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Differentiation of *Mycobacterium ulcerans*, *M. marinum*, and *M. haemophilum*: Mapping of Their Relationships to *M. tuberculosis* by Fatty Acid Profile Analysis, DNA-DNA Hybridization, and 16S rRNA Gene Sequence Analysis

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Although *Mycobacterium ulcerans*, *M. marinum*, and *M. haemophilum* are closely related, their exact taxonomic placements have not been determined. We performed gas chromatography of fatty acids and alcohols, as well as DNA-DNA hybridization and 16S rRNA gene sequence analysis, to clarify their relationships to each other and to *M. tuberculosis*. *M. ulcerans* and *M. marinum* were most closely related to one another, and each displayed very strong genetic affinities to *M. tuberculosis*; they are actually the two mycobacterial species outside the *M. tuberculosis* complex most closely related to *M. tuberculosis*. *M. haemophilum* was more distinct from *M. ulcerans* and *M. marinum*, and it appeared to be as related to these two species as to *M. tuberculosis*. These results are important with regard to the development of diagnostic and epidemiological tools such as species-specific DNA probes and PCR assays for *M. ulcerans*, *M. marinum*, and *M. haemophilum*. In addition, the finding that *M. ulcerans* and *M. marinum* are more closely related to *M. tuberculosis* than are other pathogenic mycobacterial species suggests that they may be evaluated as useful models for studying the pathogenesis of *M. tuberculosis*. *M. marinum* may be particularly useful in this regard since strains of this species grow much more rapidly than *M. tuberculosis* and yet can cause systemic disease in immunocompromised hosts.

Mycobacterium marinum, *M. haemophilum*, and *M. ulcerans* are slowly growing mycobacterial species with optimal growth temperatures of 28 to 33°C. These organisms are emerging as clinically significant pathogens and are primarily associated with skin infections (13, 21, 47), although *M. marinum* and *M. haemophilum* are capable of causing systemic disease in immunocompromised patients (21, 47). *M. marinum* is widely distributed and gives rise to infections in temperate climates. *M. marinum*, which was previously also designated *M. balnei*, was first described in Sweden and has been known as the cause of "fish tank granulomas" (35). *M. haemophilum* was only recently recognized (48). It is now regarded as a relatively common pathogen of immunosuppressed patients, with infections with this organism typically occurring following organ transplantation and in association with human immunodeficiency virus infection (29, 47).

M. ulcerans appears to have a much more limited range of distribution and is the cause of infections primarily in tropical climates. Infections with this species present characteristically as extensive skin ulceration with scarring and were initially recognized as the causative agent of Buruli ulcer in Uganda (7, 8, 12) and later in central African countries (5, 19, 39, 41). *M. ulcerans* has recently received some attention from the clinical epidemiological side due to outbreaks in central African areas (21, 37, 38), also in conjunction with human immunodeficiency virus infections (1). However, *M. ulcerans* skin infections have also been described in Australia, where outbreaks have occurred recently (16, 18, 28, 51). In contrast to other mycobacterial agents, culture filtrates and viable organisms of *M. ulcerans* strains have shown cytotoxic activity in cell culture assays, and this toxin has been implicated as the cause of both focal necrosis and focal inflammation (22, 40, 44). A defined cytotoxic substance has not yet been isolated. The natural reservoir of *M. ulcerans* remains unknown, despite attempts to find the pathogen in resident flora and fauna (41). The incidence of *M. ulcerans*, *M. marinum*, and *M. haemophilum* as causes of infection may be seriously underestimated due to the long incubation time for *M. ulcerans* and the fact that culture of clinical specimens other than skin biopsy specimens at 37°C usually does not result in the isolation of these species.

Once cultured, *M. marinum* and *M. haemophilum* are readily identified by using conventional mycobacterium characterization methods. *M. marinum* grows relatively quickly (5 to 14 days) and is easily recognized due to its photochromogenicity. *M. haemophilum* is characterized by its enhanced growth requirement for iron (42, 48), although recent evidence suggests that this requirement is not absolute (47a). *M. ulcerans* strains are often difficult to isolate from primary culture, and due to their long generation time, they need at least 4 to 6 weeks to form colonies. Definitive identification of *M. ulcerans* strains is particularly time-consuming due to the slow growth and weak phenotypic expression, except for the presence of a heat-stable catalase, in conventional tests. The characteristics of the clinical lesion(s) and knowledge about geographic location can provide a presumptive tentative diagnosis, which is important for establishing the clinical prognosis, but definitive identification has required inoculation into animals, preferably guinea pigs.

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TABLE 1. Mycobacterial isolates included in the study

Species and strain	Source	Source, origin[a]
Mycobacterium ulcerans		
NCTC 10417^T = TMC 1617	Pus from ulcer	NCTC, Australia
NCTC 10013	Leg lesion	NCTC, Australia
ATCC 35840 = TMC 1615	Skin lesions	TMC, P. L. C. Small
NCTC 10445	Leg ulcer	NCTC, Uganda
NCTC 10467	Skin lesion	NCTC, Australia
NCTC 10468	Skin lesion	NCTC, Australia
ATCC 25896	Leg ulcer	ATCC, Uganda
Mycobacterium marinum		
ATCC 927^T = CCUG 20998 = TMC 1218	Human skin lesion	TMC, Sweden
CCUG 533	Skin lesion, finger	CCUG, Sweden
F427/86	Skin lesion, leg	National Hospital, Norway
F106/91	Skin lesion, finger	National Hospital, Norway
T31/81	Water sample	NIPH, Norway
T24/83	Water sample	NIPH, Norway
T65/83	Water sample	NIPH, Norway
T25/84	Water sample	NIPH, Norway
M6	Fish isolate	P. L. C. Small, United States
H 35392/93	Human skin lesion	P. L. C. Small, United States
LS	Fish isolate	P. L. C. Small, United States
MNC195	Fish isolate	NVH, Norway
Mycobacterium tuberculosis		
ATCC 9431 = TMC 201 = H37Ra	Human lung	TMC, P. L. C. Small, United States
ATCC 9360 = H37Rv	Human lung	TMC, P. L. C. Small, United States
735/93	Sputum	T. Tønjum, Norway
22892/93	Knee lesion	T. Tønjum, Norway
18763/92	Bronchoalveolar lavage fluid	T. Tønjum, Norway
Mycobacterium haemophilum		
NCTC 11185^T = ATCC 29548	Cutaneous granuloma	NCTC, Israel
MSK 1A	Skin lesion, AIDS	Lee W. Riley, New York
MSK 15A	Skin lesion, AIDS	Lee W. Riley, New York
306S	Skin lesion	F. Portaels, Australia
777	Skin lesion	F. Portaels, Australia
309	Skin lesion	F. Portaels, Australia
780	Skin lesion	F. Portaels, Australia
3066	Skin lesion	F. Portaels, Australia
1487/93	Skin lesion	T. Tønjum, Thailand
Other mycobacterial species		
Mycobacterium scrofulaceum ATCC 19981^T = TMC 1323	Human lymphadenitis	TMC, P. L. C. Small, United States
Mycobacterium kansasii ATCC 12478^T = TMC 1204	Human inguinal sinus	TMC, P. L. C. Small, United States
Mycobacterium gordonae ATCC 14470^T = TMC 1324	Human gastric lavage	TMC, P. L. C. Small, United States
Mycobacterium avium ATCC 25291^T = TMC 724	Chicken	TMC, P. L. C. Small, United States
Mycobacterium intracellulare ATCC 13950^T = CCUG 28005 = TMC 1406	Human sputum	CCUG/E. Falsen
Mycobacterium smegmatis ATCC 19420^T = TMC 1515	Phage host	TMC, P. L. C. Small, United States

[a] NCTC, National Collection of Type Cultures; ATCC, American Type Culture Collection; TMC, Trudeau Mycobacterial Collection; CCUG, Culture Collection, University of Gothenburg, Gothenburg, Sweden; NIPH, National Institute of Public Health.

M. ulcerans, *M. marinum*, and *M. haemophilum* are closely related, but their exact taxonomic placements have not been completely delineated. Only scarce information is available on these species on the genomic level (2, 4, 20, 24). Recently, the molecular typing of *M. ulcerans* and *M. haemophilum* has been described (25, 30). Although the 16S rRNA gene sequences of all three species have been determined (31, 43, 56), this has provided inadequate information for definitive species identification since *M. ulcerans* and *M. marinum* display identical signature regions (31) and have only two single-nucleotide differences in the 3′ part of the gene (23, 43). Furthermore, their relationships to *M. tuberculosis* have not been detailed.

In the present study we have investigated the molecular and biochemical characteristics of *M. ulcerans*, *M. marinum*, and *M. haemophilum* to clarify how they are interrelated and to improve the classificatory placements of these species. We also sought to investigate the heterogeneity of strains currently assigned to these species. Information from DNA-DNA hybridization and 16S rRNA gene sequence analysis, as well as from gas chromatography of fatty acids and alcohols, was used. These findings may be useful in for the development of nucleic acid-based tests for the direct detection of these species in clinical settings and epidemiological investigations.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and conventional characterization. The strains investigated in this study are listed in Table 1. The strains were grown on Middlebrook 7H9 and 7H10 with 10% acid-albumin-dextrose-catalase (Difco)

broth and agar plates, respectively, at 31°C. For the *M. haemophilum* strains, the media were supplemented with ferric ammonium citrate and the atmosphere was enriched with 10% CO_2. The conventional characterization methods consisted of microscopy after Ziehl-Neelsen staining, evaluation of growth on Lowenstein-Jensen slants and Middlebrook medium, photochromogenicity assay, investigation of heat-stable catalase, and association with clinical presentation and geographic location (33, 53). Assignment to a species was based on conventional gas chromatography characterization (27).

Lipid analyses. Gas chromatography of fatty acids and alcohols was performed as described previously (27). A Perkin-Elmer Autosystem gas chromatograph (Perkin-Elmer Cetus, Norwalk, Conn.) equipped with a standard methyl silicone-coated fused-silica capillary column (30 m by 0.2 mm) was used, and the inlet was in the splitless mode. The temperature of both the injector and the detector was 350°C. The column was operated at 80 to 280°C; the temperature was increased by 8°C/min, and a carrier gas (He) flow rate of 1 ml/min was used. Peak areas and retention times were recorded with a Perkin-Elmer 1020 integrator.

Principal-component analysis of the fatty acid data was performed as described previously (26) by using the SIRIUS program (Pattern Recognition Systems Ltd., Bergen, Norway), which includes a SIMCA principal component analysis element. The principles of SIMCA have been described previously (55). Briefly, (i) the raw gas chromatographic data (fatty acid profile of each strain) are loaded into the computer program. (ii) These raw data are transformed into the corresponding logarithmic values after the addition of the value of 1.0 to each datum. (iii) The computer program then calculates the number of statistically significant principal components and their relative contribution to the variation within the fatty acid profiles from the strains and then creates the plots.

DNA isolation. *M. tuberculosis* cells were heat inactivated for 20 min at 80°C in 1× TE buffer (0.01 M Tris-HCl [pH 7.5], 0.001 M EDTA). The cell suspension was treated with lysozyme at 37°C for 1 to 16 h. Lysis was performed with a sodium dodecyl sulfate-proteinase K mixture at 65°C for 1 h or until lysis was obtained. The lysate was treated with NaCl and cetyltrimetylammonium bromide-NaCl at 65°C for 10 min, and chloroform extraction was performed (52). The supernatant was precipitated with isopropanol, and after washing, the pellet was resuspended in TE buffer and the mixture was heated for 10 min at 65°C.

DNA-DNA hybridization. DNA-DNA hybridization was performed essentially as described previously (49).

(i) Dot blot filters. For each strain, eight parallel dots of single-stranded genomic DNA and Tris-EDTA buffer control were blotted onto nitrocellulose paper. The filters were stored dry.

(ii) Labelling of probes. Mechanically fragmented genomic DNA probes were labelled with [^{32}P]dCTP to a specific activity of 10^8 cpm with a random priming labelling kit (Amersham International, Buckinghamshire, United Kingdom) according to the manufacturer's recommendations.

(iii) Hybridization. Hybridization was performed in a Hyb-Aid hybridization oven with a hybridization fluid consisting of denatured probe DNA, SSC (0.15 M NaCl plus 0.015 M sodium citrate), 2× Denhardt's solution, 0.5% sodium dodecyl sulfate, 0.1 mg of denatured salmon sperm DNA per ml, and 10% dextran sulfate. The modifications used to increase the stringency of the hybridization procedure were a temperature of 70°C and sodium salt concentrations in the prehybridization and hybridization fluids of 0.1 M. The posthybridization washes were also performed at 70°C and with a sodium salt concentration of 0.1 M. The hybridization results for the dot blot were obtained by overnight autoradiography (Hyperfilm MP; Amersham International) and scintillation counting (Packard Instruments and Beckman) of standardized pieces cut from the nitrocellulose filter with a self-designed vacuum cutting device.

(iv) Quantitation of the dot blot hybridization reaction. The mean counts per minute for the eight parallel dots minus the counts per minute for the control (salmon sperm DNA) was determined for each of the strains. The mean counts per minute for the autologous strains was defined as representing a DNA homology ratio or a relative binding ratio (RBR) of 100%. The mean RBR for each strain was calculated by dividing the mean counts per minute for each strain by the mean counts per minute for the autologous reaction and multiplying by 100. The 95% confidence interval of the sample mean of the RBRs was estimated (49).

(v) Selection of *M. marinum* hybridization probe. Clone pMm5 from an *M. marinum* genomic pHC79 cosmid library was selected because of its lack of affinity to *M. ulcerans* DNA by Southern hybridization. This clone was used as probe DNA in parallel with the total genomic DNA probes.

PCR. Synthetic oligonucleotides (Genosys Biotechnologies, Inc., The Woodlands, Tex.) were made from conserved areas of the 16S rRNA gene molecule that were most convenient for PCR and DNA sequencing. The nucleotide sequences of the primers used were as follows: primer 285, 5′-GAGAGTTTGAT CCTGGCTCAG-3′; primer 244, 5′-CCCACTGCTGCCTCCCGTAG-3′; primer 259, 5′-TTTCACGAACAACGCGACAA-3′; primer 248, 5′-GTGTGG GTTTCCTTCCTTGG-3′; primer 264, 5′-TGCACACAGGCCACAAGGGA-3′ (31); primer rRog, 5′-AAGGAGGTGATCCAGCCGCA-3′ (45); primer TT85, 5′-CAACCGCACCCGGAACTG-3′; and primer TT86, 5′-GTCCCGAAATAT CTCGAC-3′. Fragments were amplified in 50-μl reaction mixtures containing 10 ng of template DNA, 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 2 mM $MgCl_2$, 200 μM (each) dATP, dCTP, dGTP, and dTTP (Pharmacia LKB Biotechnology AB, Bromma, Sweden), 0.001% (wt/vol) gelatin, 0.5 μM (each) primer, and 1 U of Amplitaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.). Each sample was amplified for 25 cycles of 45 s at 95°C, 1 min at 65°C, and 1 min at 72°C in a DNA thermal cycler (TA9600; Perkin-Elmer Cetus). For the negative controls, buffer instead of DNA was added. The PCR products were visualized by electrophoresis in a 0.8% agarose gel (SeaKem GTG; FMC Bioproducts, Rockville, Md.) to monitor size and specificity. The PCR products were prepared for DNA sequence analysis by purification in Magic Wizard columns (Promega, Madison, Wis.).

DNA sequence analysis. Sequencing reactions with the Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems Inc., Foster City, Calif.) were performed with 7 μl of PCR-amplified DNA as the template and 3.2 pmol of either primer. The unincorporated dye terminators and primers were separated from the extension products by spin column purification (Centri-Sep; Princeton Separations Inc., Adelphia, N.J.). The products were dried in a vacuum centrifuge, resuspended in 4 μl of loading buffer (5:1 deionized formamide, 50 mM EDTA [pH 8.0]), heat denatured for 2 min at 90°C, and immediately loaded onto an acrylamide gel in an automated DNA sequencer (model 373A; Applied Biosystems Inc.). Both strands of the 16S rRNA gene were sequenced, and ubiquitous areas were resequenced. The DNA sequence data were compiled and were analyzed by computer with the MacVector program (version 5.0; Oxford Molecular Group PLC, Oxford, United Kingdom).

The 16S rRNA and/or 16S rRNA gene sequences 22 mycobacterial species and *Nocardia asteroides* were obtained from GenBank. The accession numbers of these respective sequences are as follows: *M. ulcerans* ATCC 19423, Z13990 and X58954; *M. marinum* FIB, X52920; *M. haemophilum* NCTC 11185^T, L24800; *M. tuberculosis* Rv, X52917; *M. terrae* ATCC 15755^T, M29568 and X52925; *M. hiberniae* ATCC 9874^T, X67096; *M. nonchromogenicum* ATCC 19530^T, M29565 and X52928, *M. celatum* ATCC 51131, L08170; *M. cookii* ATCC 49103, X53896; *M. shimoidei* ATCC 27962, X82459 and Z27069; *M. xenopi* ATCC 19250^T, X52929; *M. asiaticum* ATCC 25276^T, M29556 and X55604; *M. gordonae* ATCC 14470^T, M29563 and X52923; *M. leprae* LTB, X55587; *M. gastrii* and *M. kansasii* ATCC 12478^T, M29575; *M. scrofulaceum* ATCC 19981^T, X52924; "*M. paraffinicum*" LT, L08169; *M. szulgai* ATCC 25799^T, X52926; *M. malmoense* ATCC 29571^T, X52930; *M. intracellulare* ATCC 15985, X52927; *M. paratuberculosis* ATCC 19698^T, X52934; *M. avium* DSM 43216, X52918; and *N. asteroides* ATCC 3306, X57949. The sequences from our 16S rRNA gene sequence analysis of the *M. ulcerans*, *M. marinum*, and *M. haemophilum* strains were aligned with the selected 16S rRNA sequences retrieved from GenBank by the multisequence alignment program Pileup (11) and with Clustal personal computer software packages. The alignment was edited by removing all positions at which any sequence contained an ambiguous or undetermined nucleotide. Phylogenetic relationships were inferred by using version 3.4 of the PHYLIP software package (15). A dendrogram was constructed by the distance-based, neighbor-joining method (46) by using the sequence from *N. asteroides* as the outgroup (the nonrelated species used to root the tree). The reproducibility of the tree nodes was analyzed by bootstrapping (15).

RESULTS

Gas chromatography. The strains of *M. ulcerans*, *M. marinum*, *M. haemophilum*, and *M. tuberculosis* were clearly distinguished by their fatty acid profiles. Three categories of fatty acids were represented among the four species examined: straight-chain saturated, straight-chain monounsaturated, and methyl branched, including long-chain mycolic acid cleavage products (Table 2). *M. ulcerans* strains were characterized by a 2,4,6-trimethyl tetracosanoic acid (2,4,6Me24:0), as described previously by semiquantitative analysis (9, 10, 32). *M. marinum* was the only species containing 2,4-dimethyl hexadecanoic acid (2,4Me16:0). A feature of *M. haemophilum* not seen in the other species was higher amounts of docosanoic acid (22:0) than tetracosanoic acid (24:0), which has also been described previously (42). The *M. tuberculosis* isolates consistently contained a relatively high amount of tuberculostearic acid (10Me18:0) and hexacosanoic acid (26:0) (27, 36). Long-chain secondary alcohols characteristic of strains of the *M. avium* complex and several other mycobacterial species (27, 36) were not detected. As seen in Fig. 1 and Table 2, when analyzed by principal-component analysis (27), the overall differences in fatty acid profiles gave four distinct clusters representing the four species investigated.

DNA-DNA hybridization. Mean RBRs of DNAs from strains of *M. ulcerans*, *M. marinum*, *M. haemophilum*, and *M. tuberculosis* and reference strains of other mycobacterial species hybridized with total genomic DNA probes of the type and reference strains of the same species are shown in Fig. 2 and

TABLE 2. Fatty acid contents of *M. ulcerans*, *M. marinum*, *M. tuberculosis*, and *M. haemophilum* analyzed by gas chromatography (as methyl esters) and identified by retention characteristics

Fatty acid[a]	Range (mean) % of total amt of fatty acids			
	M. ulcerans (n = 7)	*M. marinum* (n = 12)	*M. tuberculosis* (n = 7)	*M. haemophilum* (n = 9)
14:0	0.1–1.5 (0.9)	0.4–1.5 (1.0)	0.5–4.4 (1.2)	1.7–3.4 (2.7)
15:0	0.1–0.9 (0.4)	0.2–1.1 (0.6)	0.4–1.0 (0.7)	0.2–0.6 (0.4)
16:1a	0.3–2.0 (0.9)	0.7–4.2 (1.9)	0–1.5 (0.5)	0.5–2.8 (1.0)
16:1b	0.3–4.3 (2.4)	1.3–5.8 (3.3)	1.9–5.3 (2.6)	3.3–8.2 (6.3)
16:0	17.5–23.9 (19.8)	24.1–31.2 (26.6)	24.2–41.8 (31.4)	28.6–35.9 (32.4)
10Me17:0	0.2–1.0 (0.5)	0–1.4 (0.6)	0.3–0.7 (0.6)	1.1–1.8 (1.5)
2,4Me16:0[b]		0.1–8.5 (**3.4**[b])		
17:1	0.1–1.0 (0.7)	0.5–4.4 (1.3)	0.4–1.2 (0.6)	0.3–0.7 (0.5)
17:0	0.3–1.8 (1.2)	0.5–6.4 (1.8)	0.9–4.4 (2.7)	0.2–0.4 (0.4)
18:1a	2.8–6.0 (4.0)	1.5–9.9 (6.7)	0.1–1.0 (0.5)	2.0–5.4 (4.3)
18:1b	10.3–23.4 (14.6)	21.7–39.2 (30.8)	9.5–23.6 (15.1)	19.7–23.7 (21.7)
18:0	8.7–20.5 (14.5)	3.7–10.9 (7.8)	4.8–9.2 (7.2)	4.3–8.2 (6.7)
10Me18:0	1.1–5.1 (**2.9**)	0.5–9.0 (**4.0**)	14.0–28.8 (**18.3**)	9.2–13.9 (**11.7**)
20:0	1.9–3.8 (**2.8**)	0.1–0.8 (**0.4**)	0.7–2.5 (**1.2**)	0.5–1.0 (**0.7**)
22:0	2.2–3.8 (**2.9**)	0.2–1.6 (**0.7**)	0.3–1.2 (**0.5**)	1.8–7.9 (**3.9**)
24:0	2.3–18.5 (**12.5**)	1.2–14.3 (**5.5**)	0.8–3.6 (**1.9**)	1.3–5.1 (**3.1**)
TriMe24:0	6.4–24.0 (**17.6**)	0–1.7 (**0.8**)		
26:0		0–3.7[c] (trace)	2.2–16.9 (**9.0**)	0–4.1[c] (trace)

[a] The number before the colon sign indicates the number of the carbon atoms in the chain, and the number after the colon represents the number of double bonds. The prefix denotes the number and position of the methyl side chains.
[b] Fatty acids and percentages in boldface type are useful for differentiation among species.
[c] Found in three strains.

Table 3. Total genomic DNA of the reference strain *M. ulcerans* TMC 1615, which was used as a probe, gave mean intraspecies RBRs ranging from 87 to 101%. Strain TMC 1615 DNA had mean RBRs of 25 to 47% for binding to strains of *M. marinum*, 25 to 34% for binding to *M. tuberculosis*, and 22 to 30% for binding to *M. haemophilum*. Total genomic DNA of *M. haemophilum* NCTC 11185 hybridized to the DNAs of other *M. haemophilum* strains, with mean RBRs ranging from 88 to 102%. *M. marinum* DNAs displayed more heterogeneous intraspecies RBRs than the other species investigated. Intraspecies variation in DNAs from *M. marinum* strains yielded RBRs ranging from 72 to 101%. RBRs of 30 to 41% were found between *M. marinum* and both *M. tuberculosis* and *M. ulcerans*, and RBRs of 17 to 28% were found between *M. marinum* and *M. haemophilum*. Toward *M. tuberculosis* DNAs, *M. ulcerans*, *M. marinum*, and *M. haemophilum* DNAs had RBRs of 32 to 38, 25 to 38, and 22 to 26%, respectively. Cosmid clone pMm5 from *M. marinum* detected all *M. marinum* DNAs tested (RBR range, 89 to 104%). This probe also discriminated better between DNAs from *M. marinum* and the other mycobacterial species investigated (including those from *M. ulcerans*) than did total DNA because it did not have heterologous RBRs higher than 19% (Fig. 2).

16S rRNA gene sequence analysis. The DNA sequence of 1469 to 1482 bp of the 16S rRNA genes of five strains were analyzed, namely, *M. ulcerans* TMC 1615 (reference strain), *M. marinum* CCUG 20998 (type strain), T24/83, and MNC195, and *M. haemophilum* NCTC 11185 (type strain). The 16S rRNA gene sequences between these species and *M. tuberculosis* were mutually conserved except at base pairs 129, 130, 186 to 192, 193, 198, 200, 201, 456, 463, 1248, and 1289, according to the numbering for the *Escherichia coli* 16S rRNA gene (6, 14). The 16S rRNA gene sequences of the three *M. marinum* strains examined were identical. The sequences of the *M. ulcerans* and *M. marinum* strains were identical in the 16S rRNA gene signature region and throughout the gene had only two single-nucleotide differences between them (nucleotides 1248 and 1289). The 16S rRNA gene sequences for *M. ulcerans* TMC 1615, *M. marinum* CCUG 20998^T, T24/83, and MNC195, and *M. haemophilum* NCTC 11185^T obtained in this study were otherwise identical to the sequences in GenBank. The 16S rRNA gene sequences determined for *M. ulcerans*, *M. marinum*, and *M. haemophilum* were compared to the 16S rRNA gene sequences of 19 other species of slowly growing mycobacteria from GenBank. A phylogenetic tree constructed on the basis of these data is presented in Fig. 3.

DISCUSSION

Detection of heat-stable catalase is the only phenotypic characteristic available for the identification of *M. ulcerans*, and definitive identification has relied on injection of strains into guinea pigs or mouse footpads. Two of the isolates analyzed in this study, TMC 1617 (the type strain) and ATCC 10013, showed increased growth rates, and they were deficient in toxin production and had attenuated virulence when they were injected intradermally into guinea pigs. This led to the concern that these isolates had been misidentified. However, gas chromatography, DNA-DNA hybridization, and heat-stable catalase production confirmed their identities as *M. ulcerans*. Since these isolates were originally obtained from patients with clinical cases of ulcerating disease, it is clear that *M. ulcerans*, like many pathogenic bacteria, can lose their virulence through subculture.

The gas chromatographic results (Fig. 1; Table 2) revealed that *M. ulcerans*, *M. marinum*, *M. haemophilum*, and *M. tuberculosis* were clearly distinguishable from each other by their fatty acid and alcohol profiles. It is interesting that the *M. haemophilum* strains appeared as a very homogeneous cluster, despite the diverse sources of the strains (Table 1). The seven strains of *M. tuberculosis* examined comprised a fairly homogeneous group, with reference strain H37Rv being a typical member, whereas the *M. marinum* cluster emerged as a more heterogeneous group. The inter- and intraspecies affinities revealed by these phenotypic characteristics were convincingly confirmed by the nucleic acid analysis.

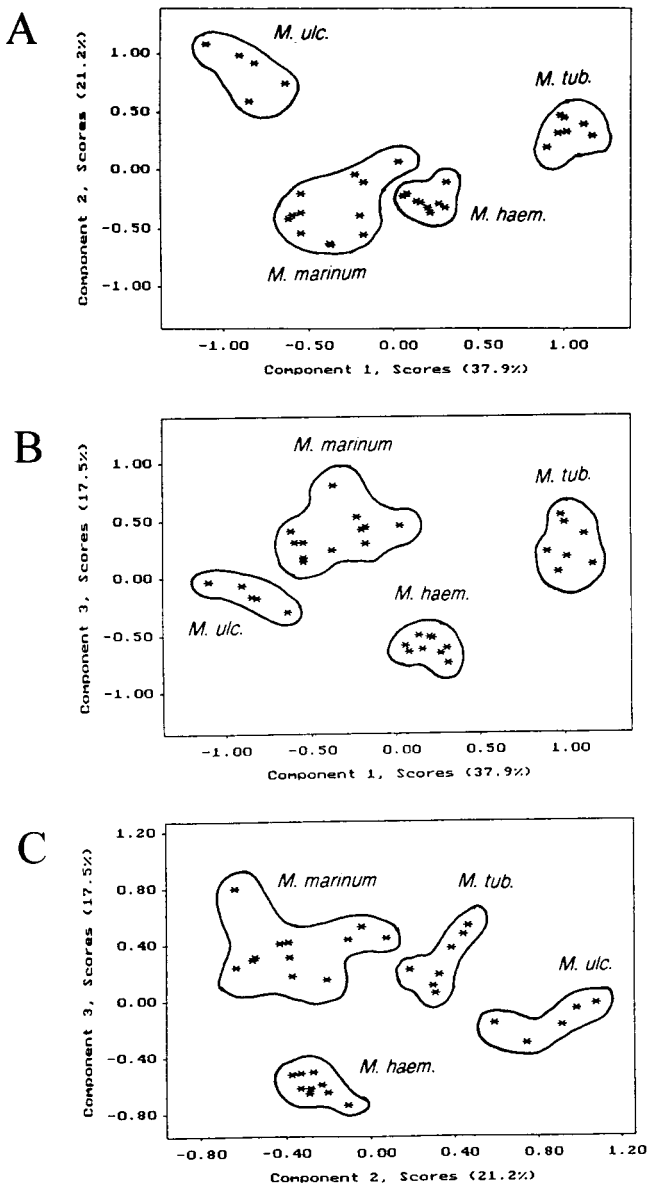

FIG. 1. Principal-component plots based on the fatty acid profiles of the individual mycobacterial strains. (A) Component 1 versus component 2. (B) Component 1 versus component 3. (C) Component 2 versus component 3. Each point represents one strain. *M. tub.*, *M. tuberculosis*; *M. ulc.*, *M. ulcerans*, *M. haem.*, *M. haemophilum*.

DNA-DNA hybridization demonstrated a particularly close relationship between *M. ulcerans*, *M. marinum*, and *M. tuberculosis*. *M. ulcerans* and *M. marinum* were just sufficiently different to be considered separate species (Fig. 2 and Table 3) (54), and their relationships to *M. tuberculosis* were found to be closer than was previously recognized. The strong affinities of *M. marinum* versus *M. tuberculosis* and *M. ulcerans* versus *M. tuberculosis* have apparently been overlooked, probably because of the few hybridization data that have been available for these species. Moreover, the sparse data that did exist were based on DNAs from single isolates (20, 24). To distinguish between bacterial DNAs with high guanine-plus-cytosine contents, such as for mycobacteria, DNA-DNA hybridization conditions need to be highly stringent, as provided by the 0.1 M sodium salt concentration and the 70°C hybridization and wash temperature used in this study. The high G+C content and the consequent high denaturation and annealing temperatures used may lead to a shift in hybridization kinetics. This may make it difficult to compare these hybridization results directly to the results of similar studies comparing bacterial DNAs with lower G+C contents. Cosmid clone pMm5 from *M. marinum* detected all *M. marinum* DNAs tested and also discriminated better between *M. marinum* and the other mycobacterial DNAs investigated, displaying promising results for the development of an *M. marinum* species-specific DNA probe (Fig. 2 and Table 3).

In this study we determined the nearly complete DNA sequences of the 16S rRNA genes from *M. ulcerans* TMC 1615, *M. haemophilum* NCTC 11185, and three *M. marinum* isolates, CCUG 20998, T24/83, and MNC195. When these sequences were compared to the 21 16S rRNA gene sequences of slowly growing mycobacteria from GenBank, a phylogenetic tree could be constructed (Fig. 3). Evidently, *M. marinum* and *M. ulcerans* are more closely related to *M. tuberculosis* than are any of the mycobacterial species commonly used as model systems for studying tuberculosis, such as *M. avium* and *M. intracellulare*. *M. marinum* replicates within macrophages and resides in an endosomal compartment which is developmentally retarded and which does not fuse with the lysosomal compartment, and like *M. tuberculosis*, it readily kills infected macrophages (3). This species may therefore provide an improved model for studying the interaction of *M. tuberculosis* with macrophages.

M. marinum and *M. ulcerans* have identical sequences in the mycobacterial 16S rRNA gene signature region (31). The only 16S rRNA sequence differences found were A versus G at position 1248 for *M. marinum* and *M. ulcerans*, respectively, and A versus C at position 1289, respectively, as described previously (23, 43). The *M. ulcerans* strains analyzed in this study were type 2 strains belonging to the Institute of Tropical Medicine (ITM) and originated in Australia (43). Considerable phenotypic variation was found among the *M. marinum* isolates examined with regard to colony morphology, growth temperature limitation, and pigmentation. The isolates analyzed were obtained from humans with infections, fish, and water. Not surprisingly, therefore, our hybridization data showed that the *M. marinum* group appeared to be more heterogeneous than the groups of the other mycobacterial species examined. However, the 16S rRNA gene sequences from all three *M. marinum* isolates were identical.

Despite the high degree of homology at the 16S rRNA gene sequence level, DNAs from strains of *M. ulcerans* and *M. marinum* analyzed with the *M. tuberculosis* complex-specific PCR test Amplicor (Roche, Basel, Switzerland), which uses the 16S rRNA gene signature region as a target template (50), repeatedly gave negative results (50a). The observed lack of cross-reactivity of nucleic acids from *M. ulcerans* and *M. marinum* in this established diagnostic test adds to the specificity criteria of this assay. However, a corresponding distinction between *M. ulcerans* and *M. marinum* by 16S rRNA gene analysis is more difficult due to the existence of identical signature regions and only two single-nucleotide differences at the 3′ end of the gene. Correspondingly, negative test results and a high level of specificity were obtained when analyzing *M. ulcerans* and *M. marinum* DNAs and cultures with the *M. tuberculosis* complex-specific LCx MTB assay (Abbott Laboratories, Chicago, Ill.), which uses the gene encoding the protein antigen b as a target for identification (34). However, new

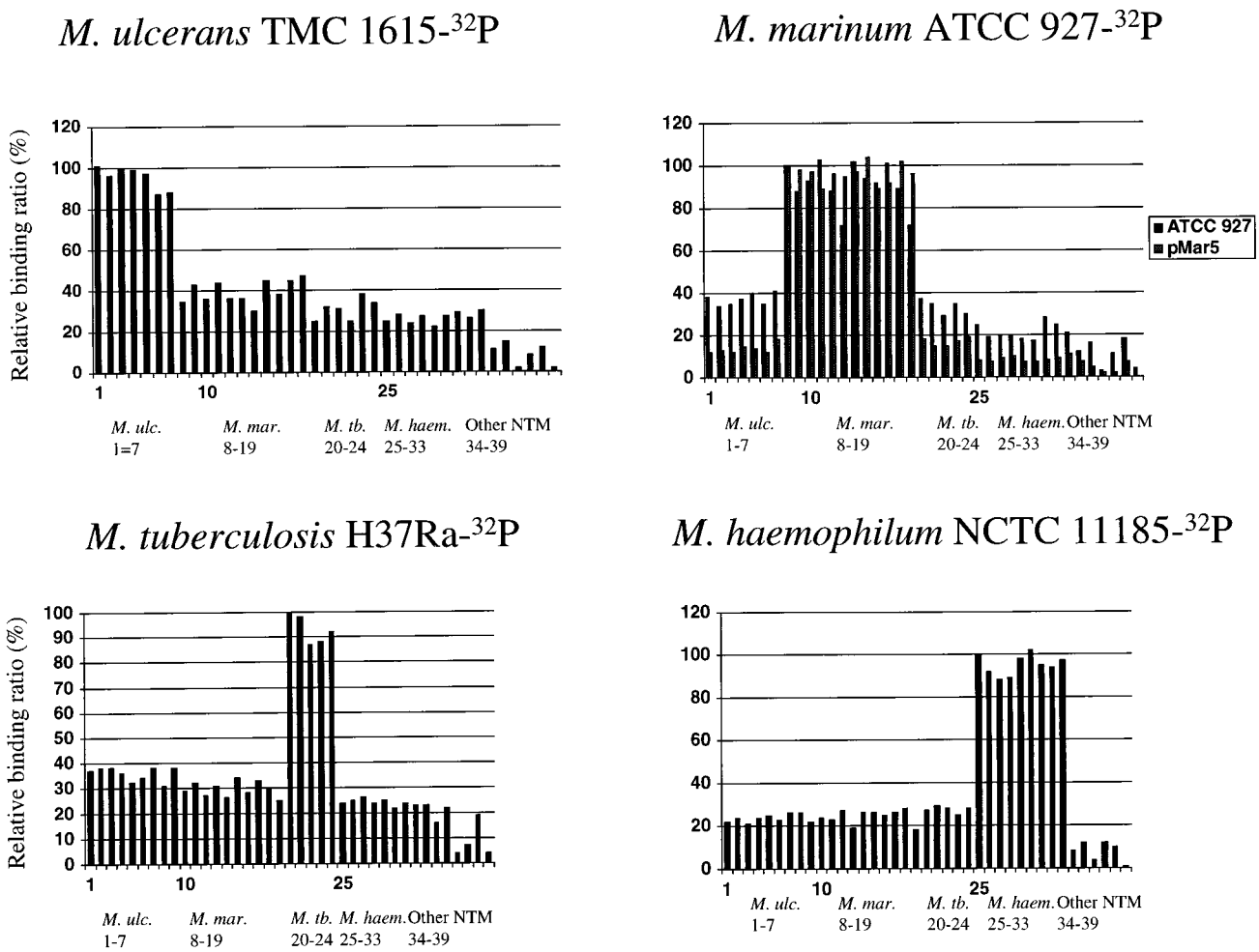

FIG. 2. Graphic presentation of the mean RBRs for *M. ulcerans* (*M. ulc.*), *M. marinum* (*M. mar.*), *M. tuberculosis* (*M. tb.*), and *M. haemophilum* (*M. haem.*) with total genomic DNAs as radioactively labelled probes. For *M. marinum* the results obtained by using the genomic DNA probe (left bars) and the cosmid clone pMm5 (right bars) are shown.

targets for discriminating between *M. ulcerans* and *M. marinum* will be sought, and the probe pMm5 may be a candidate.

Fox and coworkers (17) showed that among *Bacillus* species, the 16S rRNA gene sequence data are not always reliable criteria for species designation. In most cases, organisms with fewer than 15 differences in their 16S rRNA sequences will be defined as a single species by other criteria such as the results of DNA-DNA hybridization. However, even though *Bacillus psychrophilus* and *Bacillus globisporus* share 99.8% similarity in their 16S rRNA sequences, they are only 23% homologous by DNA-DNA hybridization and thus are clearly separate species (17). A similar case is that of *M. marinum* and *M. ulcerans*. These two organisms share more than 99.8% similarity in their 16S rRNA sequences, but DNA-DNA hybridization results

TABLE 3. RBRs for hybridization of *M. ulcerans*, *M. marinum*, *M. tuberculosis*, and *M. haemophilum* obtained by using total genomic DNA as radioactively labelled probe

Filter DNA source	Mean (range) RBR with the following DNA probes[a]:				
	M. ulcerans TMC 1615^T genomic DNA	*M. marinum* CCUG 20998^T genomic DNA	*M. tuberculosis* H37Ra^T genomic DNA	*M. haemophilum* NCTC 11185^T genomic DNA	*M. marinum* pMm5 plasmid DNA
M. ulcerans (n = 7)	95 (87–101)	37 (35–41)	36 (32–38)	24 (21–26)	14 (12–18)
M. marinum (n = 12)	38 (25–47)	85 (72–101)	30 (25–38)	24 (18–27)	96 (89–104)
M. tuberculosis (n = 5)	32 (25–34)	33 (29–37)	93 (87–100)	27 (25–29)	17 (15–19)
M. haemophilum (n = 9)	29 (22–30)	21 (17–28)	24 (22–26)	95 (88–102)	9 (7–11)
Other NTM[b] (n = 6)	9 (2–15)	11 (2–18)	13 (4–22)	9 (4–12)	4 (0–7)

[a] The sample mean and range RBRs for eight parallel DNAs from the total number of strains indicated in parentheses in the filter DNA source column are presented. The 95% confidence interval was calculated for all RBRs (data not shown).

[b] NTM, nontuberculous mycobacteria.

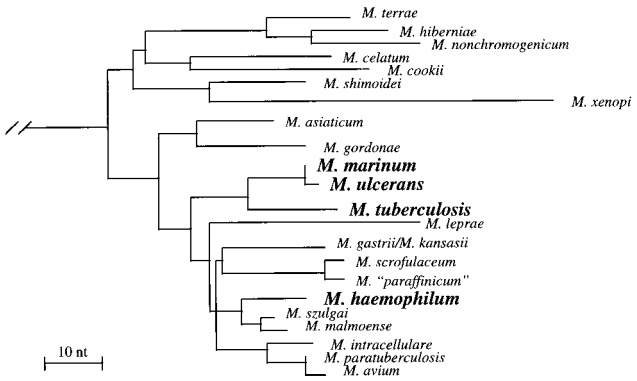

FIG. 3. Phylogenetic tree based on the alignment of partial 16S rRNA gene sequences illustrating the positions of strains of *M. ulcerans*, *M. marinum*, *M. tuberculosis*, and *M. haemophilum* and other slowly growing mycobacterial species. The tree was rooted by use of *N. asteroides* as an outgroup. The bar indicates a 10-nucleotide (10-nt) difference.

showed RBRs of 37 to 38% (range, 25 to 47%; Table 3). This is less than the 50 to 70% DNA hybridization level expected within a single species (54).

Although helpful in assigning classificatory placements for many mycobacterial and other bacterial species (6, 31), 16S rRNA gene sequence analysis may not reflect phylogenetic relations in, for example, slowly growing mycobacteria or in bacterial groups which may exhibit more recent evolutionary divergence in this part of the genome (17). The high degree of conservation of the mycobacterial 16S rRNA gene may explain why analysis of the 16S rRNA genes of *M. ulcerans*, *M. marinum*, and *M. haemophilum* is not so useful for discriminating between these species. Although the 16S rRNA gene sequences of all three species have been known for some time (23, 45, 56), the taxonomic relationships of these organisms to each other and to *M. tuberculosis* have not definitively been described previously.

At the time that this study was started limited data on the taxonomic relationship of *M. haemophilum* to other slowly growing mycobacteria were available. The gas chromatography and DNA hybridization data presented in this study confirm that *M. haemophilum* is a separate species which is clearly distinct from *M. ulcerans* and *M. marinum*, and *M. haemophilum* appears to be as related to *M. ulcerans* and *M. marinum* as to *M. tuberculosis*. Sufficient *M. leprae* DNA was not available to compare *M. haemophilum* with *M. leprae* by DNA-DNA hybridization, but the 16S rRNA gene sequence analysis suggests a relatively close relationship between *M. haemophilum* and *M. leprae*. On the basis of the affinities demonstrated by 16S rRNA gene sequence analysis, the relationships of *M. haemophilum* to *M. malmoense* and *M. szulgai* should also be further detailed. In our analyses, the total genomic DNA of *M. haemophilum* strains appeared to be quite homogeneous, despite their various origins, and other markers are required for further subtyping, such as the insert sequence element that has been described previously (30).

In summary, gas chromatography of fatty acids and alcohols, DNA-DNA hybridization, and 16S rRNA gene sequence analysis showed that *M. ulcerans* and *M. marinum* are most closely related and that these species are more closely related to *M. tuberculosis* than are any other mycobacterial species outside the *M. tuberculosis* complex. *M. haemophilum* was more distantly related to both *M. ulcerans* and *M. marinum* and to *M. tuberculosis*. These findings will be helpful in addressing major obstacles such as how to develop rapid and reliable diagnostic tests for these slowly growing mycobacterial species. They can also enable us to design tools for use in environmental screening for the reservoir of *M. ulcerans.*

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