Biochemical Properties and Fatty Acid Composition of *Mycobacterium haemophilum*: Study of 16 Isolates from Australian Patients

F. PORTAELS,^{1*} D. J. DAWSON,² L. LARSSON,³ AND L. RIGOUTS¹

Laboratory of Mycobacteriology, Department of Microbiology, Institute of Tropical Medicine, Nationalestraat 155, 2000 Antwerp, Belgium¹; Laboratory of Microbiology and Pathology, Queensland Health, Brisbane, Australia²; and Department of Medical Microbiology, University of Lund, Lund, Sweden³

Received 25 June 1992/Accepted 6 October 1992

The biochemical properties and fatty acid compositions of 16 strains of *Mycobacterium haemophilum* from Australian patients were studied. The strains proved to be indistinguishable from each other but could readily be differentiated from other slowly growing mycobacteria with similar cultural features. Mycolic acid analyses revealed the presence of α -, methoxy-, and ketomycolates. The fatty acid composition supports the validity of the fact that *M. haemophilum* is a distinct species. The fatty acid composition was consistent among the 16 strains, but it was unusual in that there was some resemblance to the fatty acid composition of *M. leprae*. The wide range of pHs (5.4 to 7.4) that supported growth of *M. haemophilum* on artificial medium is in keeping with suggestions that *M. haemophilum* exists in an environmental habitat.

Mycobacterium haemophilum was first described in 1978 as a fastidious organism that caused skin lesions in an Israeli patient receiving immunosuppressive therapy for Hodgkin's disease (20). It is now regarded as a relatively common

pathogen of immunosuppressed patients, typically following organ transplantation and in association with human immunodeficiency virus infection (1, 13). The organism has also been implicated in lymph node infections in apparently

TABLE 1. Characteristics of *M. haemophilum* and other nonpigmented slowly growing mycobacteria^a

Characteristic	<i>M. haemophilum</i>	Cluster 4 ^b	<i>M. gastr</i> ^{b,c}	<i>M. shimoidei</i>	<i>M. avium</i> ^{b,c}	<i>M. malmoense</i> ^{b,c}	<i>M. ulcerans</i> ^c
Colony morphology ^d	SmK/R		SmK/R	Rf	SmS/SmT	SmS/SmT	R
Growth on Löwenstein-Jensen medium	—	+	+	+	+	+	+
Growth at 37°C	—	+	+	+	+	+	F
Growth in presence of							
Isoniazid (10 µg/ml)	+	F	—	—	+	—	M
Hydroxylamine (250 µg/ml)	+	+	+	—	+	M	F
p-Nitrobenzoate (500 µg/ml)	—	+	—	+	+	+	—
Optimal pH range ^e	5.4–7.4		5.4–6.5		5.4–6.5	5.4–6.9	5.4–7.4
Enzymatic properties							
Urease	—	—	+	—	—	M	F
Tween hydrolysis (10 days)	—	—	+	+	—	+	—
Acid phosphatase	+	M	+	+	—	—	—
Mycolate types ^f							
α -Mycolates	+		+	+	+	+	+
α' -Mycolates	—		—	+	—	+	—
Methoxymycolates	+		+	—	—	—	+
Ketomycolates	+		+	+	+	+	+
ω -Carboxymycolates	—		—	+	+	—	—

^a +, >85% of strains positive; M, 50 to 85% of strains positive; F, 15 to 49% of strains positive; —, <15% of strains positive; blank spaces, not tested. SmK/R, smooth *M. kansasii*/rough; Rf, rough; SmS/SmT, smooth scotochromogenic/smooth transparent.

^b Data from Wayne et al. (25).

^c Data from Jenkins et al. (11).

^d For colony morphology descriptions, see Jenkins et al. (11).

^e Data from Portaels and Pattyn (18).

^f Terminology of Dobson et al. (10).

* Corresponding author.

FIG. 1. Results of one-dimensional thin-layer chromatography analysis after double development with petroleum ether-acetone (95/5; vol/vol) (plate I) and a single development with dichloromethane (plate II). Lanes a, *M. avium*; lanes b, *M. gordonae*; lanes c, *M. simiae*; lanes d through h, *M. haemophilum*; lanes i, *M. shimoidei*; lanes j, *M. avium*; lanes k, *M. simiae*; lanes l, *M. marinum*. A, α -mycolates; A', α' -mycolates; B, methoxymycolates; C, ketomycolates; D, wax ester mycolates; E, eicosanol; F, nonhydroxylated fatty acid methyl esters.

healthy children (8, 22) and in a nonimmunosuppressed 65-year-old woman (15).

M. haemophilum is a nonpigmented mycobacterium which grows slowly at temperatures of about 30°C. Because of its low enzymatic activity, some investigators (26) have suggested that this organism be classified as an inert subspecies of *M. avium*, like *M. paratuberculosis* and *M. leprae-murium*. It is unusual in that it requires medium enriched with either blood products, such as hemin, or iron-contain-

ing compounds, such as ferric ammonium citrate (FAC), for growth. This fact has undoubtedly caused it to be overlooked in many laboratories. Also, unlike most of the other mycobacterial pathogens, its characteristics have not been well studied. The availability of a collection of 16 isolates of *M. haemophilum* from Australian patients provided us with the opportunity to further investigate its biochemical properties and fatty acid composition. This report presents our findings.

MATERIALS AND METHODS

The 16 strains of *M. haemophilum* were grown from either skin lesions or lymph nodes from Australians (Queensland Health, Brisbane, Queensland, Australia) by using Löwenstein-Jensen medium supplemented with FAC. The strains were isolated from nine patients who were immunosuppressed because of renal transplantation, long-term steroid use, or AIDS and six patients who were considered otherwise healthy. Identification was established on the basis of the organisms' (i) requirements for FAC, (ii) preference for 30°C, (iii) unreactivities in conventional biochemical tests, and (iv) agglutination by a type-specific antiserum (9). Strains POW (ATCC 33207) and RM (ATCC 33208) formed part of a previous study by Dawson and Jennis (9), in which it was shown that the strains are indistinguishable from the prototype strain (ATCC 29548).

Cultural, biochemical, and physiological properties, including growth at a range of pHs, were determined as described previously (11, 18). Lipid analyses were performed on freeze-dried bacteria harvested from subcultures in Dubos broth medium supplemented with Dubos oleic-albumin complex (100 ml/liter) and 1.5% FAC that were incubated at 30°C for 3 months.

The mycolic acid compositions were analyzed by one-dimensional thin-layer chromatography after heating the freeze-dried material in alkaline methanolic solution and then esterifying the liberated acids with iodomethane (5, 10). *M. avium*, *M. gordonae*, *M. marinum*, *M. shimoidei*, and *M. simiae* (one strain each) were used as reference strains.

The fatty acids were studied by gas chromatography (GC) after heating the freeze-dried material in methanolic hydrogen chloride and extracting the fatty acid methyl esters (12). The extracts were introduced and separated on a narrow-bore fused-silica capillary column by using a nonpolar stationary phase. Peak identification was performed by GC-mass spectrometry analysis in the electron impact mode (24).

RESULTS

The 16 strains that were previously identified as *M. haemophilum* had identical cultural, physiological, and biochemical characteristics. Results for *M. haemophilum* are given in Table 1, along with those for other slowly growing nonpigmented mycobacteria which share several properties with *M. haemophilum*, e.g., low catalase activity, negative niacin production, negative nitrate reduction, resistance to 1 µg of thiophen-2-carboxylic acid hydrazide per ml, and susceptibility to 5% sodium chloride. The optimal pH range for growth of the *M. haemophilum* strains was 5.4 to 7.4.

Results of thin-layer chromatographic analysis of mycolic acids are shown in Fig. 1. All strains studied contained α-mycolates, methoxymycolates, and ketomycolates. Also, the GC and GC-mass spectrometry analyses gave identical results with all 16 strains of *M. haemophilum*. Straight-chain fatty acids ranging from 12 to 24 carbon atoms and 10-methyl-substituted acids containing 17 and 19 (tuberculoostearic acid) carbon atoms were identified. Notably, all strains contained more docosanoic acid (C_{22:0}) than eicosanoic (C_{20:0}) and tetracosanoic (C_{24:0}) acids (Fig. 2). The GC-mass spectrometry analyses further revealed the presence of a compound that eluted approximately 0.5 min after the tricosanoic acid methyl ester. This compound produced a mass spectrum containing *m/z* 101 as the base peak and *m/z*

FIG. 2. Gas chromatogram (A) and extracted ion current profile (*m/z* 74) (B) of *M. haemophilum*. Peak designations indicate the number of carbon atoms:number of double bonds. TSA, tuberculoostearate; FS, full scale.

394 as the molecular ion peak, indicative of a monounsaturated branched-chain C₂₅ fatty acid methyl ester (Fig. 3).

DISCUSSION

Results of our study showed that a group of 16 strains which were previously identified as *M. haemophilum* on the basis of a limited number of properties are indistinguishable by an extended range of tests, including fatty acid composition analysis. Furthermore, the results support the validity of the fact that *M. haemophilum* is a distinct species. It can be differentiated from other species of nonpigmented slowly growing mycobacteria by at least two of a standard set of properties (Table 1).

We have shown (10) that the mycolic acid composition of *M. haemophilum* is similar to those of other slowly growing mycobacteria (e.g., *M. tuberculosis*, *M. gordonae*, *M. marinum*, *M. szulgai*, and *M. ulcerans*), in that it possesses α-, methoxy-, and ketomycolates. These findings confirm the results of Damato and Collins (6), who found the same mycolic acid patterns for *M. haemophilum* and *M. szulgai*. The cellular fatty acids of *M. haemophilum* have also been studied by others; however, in one report (21) tuberculoostearic acid was not detected, and in another report (6), the fatty acids were not identified. The abundant relative amount of C_{22:0} fatty acid which we found in *M. haemophilum* has previously been reported as a feature typical of *M. leprae* (14). It is noteworthy that *M. haemophilum* has been shown to possess a specific phenolic glycolipid antigen (3) which closely resembles the corresponding lipid of *M. leprae* (2).

FIG. 3. Extracted ion current profile of a selected time interval in analysis of *M. haemophilum* indicative of methyl esters of branched-chain (m/z 101) and straight-chain (m/z 74) fatty acids (A); mass spectrum of the branched-chain fatty acid methyl ester with a retention time of 13.94 min (B). FS, full scale.

Other mycobacterial taxa which have unusual growth requirements in vitro include *M. lepraemurium* (17), *M. paratuberculosis* (19), "wood-pigeon mycobacteria" (19), now called *M. avium* subsp. "*silvaticum*" (23), and some armadillo-derived mycobacteria (16). While these taxa have been regarded as probably distinct species, recent nucleic acid analyses suggest that *M. lepraemurium*, *M. paratuberculosis*, and *M. avium* subsp. "*silvaticum*" are variants of *M. avium* (25). The demonstrated phenetic divergences are likely due to adaption to different hosts through almost absolute parasitism. While we await results of genetic studies such as analysis of 16S rRNA from *M. haemophilum*, it seems reasonable to speculate that it is a distinct species. Any further evidence of its relatedness to *M. leprae* will be of great interest.

It is likely that *M. haemophilum* is an ubiquitous organism (1, 4) of environmental origin that causes infections in only a small proportion of exposed humans. Its occurrence in

children's lymph nodes and surface wounds (1, 7) supports this contention. In the present study, we showed that *M. haemophilum* grows over a wide pH range, as do most saprophytic mycobacteria (18). It may show a preference for iron-rich natural environments. No thorough search of the environment for *M. haemophilum* has yet been carried out.

The present investigation has increased the available data on one of the more recently recognized mycobacterial pathogens. *M. haemophilum* is apparently a potential pathogen of global distribution, and it is likely that further cases of *M. haemophilum* infection will be identified as laboratories become more aware of the need to use appropriate media. Consideration of *M. haemophilum* must be given in any case of surface lesions containing noncultivable mycobacteria. Once isolated, recognition is not difficult. A requirement for FAC (or blood, hemin, etc.) and a preference for temperatures of about 30°C are adequate for a presumptive identification of *M. haemophilum*. Better data regarding its drug

susceptibility, genetic relatedness to other species, and habitat are still required.

REFERENCES

1. Becherer, P., and R. L. Hopfer. 1992. Infection with *Mycobacterium haemophilum*. Clin. Infect. Dis. 14:793.
2. Besra, G. S., M. McNeil, D. E. Minnikin, F. Portaels, M. Ridell, and P. J. Brennan. 1991. Structural elucidation and antigenicity of a novel phenolic glycolipid antigen from *Mycobacterium haemophilum*. Biochemistry 30:7772-7777.
3. Besra, G. S., D. E. Minnikin, L. Rigouts, F. Portaels, and M. Ridell. 1990. A characteristic phenolic glycolipid antigen from *Mycobacterium haemophilum*. Lett. Appl. Microbiol. 11:202-204.
4. Branger, B., A. Gouby, R. Oulès, J. P. Balducchi, G. Mourad, J. Fourcade, C. Mion, F. Duntz, and M. Ramuz. 1985. *Mycobacterium haemophilum* and *Mycobacterium xenopi* associated infection in a renal transplant patient. Clin. Nephrol. 23:46-49.
5. Daffé, M., M. A. Lanéele, C. Asselineau, V. Lévy-Frébault, and H. David. 1983. Intérêt taxonomique des acides gras des mycobactéries: proposition d'une méthode d'analyse. Ann. Microbiol. (Inst. Pasteur). 134:241-256.
6. Damato, J. J., and M. T. Collins. 1984. Radiometric studies with gas-liquid and thin-layer chromatography for rapid demonstration of hemin dependence and characterization of *Mycobacterium haemophilum*. J. Clin. Microbiol. 20:515-518.
7. Dawson, D. J. Unpublished data.
8. Dawson, D. J., Z. M. Blacklock, and D. W. Kane. 1981. *Mycobacterium haemophilum* causing lymphadenitis in an otherwise healthy child. Med. J. Aust. 2:289-290.
9. Dawson, D. J., and F. Jennis. 1980. Mycobacteria with a growth requirement for ferric ammonium citrate, identified as *Mycobacterium haemophilum*. J. Clin. Microbiol. 11:190-192.
10. Dobson, G., D. E. Minnikin, S. M. Minnikin, J. H. Parlett, M. Goodfellow, M. Ridell, and M. Magnusson. 1985. Systematic analysis of complex mycobacterial lipids, p. 237-265. In M. Goodfellow and D. E. Minnikin (ed.), Chemical methods in bacterial systematics. Academic Press, Inc. (London), Ltd., London.
11. Jenkins, P. A., S. R. Pattyn, and F. Portaels. 1982. Diagnostic bacteriology, p. 441-469. In C. Ratledge and J. L. Stanford (ed.), The biology of the mycobacteria. Academic Press, Inc. (London), Ltd., London.
12. Jimenez, J., and L. Larsson. 1986. Heating cells in acid methanol for 30 min without freeze-drying provides adequate yields of fatty acids and alcohols for gas chromatographic characterization of mycobacteria. J. Clin. Microbiol. 24:844-845.
13. Kristjansson, M., V. M. Bieluch, and P. D. Byeff. 1991. *Mycobacterium haemophilum* infection in immunocompromised patients: case report and review of the literature. Rev. Infect. Dis. 13:906-910.
14. Kusaka, T., and S. Izumi. 1983. Gas chromatography of constitutive fatty acids in *Mycobacterium leprae*. Microbiol. Immunol. 27:409-414.
15. McBride, M. E., A. H. Rudolph, J. A. Tschen, P. Cernoch, J. Davis, B. A. Brown, and R. J. Wallace. 1991. Diagnostic and therapeutic considerations for cutaneous *Mycobacterium haemophilum* infections. Arch. Dermatol. 127:276-277.
16. Portaels, F., C. Asselineau, I. Baess, M. Daffé, G. Dobson, P. Draper, D. Gregory, R. M. Hall, T. Imaeda, P. A. Jenkins, M. A. Lanéele, L. Larsson, M. Magnusson, D. E. Minnikin, S. R. Pattyn, G. Wieten, and P. R. Wheeler. 1986. A cooperative taxonomic study of mycobacteria isolated from armadillos infected with *Mycobacterium leprae*. J. Gen. Microbiol. 132:2693-2707.
17. Portaels, F., and S. R. Pattyn. 1981. Parameters influencing the in vitro growth of *Mycobacterium lepraemurium*. Int. J. Lepr. 49:194-197.
18. Portaels, F., and S. R. Pattyn. 1982. Growth of mycobacteria in relation to the pH of the medium. Ann. Microbiol. (Inst. Pasteur). 133:213-221.
19. Saxegaard, F., and I. Baess. 1988. Relationship between *Mycobacterium avium*, *Mycobacterium paratuberculosis* and 'wood pigeon mycobacteria.' APMIS 96:37-42.
20. Sompolinsky, D., A. Lagziel, D. Naveh, and T. Yankilevitz. 1978. *Mycobacterium haemophilum* sp. nov., a new pathogen of humans. Int. J. Syst. Bacteriol. 28:67-75.
21. Sompolinsky, D., A. Lagziel, and I. Rosenberg. 1979. Further studies of a new pathogenic mycobacterium (*M. haemophilum* sp. nov.). Can. J. Microbiol. 25:217-225.
22. Thibert, L., F. Lebel, and B. Martineau. 1990. Two cases of *Mycobacterium haemophilum* infection in Canada. J. Clin. Microbiol. 28:621-623.
23. Thorel, M. F., M. Krichevsky, and V. V. Levy-Frebault. 1990. Numerical taxonomy of mycobactin-dependent mycobacteria, emended description of *M. avium*, and description of *M. avium* subsp. *avium*, supsp. nov., *M. avium* subsp. *paratuberculosis* subsp. nov., and *M. avium* subsp. *silvaticum* subsp. nov. Int. J. Syst. Bacteriol. 40:254-260.
24. Valère-Guillén, P., F. Martin-Luengo, L. Larsson, J. Jimenez, I. Juhlin, and F. Portaels. 1988. Fatty and mycolic acids of *Mycobacterium malmoense*. J. Clin. Microbiol. 26:153-154.
25. Wayne, L. G., R. C. Good, M. I. Krichevsky, Z. Blacklock, H. L. David, D. J. Dawson, W. Gross, J. Hawkins, V. Lévy-Frébault, C. McManus, F. Portaels, S. Rusch-Gerdes, K. H. Schroder, V. A. Silcox, M. Tsukamura, L. Van den Breen, and M. A. Yakus. 1991. Fourth report of the cooperative, open-ended study of slowly growing mycobacteria by the International Working Group on Mycobacterial Taxonomy. Int. J. Syst. Bacteriol. 41:463-472.
26. Wayne, L. G., and H. A. Sramek. 1992. Agents of newly recognized or infrequently encountered mycobacterial diseases. Clin. Microbiol. Rev. 5:1-25.