, St. Louis, MO, USA). For this purpose, 2 g of N-Nitroso-N-methylurea were dissolved in a precooled solution containing 30 ml of diethyl ether, 2.4 g of KOH and 6 ml of distilled water. The mixture was agitated for 5 to 10 min, and then the supernatant was removed and placed in a new tube cooled in ice containing potassium hydroxide pellets. A 1-ml portion of this solution was poured into each tube containing the dried lipids, and methylation was achieved within 5 min. Diazomethane was then evaporated. Lipid extracts were resuspended in 0.5 ml of *n*-hexane, and then 10 μl were applied to silica gel plates and 1 μl of the same lipid extract was injected in a gas chromatograph as described below. In order to avoid handling diazomethane, methyl esters of fatty acids can be obtained with anhydrous hydrogen chloride in methanol, or boron trifluoride in methanol [19].

2.4. TLC of mycolic acid methyl esters

Analytical one-dimensional TLC was performed using sheets of precoated silica gel 60 TLC plates (0.25 mm thick, 20×20 cm; DC-Fertigplatten Kieselgel 60; Merck, Darmstadt, Germany). A triple development with 100 ml of a monophasic mixture of *n*-hexane–ether (85:15, v/v) was performed. The separate components were revealed as dark blue spots by spraying with 10% (w/v) molybdophosphoric acid (Merck) in ethanol followed by charring. Identification of mycolates on TLC was performed by comparison with the mycolate patterns of reference strains.

2.5. GLC of cellular fatty acid methyl esters and alcohols

Fatty acid methyl esters and alcohols were analyzed on a fused-silica capillary column (15 m×0.25

mm I.D.) with cross-linked methyl silicone (HP-1, Hewlett-Packard, Palo Alto, CA, USA) as the stationary phase; the column was inserted in a Hewlett-Packard 5890A gas chromatograph equipped with a flame ionization detector. The column was programmed at 175 to 300°C at 8°C/min and maintained at 300°C for 15 min. The injector and detector temperatures were 275 and 315°C, respectively. The carrier gas was helium with a flow-rate of approximately 1 ml/min, and the split ratio was approximately 1:50. The chromatograms were integrated by using a HP3396 Series II integrator. The peaks were identified by comparing retention times with authentic methyl ester and alcohol standards (Supelco and Sigma). The identities of some compounds were also confirmed by mass spectrometry. A Hewlett-Packard Model 5988A mass spectrometer interfaced to a Hewlett-Packard 5890A gas chromatograph was used.

3. Results and discussion

Clinical and environmental isolates (a total of 31 strains) grew in less than 7 days (100%), were non-pigmented (100%), gave a positive 3-day arylsulfatase reaction (100%) and grew on MacConkey agar without crystal violet at 28°C (83%). Like *M. chelonae*, our strains were nitrate negative (80%), unable to grow in Lowenstein-Jensen containing 5% NaCl (100%) and utilized citrate as the sole carbon source (85%). Nevertheless, in contrast to *M. chelonae* and *M. abscessus*, isolates were susceptible to cephalothin (disk of 30 μg) (94%) and grew on media containing mannitol as the sole carbon source (93%). The isolates were uniform in 49 other physiological, biochemical and growth characteristics [6,15,16]. Phenotypical characteristics shown by our isolates are identical to those described for *M. mucogenicum* strains [4].

The amplified region of the 16S rRNA gene was a 603 base pairs fragment containing a species-specific character for mycobacteria. Analysis of the signature sequence of the 16S rRNA gene, corresponding to the *E. coli* nucleotides at positions 175 to 202, which is unique for most established mycobacterial species [17,18], showed that the four selected *M. mucogenicum* isolates display a unique sequence that differed from those of all described species of mycobacteria, but was identical to *M. mucogenicum* ATCC 49650^T [18].

3.1. Mycolate patterns obtained by TLC

Reference strains and clinical and environmental isolates of *M. mucogenicum* showed the same pattern of mycolates, mycolate I (R_F 0.48) and mycolate VI (R_F 0.25) as reference strains *M. xenopi* and *M. terrae* (Figs. 1 and 2). This last type of mycolate was accompanied by secondary long-chain alcohols released from dicarboxylic mycolate during saponification [8,9,20]. This mycolate composition was distinct

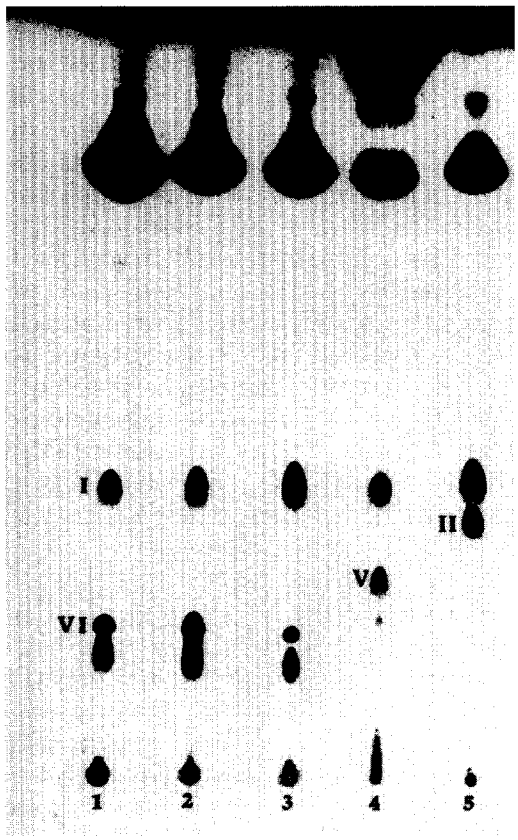

Fig. 1. Thin-layer chromatography of methyl mycolates from *M. mucogenicum* ATCC 49650^T (lane 1), *M. xenopi* ATCC 19250^T (lane 2), *M. terrae* ATCC 15755^T (lane 3), *M. fortuitum* ATCC 6841^T (lane 4) and *M. chelonae* NCTC 946^T (lane 5). I, α-mycolates; II, α'-mycolates; V, epoxymycolates; VI, dicarboxymycolates.

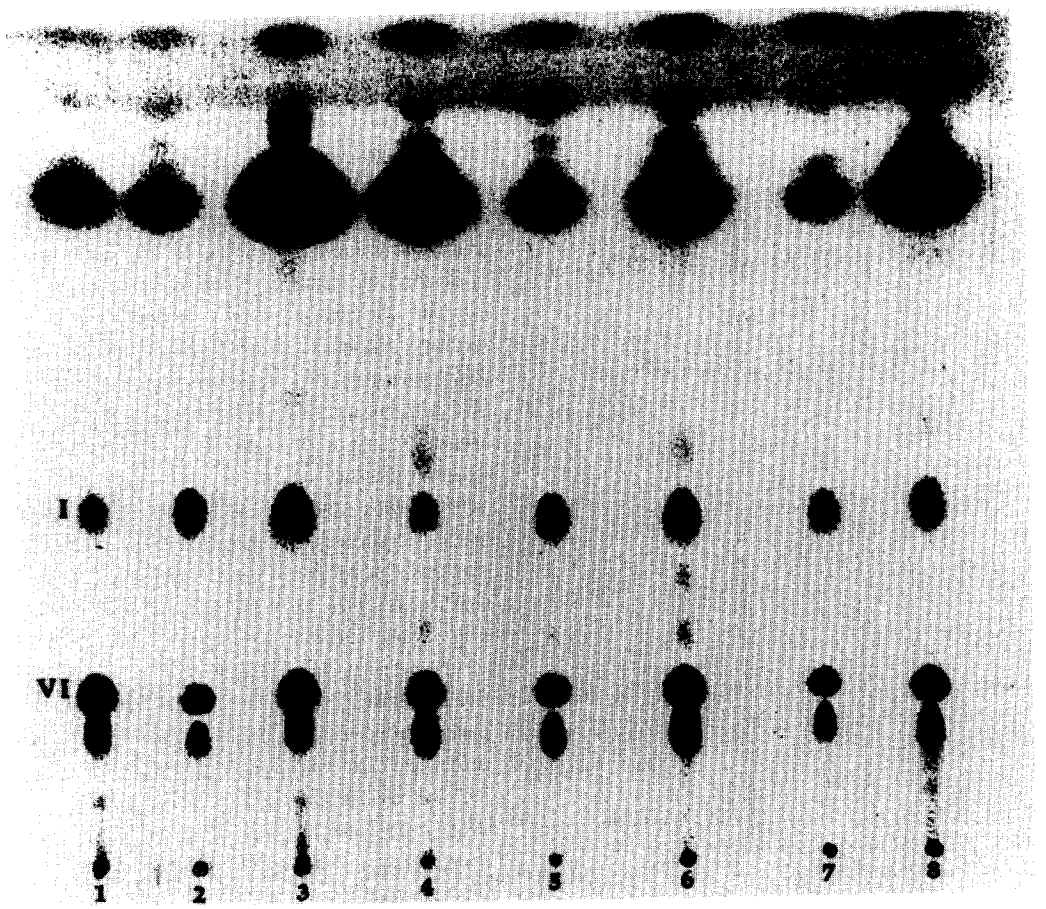

Fig. 2. Thin-layer chromatography of methyl mycolates from *M. mucogenicum* ATCC 49650^T (lane 1), ATCC 49649 (lane 2) and ATCC 49651 (lane 3). *M. mucogenicum* environmental and clinical isolates, CR-1 (lane 4), CR-2 (lane 5), CR-20 (lane 6), CR-46 (lane 7) and CR-51 (lane 8). I, α-mycolates; VI, dicarboxymycolates.

from that of *M. chelonae* NCTC 946^T which contained only mycolates I and II (R_f 0.43), and from *M. fortuitum* ATCC 6841^T composed of mycolate I and mycolate V (R_f 0.34). In a previous study [10], we examined by TLC 102 other mycobacterial strains belonging to the four species which make up the *M. fortuitum* complex (28 *M. chelonae*, 6 *M. abscessus*, 42 *M. fortuitum* and 26 *M. peregrinum*). Mycolate VI was not detected in any of these strains. All the strains belonging to *M. chelonae* and *M. abscessus* species showed a mycolate pattern composed of mycolates I and II. Likewise, *M. fortuitum* and *M. peregrinum* strains showed a pattern composed of mycolates I and V. In addition to these mycolates some strains of *M. fortuitum* and *M. peregrinum* showed mycolates II and VII as minor compounds. These results agree with those obtained by other authors [8,9,11,13].

3.2. Analysis of fatty acid methyl esters and alcohols

Gas chromatograms from all strains tested revealed the presence of tetradecanoic ($C_{14:0}$), hexadecenoic ($C_{16:1}$), hexadecanoic ($C_{16:0}$), octadecenoic ($C_{18:1}$), octadecanoic ($C_{18:0}$), 10-methyloctadecanoic (TBS, tuberculostearic acid), docosanoic ($C_{22:0}$) and tetracosanoic acids ($C_{24:0}$) (Fig. 3). Gas chromatograms of all the *M. mucogenicum* strains presented two additional peaks which were absent in *M.*

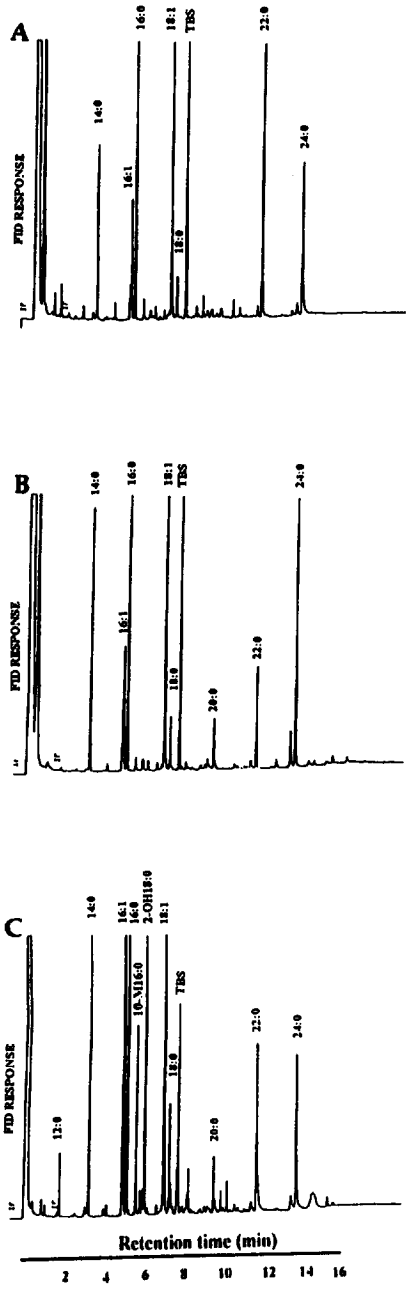

Fig. 3. Gas chromatograms of fatty acid methyl esters and alcohols of *M. fortuitum* ATCC 6841^T (A); *M. chelonae* NCTC 946^T (B) and *M. mucogenicum* ATCC 49650^T (C). The numbers above the peaks indicate the number of carbon atoms, followed by the number of double bonds. 10-M16:0, 10-methylhexadecanoate; 2-OH18:0, 2-octadecanol; TBS, tuberculostearate; FID, Flame ionization detector.

chelonae and *M. fortuitum* strains. These two compounds were identified by gas chromatography–mass spectrometry as 10-methylhexadecanoic acid (10-M $C_{16:0}$) and 2-octadecanol (2-OH $C_{18:0}$). As stated above, secondary long-chain alcohols (octadecanol, eicosanol and docosanol) were released from dicarboxymycolates during saponification. These alcohols can easily be detected by GLC [20–24]. Thus, a secondary alcohol, 2-octadecanol, was detected in all the *M. mucogenicum* tested. As expected, secondary alcohols were not detected in strains of *M. chelonae* and *M. fortuitum* since these species, like *M. abscessus* and *M. peregrinum*, do not display dicarboxymycolates [8–10]. In previous extensive studies performed by us and other authors [22–27], appreciable amounts of 10-methylhexadecanoic acid were detected in only two photochromogenic species, *Mycobacterium marinum* and *Mycobacterium szulgai*; this compound was not detected in any of the members of the *M. fortuitum* complex.

M. xenopi and *M. terrae* are two slow-growing mycobacteria which are distantly related to *M. mucogenicum*. Normally, chromatographic procedures are not used to differentiate these species from *M. mucogenicum*, since more simple observations such as growth rate, pigmentation and colonial morphology can accomplish this purpose. In the present study we used type strains of *M. xenopi* and *M. terrae* only to provide a pattern for identification on TLC of dicarboxylic mycolate. However, gas chromatograms of non-hydroxylated fatty acids and alcohols of *M. xenopi* and *M. terrae* could be used to discriminate between these species and *M. mucogenicum* since, as we reported previously [20], gas chromatograms of *M. xenopi* strains show two characteristic peaks corresponding to 2-docosanol (2-OH $C_{22:0}$) and hexacosanoic acid ($C_{26:0}$) which are absent in gas chromatograms of *M. mucogenicum* strains. On the other hand, *M. terrae* strains are devoid of 10-M $C_{16:0}$ [23], a compound present in all the *M. mucogenicum* strains investigated.

Our study showed that the determination of the presence of dicarboxymycolates (type VI) by TLC, or 2-octadecanol and 10-methylhexadecanoic acid by GLC, in the *M. mucogenicum* strains, allows the clear differentiation of these from other members of the *M. fortuitum* complex. TLC or GLC can both be used for differentiation of *M. mucogenicum* strains.

TLC is an easy and inexpensive technique; GLC requires appropriate equipment and skilled personnel, but is routinely used in many clinical microbiology laboratories to identify different bacterial groups (anaerobic bacteria, non-fermentative gram-negative bacteria, *Campylobacter, Helicobacter, Legionella*, etc...).

The molecular type of mycolates containing *M. mucogenicum* strains and the profile of non-hydroxylated fatty acids and alcohols have been established for the first time in the present investigation. These findings increase the available data on one recently recognized mycobacterial pathogen. These data support the conclusion of previous phenotypic and genetic studies [4,18], namely, that the *M. mucogenicum* is a new species of the *M. fortuitum* complex.

Acknowledgments

We are grateful to M. A. Lanéelle for her helpful comments and suggestions throughout this study.

References

[1] J.D. Band, J.I. Ward, D.W. Fraser, N.J. Peterson, V.A. Silcox, R.C. Good, P.R. Ostrey and J. Kennedy, J. Infect. Dis., 145 (1982) 9.
[2] G. Bolan, A.L. Reingold, L.A. Carson, V.A. Silcox, C.L. Woodley, P.S. Hayes, A.W. Hightower, L. McFarland, J.W. Brown III, N.J. Petersen, M.S. Favero, R.C. Good and C.V. Broome, J. Infect. Dis., 152 (1985) 1013.
[3] P.W. Lowry, C.M. Beck-Sague, L.A. Bland, S.M. Aguero, M.J. Arduino, A.N. Minuth, R.A. Murray, J.M. Swenson and W.R. Jarvis, J. Infect. Dis., 161 (1990) 85.
[4] R.J. Wallace, Jr., V.A. Silcox, M. Tsukamura, B.A. Brown, J.O. Kilburn, W.R. Butler and G. Onyi, J. Clin. Microbiol., 31 (1993) 3231.
[5] R.J. Wallace, Jr., Eur. J. Clin. Microbiol. Infect. Dis., 13 (1994) 953.
[6] A. Silcox, R.C. Good and M.M. Floyd, J. Clin. Microbiol., 14 (1981) 686.
[7] A. Brown, R.J. Wallace, Jr, G.O. Onyi, V.D. Rosas and R.J. Wallace III, Antimicrob. Agents Chemother., 36 (1992) 180.
[8] M. Daffé, M.A. Lanéelle, C. Asselineau, V. Lévy-Frébault and H. David, Ann. Microbiol. (Inst. Pasteur), 134B (1983) 241.
[9] D.E. Minnikin, S.M. Minnikin, J.H. Partlett, M. Goodfellow and M. Magnusson, Arch. Microbiol., 139 (1984) 225.
[10] M. Luquin, M.A. Lanéelle, V. Ausina, M. García-Barceló, F. Belda, C. Alonso and G. Prats, Int. J. Syst. Bacteriol., 41 (1991b) 390.
[11] V. Ausina, M. Luquin, L. Margarit and G. Prats, in M. Casal (Editor), Mycobacteria of Clinical Interest, Elsevier, Amsterdam, 1986, p. 70.
[12] H.P. Hinrikson and G.E. Pfyffer, Med. Microbiol. Lett., 3 (1994) 49.
[13] D.E. Minnikin, S.M. Minnikin, J.H. Parlett and M. Goodfellow, Zbl. Bakt. Hyg. A, 259 (1985) 446.
[14] V. Lévy-Frébault and F. Portaels, Int. J. Syst. Bacteriol., 42 (1992) 315.
[15] M. Tsukamura, Tubercle, 48 (1967) 311.
[16] A. Vestal, Publication (CDC), (1975) 76.
[17] P. Kirschner, B. Springer, U. Vogel, A. Meier, A. Wrede, M. Kiekenbeck, F.C. Bange and E.C. Böttger, J. Clin. Microbiol., 31 (1993) 2882.
[18] B. Springer, E.C. Böttger, P. Kirschner and R.J. Wallace, Int. J. Syst. Bacteriol., 45 (1995) 262.
[19] W. Christie, Lipid Analysis, Pergamon Press. Oxford, 1973, p. 85.
[20] M. Luquin, F. López and V. Ausina, J. Clin. Microbiol., 27 (1989) 1403.
[21] M. García-Barceló, M. Luquin, F. Belda and V. Ausina, J. Chromatogr., 617 (1993) 299.
[22] L. Larsson, E. Jantzen and J. Johnson, Eur. J. Clin. Microbiol., 4 (1985) 483.
[23] M. Luquin, V. Ausina, F. López-Calahorra, F. Belda, M. García-Barceló, C. Celma and G. Prats, J. Clin. Microbiol., 29 (1991a) 120.
[24] J.J. Parez, M. Fauville-Dufaux, J.L. Dossogne, E. de Hoffmann and F. Pouthier, Eur. J. Clin. Microbiol. Infect. Dis., 13 (1994) 717.
[25] M.A. Lambert, C.W. Moss, V.A. Silcox and R.C. Good, J. Clin. Microbiol., 23 (1986) 731.
[26] P.A. Tisdall, G.D. Roberts and J.P. Anhalt, J. Clin. Microbiol., 10 (1979) 506.
[27] P.A. Tisdall, D.R. DeYoung, G.D. Roberts and J.P. Anhalt, J. Clin. Microbiol., 16 (1982) 400.