Mycobacterium brumae sp. nov., a Rapidly Growing, Nonphotochromogenic Mycobacterium

M. LUQUIN,^{1,2*} V. AUSINA,^{1,2} V. VINCENT-LÉVY-FRÉBAULT,³ M. A. LANÉELLE,⁴ F. BELDA,^{1,2}
M. GARCÍA-BARCELÓ,^{1,2} G. PRATS,¹ AND M. DAFFÉ⁴

Servicio de Microbiología, Hospital de la Santa Cruz y San Pablo, Departamento de Genética y Microbiología, Universidad Autónoma de Barcelona, 08025 Barcelona,¹ and Servicio de Microbiología, Hospital Germans Trias i Pujol, Departamento de Genética y Microbiología, Universidad Autónoma de Barcelona, 08915 Badalona,² Spain, and Unité de la Tuberculose et des Mycobactéries, Institut Pasteur, 75724 Paris Cedex 15,³ and Département III du LPTF du Centre National de la Recherche Scientifique et Université Paul Sabatier, 31062 Toulouse Cedex,⁴ France

Strains of a new species of rapidly growing, nonphotochromogenic mycobacteria, *Mycobacterium brumae*, have been isolated from water, soil, and human sputum samples in Barcelona, Spain. The inclusion of this organism in the genus *Mycobacterium* is based on its acid-alcobol fastness, its DNA G+C content, its mycolate pattern, and its mycolate pyrolysis esters. A study of 11 strains showed that they form a homogeneous group with an internal phenotypic similarity value of $94.9 \pm 3.79\%$. The results of a comparison with 39 other mycobacterial species and subspecies are also presented. DNA relatedness studies showed that the *M. brumae* strains studied form a single DNA hybridization group which is less than 30% related to 15 other species of the genus *Mycobacterium*. Thin-layer chromatographic analysis showed that only α -mycolates are present. Unlike *Mycobacterium fallax* and *Mycobacterium triviale* α -mycolates, *M. brumae* α -mycolates release only 22-carbon atom esters after pyrolysis. Strain CR-270 is the type strain; a culture of this strain has been deposited in the Collection Nationale de Cultures de Microorganismes de l'Institut Pasteur, Paris, France, as strain CIP 103465.

From 1983 to 1987, in the course of a search for mycobacteria in environmental samples, we isolated 10 strains with homogeneous properties belonging to the genus *Mycobacterium*. Later, in 1991, a similar strain was isolated from a human sputum sample. These organisms were rapidly growing, nonphotochromogenic mycobacteria that differed from all known species and are considered members of a new species. These strains had a colonial morphology similar to that of *Mycobacterium fallax* and the same pattern of mycolates (only α -mycolates) as determined by thin-layer chromatography (TLC) (16). *Mycobacterium triviale* is the only slowly growing *Mycobacterium* species with this pattern of mycolates (4, 16). Mycolic acids are useful taxonomic markers for the differentiation of mycobacteria at both the genus level and the species level. Mycobacterial mycolic acids are characteristically 2-branched, 3-hydroxy fatty acids with very long chains (up to 90 carbon atoms long) which are major lipid constituents in the cell wall (4, 7). By TLC they may be separated into seven types according to the content and position of different functional groups in their carbon chains (18, 19). Mycolic acid methyl esters release by pyrolysis straight esters with carbon chain lengths ranging from C₂₂ to C₂₆ (4, 12, 17). Within the genus *Mycobacterium*, variation in the pyrolysis ester chain lengths is an additional differential characteristic that is especially useful for subdividing groups of species that produce the same TLC pattern. The strains of the new species could be clearly distinguished from *M. fallax* and *M. triviale* by the chain lengths of their pyrolysis esters. Esters containing 22 and 24 carbon atoms were released by α -mycolates of *M. fallax*, and tetracosanoate was the only ester liberated by *M. triviale* α -mycolates (14, 16, 17, 22). In contrast, the α -mycolates of the

new strains give only docosanoate when they are subjected to pyrolytical cleavage. A subsequent structural analysis showed that there are great differences in the compositions of the α -mycolates of these strains and the α -mycolates of *M. fallax* and *M. triviale*. Genotypically, there was clear evidence for differentiation of these organisms, since the levels of DNA-DNA hybridization between the strains of the new taxon and other related species were less than 30%. In this paper, we describe the characteristics of these strains and designate a new species, *Mycobacterium brumae*.

MATERIALS AND METHODS

Bacterial strains. A total of 11 strains were studied. Eight of these (strains CR-103, CR-104, CR-142, CR-210, CR-267, CR-268, CR-270^T [T = type strain], and CR-271) were isolated from water samples taken from the Llobregat River in Barcelona, Spain; two strains (strains CR-130 and CR-269) were isolated from soil; and one strain (strain CR-148) was isolated from a human sputum sample. In addition to these 11 isolates, we studied the following strains of species which are on the Approved Lists of Bacterial Names (24) or in the *Index of the Bacterial and Yeast Nomenclatural Changes* (20): *Mycobacterium agri* ATCC 27406^T; *Mycobacterium aichiense* ATCC 27280^T; *Mycobacterium aurum* ATCC 23366^T; *Mycobacterium austroafricanum* ATCC 33464^T; *Mycobacterium chelonae* subsp. *chelonae* NCTC 946^T and CIP 801159; *Mycobacterium chelonae* subsp. *abscessus* ATCC 19977^T; *Mycobacterium chitae* ATCC 19627^T; *Mycobacterium chubuense* ATCC 27278^T; *Mycobacterium diernhoferi* ATCC 19340^T; *Mycobacterium duvalii* NCTC 358^T; *Mycobacterium fallax* CIP 8139^T, CIP 1390007, and CIP 1390014; *Mycobacterium flavescens* ATCC 14474^T; *Mycobacterium fortuitum* ATCC 6841^T and ATCC 14467; *Mycobacterium gadium* ATCC 27726^T; *Mycobacterium gastri*

* Corresponding author.

ATCC 15754^T; *Mycobacterium gilvum* NCTC 10742^T; *Mycobacterium gordonae* ATCC 14470^T and CIP 0210008; *Mycobacterium kansasii* ATCC 12478^T; *Mycobacterium kosmosense* ATCC 33013^T; *Mycobacterium marinum* ATCC 927^T; *Mycobacterium morioakaense* ATCC 43059^T; *Mycobacterium neoaurum* ATCC 25795^T; *Mycobacterium obuense* ATCC 27023^T; *Mycobacterium parafortuitum* ATCC 19686^T; *Mycobacterium phlei* ATCC 11758^T; *Mycobacterium porcinum* ATCC 33776^T; *Mycobacterium poriferae* ATCC 35087^T; *Mycobacterium pulveris* ATCC 35154^T; *Mycobacterium rhodesiae* ATCC 27024^T; *Mycobacterium senegalense* NCTC 10956^T; *Mycobacterium smegmatis* ATCC 19420^T; *Mycobacterium sphagni* ATCC 33027^T; *Mycobacterium terrae* ATCC 15755^T; *Mycobacterium thermoresistibile* ATCC 19527^T; *Mycobacterium tokaiense* ATCC 27282^T; *Mycobacterium triviale* ATCC 23292^T; *Mycobacterium tuberculosis* ATCC 27294^T and ATCC 25177; and *Mycobacterium vaccae* ATCC 15483^T. In addition, the frequency distribution of the test results and the results of a lipid analysis for mycobacterial isolates obtained from human host and environmental sources were included for comparative purposes. These isolates included 13 *M. chelonae* subsp. *chelonae* strains, 6 *M. chelonae* subsp. *abscessus* strains, 8 *M. fallax* strains, 7 *M. flavescens* strains, 12 *M. fortuitum* strains, 9 *M. gastri* strains, 28 *M. gordonae* strains, 21 *M. kansasii* strains, 4 *M. marinum* strains, 7 *M. smegmatis* strains, 13 *M. terrae* strains, 11 *M. triviale* strains, and 47 *M. tuberculosis* strains. We also included in this study six strains of a recently described new species, *Mycobacterium alvei* (1).

Characterization of strains. Colony morphology, the ability to grow at various temperatures (25, 30, 37, 45, and 52°C), pigment production, and photoreactivity were determined as previously described (9, 10, 28–30). The niacin test was performed with test strips (Difco Laboratories, Detroit, Mich.). Strains were tested for iron uptake by using a modification of the procedure of Szabo and Vandra (26), as described by Silcox et al. (23). The catalase test was performed by the method of Kubica and Pool (11). The nitrate reductase and arylsulfatase tests were performed as described by Vestal (28). The β -glucosidase, urease, penicillinase, and trehalase tests were performed as recommended by David and Jahan (5) and David et al. (6). Tests for resistance to hydroxylamine and sodium chloride, hydrolysis of Tween 80, and β -galactosidase were performed as described by Wayne et al. (29, 30). Tests to determine the use of glucose, inositol, mannitol, and sodium citrate as sole carbon sources in the presence of ammoniacal nitrogen were performed by using the procedure of Silcox et al. (23). Tests to determine degradation of salicylate, tolerance to picric acid, and acid formation from carbon sources were performed as described by Tsukamura (27). Growth in the presence of different antibiotics was determined by using the method of Canetti et al. (2).

Lipid analysis. To isolate lipid components, all of the strains were cultivated on plates containing Middlebrook 7H10 agar and incubated at 30 or 37°C in the presence of 5% CO₂. Fatty acid esters were prepared as previously described (4). Mycolates were obtained by precipitating them twice with methanol from the methyl ester mixture. We then checked by TLC that the pellet contained only mycolates. The mycolates were fractionated by argentation chromatography by using increasing proportions of diethyl ether in petroleum ether (22). This separation was followed by TLC on silver nitrate-impregnated commercial plates developed

with petroleum ether-diethyl ether (6:4, vol/vol). The spots were visualized by charring after the plates were sprayed with a 10% solution of molybdophosphoric acid in ethanol. Oxidative cleavage, isolation of oxidation products, and structural analysis were performed as previously described (13). The patterns of nonhydroxylated fatty acids were determined by capillary gas chromatography as previously described (17).

Instrumentation. Gas chromatography was conducted with a Hewlett-Packard model 5890A apparatus equipped with a fused silica capillary column with SPB1 as the stationary phase. The temperature of the injector was raised from 260 to 350°C for pyrolytical conditions. Infrared spectra were recorded with a Perkin-Elmer model FTIR 1600 apparatus. ¹H nuclear magnetic resonance spectra were obtained with a 200-MHz Bruker instrument by using CDCl₃ as the solvent. Polarimetry was conducted with a Perkin-Elmer model 141 polarimeter. Mass spectra were determined by using an electron impact ion source and a Varian model 311A spectrometer.

DNA-DNA hybridization. For DNA extraction and subsequent DNA-DNA hybridization, selected strains (see Table 4) were cultivated in 1 to 3 liters of nutrient broth (Difco) supplemented with a powder base containing 7H9 Middlebrook broth medium (Difco), 1% glycerol, and 0.05% Tween 80. Flasks were incubated at 30 or 37°C with gentle agitation for 10 to 21 days. DNA was extracted as previously described (14, 15). Briefly, the bacteria were converted to wall-deficient forms and then lysed by adding sodium dodecyl sulfate, as previously described, and proteinase K (Boehringer, Mannheim, Germany) instead of pronase. The DNA was then purified by using the usual methods. DNA from the type strain of the proposed new mycobacterial taxon, strain CIP 103465, was labeled in vitro by nick translation (nucleotides were obtained from the Radiochemical Centre, Amersham, England) as previously described (8), except that we modified the concentrations of some of the reagents, as follows: 3 μ l of DNA (800 μ g/ml), 40 μ l of S1 nuclease (1 U/ml; Sigma Chemical Co., St. Louis, Mo), and 15 μ l of DNase I (10⁻⁸ g/ml). Unlabeled DNAs were sheared by sonication in order to get DNA fragments that were 500 to 800 bp long. The molecular weight of the sonicated DNA was checked by gel electrophoresis by using phage lambda hydrolyzed by restriction enzyme HindIII. The S1 nuclease method (3) in which the S1 nuclease-trichloroacetic acid procedure (8) was used was modified as previously described (14). The temperature (*T_m*) at which 50% of the DNA became hydrolyzable by S1 nuclease was determined by using the procedure developed by Crosa et al. in 1973 (3) and modified as described previously (14, 15).

Numerical taxonomy analysis. The data obtained from our study of 59 taxonomic properties were recorded as positive or negative, and the matching coefficient (*M* value) was calculated by using the following equation: $M \text{ value} = (ns \times 100)/(ns + nd)$, where *ns* is the number of characters for which two strains gave the same results (both positive or both negative) and *nd* is the number of characters for which the strains gave different results (one positive and one negative) (25). The computer which we used was an IBM model 3083/XE/VM/SP-HPC computer. The program which we used is included in the Clustan 2 packet of programs (Computing Laboratory, University of St. Andrews, St. Andrews, Scotland).

FIG. 1. Mature *M. brumae* CIP 103465^T colonies on Middlebrook 7H10 medium. (A) Micrograph. Magnification, $\times 120$. (B) No magnification.

RESULTS AND DISCUSSION

Cells of the 11 new strains grown on Löwenstein-Jensen agar and on Middlebrook 7H10 agar were rod shaped, gram positive, and acid-alcohol fast and were able to form clumps and cords. Spores, capsules, and true branching were not observed. At 25, 30, and 37°C, growth occurred within 4 to 5 days on Löwenstein-Jensen and Middlebrook 7H10 media and on nutrient agar. Dilute inocula on Middlebrook agar yielded large, eugonic, buff-colored, rough colonies (Fig. 1). The phenotypic properties of the 11 *M. brumae* strains are shown in Table 1. These strains constituted a homogeneous group with a level of internal similarity of $94.9 \pm 3.79\%$. Comparisons of *M. brumae* CIP 103465^T with the type strains of 39 other mycobacterial species revealed degrees of similarity of 80% with *M. alvei*, 75.8% with *M. fallax*, and

75% with *M. moriokaense* (Table 2). Characteristics which differentiate *M. brumae* from phenotypically related *Mycobacterium* species are shown in Table 3.

Lipid analysis. Gas chromatographic analysis revealed the presence of hexadecanoate, octadecenoate, and tuberculostearate (10-methyloctadecanoate) as the major fatty acid methyl esters in lipid extracts of the 11 *M. brumae* strains (Fig. 2). All *M. brumae* strains exhibited a TLC pattern consisting of only one spot corresponding to α -mycolate (Fig. 3). When pyrolytical conditions were used, the gas chromatographic profile showed an increase of methyl docosanoate (C_{22}), whereas *M. fallax* mycolates liberated by pyrolysis do- and tetracosanoate (C_{22} and C_{24}) and *M. triviale* liberated only tetracosanoate (4, 16, 17, 22) (Fig. 2). The infrared spectrum of the purified mycolates from *M.*

TABLE 1. Properties of the *M. brumae* type strain and 10 additional strains

Characteristic	Type strain ^a	Ten other strains ^b
Morphology		
Rods (>1 µm long)	+	100
Coccobacillary (<1 µm long)	—	0
Cord formation	+	100
Smooth colonies	—	0
Rough colonies	+	100
Pigmentation in the dark	—	0
Pigmentation in the light	—	0
Growth at:		
25°C	+	100
30°C	+	100
30°C in <5 days	+	100
37°C	+	100
37°C in <5 days	+	100
45°C	—	0
52°C	—	0
Growth on nutrient agar at:		
30°C	+	100
37°C	+	100
Growth on MacConkey agar without crystal violet	—	0
Iron uptake	+	80
Niacin	—	0
Enzymatic activities		
Arylsulfatase (3 days)	—	0
Catalase (22°C)	+	100
Catalase (68°C)	+ ^w	100 ^c
β-Glucosidase (3 h)	+	100
β-Galactosidase (18 h)	—	0
Nitrate reductase (2 h)	+	100
Penicillinase (5 h)	+	100
Trehalase (5 h)	+	100
Urease (18 h)	+	100
Tween hydrolysis (10 days)	+	100
Salicylate degradation (7 days)	—	0
Acid produced from:		
Glucose	+	100
L-Arabinose	—	0
Dulcitol	—	0
Fructose	+	100
meso-Inositol	+	100
Mannitol	—	0
L-Rhamnose	—	0
D-Xylose	—	20
Trehalose	+	100
Use of the following compounds as sole carbon sources:		
Glucose	+	100
meso-Inositol	+	100
Mannitol	—	0
Fructose	+	100
Sodium citrate	+	100
Growth in the presence of:		
Picric acid (0.2%)	+	50
NaCl (5%)	—	0
NH ₂ OH (500 mg/liter)	—	20
D-Cycloserine (30 mg/liter)	—	40
Ethambutol (2 mg/liter)	—	0
Streptomycin (4 mg/liter)	+	100
Kanamycin (20 mg/liter)	—	0
Capreomycin (40 mg/liter)	—	0
Isoniazid (0.1 mg/liter)	+	100
Isoniazid (1 mg/liter)	+	80
Isoniazid (10 mg/liter)	—	0
Rifampin (40 mg/liter)	+	80
p-Aminosalicylic acid (0.5 mg/liter)	+	100
Thiacetazone (2 mg/liter)	+	100
Thiacetazone (10 mg/liter)	+	100

TABLE 2. Levels of similarity between *M. brumae* CR-270^T (= CIP 103465^T) and type strains of 39 mycobacterial species and subspecies

Species or subspecies	% Similarity to strain CIP 103465 ^T
<i>M. alvei</i>	80.0
<i>M. fallax</i>	75.8
<i>M. morioakaense</i>	75.0
<i>M. austroafricanum</i>	74.3
<i>M. duvalii</i>	73.0
<i>M. gilvum</i>	73.0
<i>M. smegmatis</i>	72.7
<i>M. agri</i>	72.7
<i>M. diernhoferi</i>	72.5
<i>M. gadium</i>	72.2
<i>M. parafortuitum</i>	72.0
<i>M. fortuitum</i>	71.2
<i>M. chitae</i>	69.7
<i>M. neoaurum</i>	69.4
<i>M. senegalense</i>	68.2
<i>M. aurum</i>	68.0
<i>M. aichiense</i>	67.5
<i>M. rhodesiae</i>	67.5
<i>M. porcinum</i>	66.7
<i>M. flavescens</i>	66.7
<i>M. marinum</i>	66.7
<i>M. chelonae</i> subsp. <i>chelonae</i>	66.7
<i>M. poriferae</i>	65.9
<i>M. sphagni</i>	65.9
<i>M. thermoresistibile</i>	65.6
<i>M. kansasii</i>	65.2
<i>M. chubuense</i>	65.0
<i>M. tokaiense</i>	65.0
<i>M. pulveris</i>	64.3
<i>M. komossense</i>	63.8
<i>M. chelonae</i> subsp. <i>abscessus</i>	63.6
<i>M. triviale</i>	62.1
<i>M. phlei</i>	61.5
<i>M. vaccae</i>	60.6
<i>M. tuberculosis</i>	60.6
<i>M. gastri</i>	57.6
<i>M. terrae</i>	57.6
<i>M. obuense</i>	57.5
<i>M. gordonae</i>	53.0

brumae revealed an absorption band at 965 cm⁻¹ characteristic of *trans* double bonds, while absorption due to cyclopropane rings was not observed. The ¹H nuclear magnetic resonance spectrum confirmed this hypothesis by the presence of signals assignable to the resonance of olefinic protons at δ = 5.35 ppm and the absence of signals between δ = 0 and δ = 0.7 ppm, corresponding to cyclopropane ring protons resonances. Argentation chromatography fractionated this mycolate spot into three main compounds; each class was subjected to oxidative cleavage, and the oxidation products were analyzed by gas chromatography. These analyses gave the composition shown in Fig. 4. The I_A α-mycolates were defined as diunsaturated esters with two *trans* double bonds, as suggested by infrared and ¹H nuclear magnetic resonance spectroscopy (multiplet of the double bond at δ = 5.35 ppm and additional methyl groups near the

^a —, negative reaction; +, positive reaction; +^w, weak reaction.^b The values are the percentages of strains that are positive.^c Weak reactions.

TABLE 3. Differentiation of *M. brunae* CIP 103456^T from other phenotypically related species of mycobacteria

Characteristic	<i>M. brunae</i> CIP 103465 ^T	<i>M. alvei</i> CIP 103464 ^T	<i>M. fallax</i> CIP 8139 ^T	<i>M. mortuaense</i> ATCC 43059 ^T	<i>M. austro-</i> <i>afghanum</i> ATCC 33464 ^T	<i>M. daubii</i> NCTC 358 ^T	<i>M. gilvum</i> NCTC 10742 ^T	<i>M. smeg-</i> <i>matris</i> ATCC 19420 ^T	<i>M. agri</i> ATCC 27406 ^T	<i>M. diern-</i> <i>hoferi</i> ATCC 19340 ^T	<i>M. gadium</i> ATCC 27726 ^T	<i>M. para-</i> <i>fortuitum</i> ATCC 19686 ^T	<i>M. fortu-</i> <i>turni</i> ATCC 6841 ^T
Morphology	+	+	-	+	+	+	+	+	+	+	+	+	+
Rods (>1 µm long)	+	-	+	-	-	-	-	-	-	-	-	-	-
Coccobacillary (<1 µm long)	+	-	+	-	-	ND	ND	-	-	-	ND	-	-
Cord formation	+	-	-	-	+	+	+	-	+	+	+	-	-
Smooth colonies	+	+	+	+	-	+	-	+	+	-	+	+	+
Rough colonies	-	-	-	-	+	+	+	-	-	-	+	-	-
Pigmentation in the dark	-	-	-	-	+	+	+	-	-	-	+	+	-
Pigmentation in the light	-	-	-	-	+	+	+	-	-	-	+	+	-
Growth at:													
37°C in <5 days	+	-	-	+	+	+	+	+	+	+	+	+	+
45°C	-	-	-	-	-	-	-	+	+	-	-	-	-
Growth on MacConkey agar without crystal violet	-	-	-	ND	ND	ND	ND	-	-	-	ND	-	+
Iron uptake	+	-	-	ND	ND	ND	ND	+	-	+	-	+	+
Enzymatic activities													
Arylsulfatase (3 days)	-	+	-	+	+	-	-	-	-	-	-	-	+
Catalase (68°C)	+ ^w	+	-	+	+	+	+	+	+	+	+	+	+
β-Galactosidase (18 h)	-	+	-	-	-	-	-	+	+	+	+	+	-
Urease (18 h)	+	+	+	+	+	+	ND	+	+	+	+	+	+
Tween hydrolysis (10 days)	+	+	+	-	-	-	+	+	-	-	+	-	+
Salicylate degradation (7 days)	-	-	-	-	-	-	+	+	-	-	+	-	+
Use of the following compounds as sole carbon sources:													
<i>meso</i> -Inositol	+	-	-	+	+	-	+	+	-	+	+	-	-
Mannitol	-	-	-	+	+	+	+	-	-	+	+	+	-
Sodium citrate	+	-	-	-	+	-	-	-	-	-	+	-	-
Growth in the presence of:													
Picric acid (0.2%)	+	-	-	+	+	ND	ND	+	+	+	-	+	+
NaCl (5%)	-	-	-	+	+	ND	ND	+	+	+	+	-	+
NH ₂ OH (500 mg/liter)	-	+	+	-	-	-	-	-	-	-	-	-	+
Mycolate contents													
α-Mycolates	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxygenated mycolates	-	+	+	+	+	+	+	+	+	+	+	+	+

^a -, negative reaction; +, positive reaction; +^w, weak reaction; ND, not determined.

FIG. 2. Gas chromatogram of fatty acid methyl esters and mycolic acid cleavage products of *M. brumae* CIP 103465^T (A), *M. fallax* CIPT 1390005^T (B), and *M. triviale* ATCC 23292^T (C). The numbers above the peaks indicate the number of carbon atoms, followed by the number of double bonds. TBS, tuberculostearate (10-methyloctadecanoate); FID, flame ionization detector.

double bond at $\delta = 0.94$ ppm) (13) and by the negative value of the molecular rotation of the intact mycolate (13). The main mycolic acid of this series contained 76 carbon atoms. The I_B α -mycolates consisted of a mixture of compounds, each bearing one *cis* double bond and one *trans* double bond. The major mycolic acid of this series contained 75 carbon atoms. The I_C α -mycolates contained only *cis* diunsaturated esters. 1H nuclear magnetic resonance of this fraction showed a well-defined triplet at $\delta = 5.35$ ppm (instead of a multiplet) assignable to the resonance of olefinic protons. These α -mycolates consisted of mycolic acid homologs containing 68 to 76 carbon atoms (the main component was an acid with 70 carbon atoms). The chain length and the

existence of double bonds in these mycolates indicate that they are related to *M. fallax* mycolates (22). However, the differences in the number of double bonds (tetraunsaturated α -mycolates have been described in *M. fallax*) and in the chain length of the pyrolysis esters (16, 22) suggest that the new strains belong to a species different from *M. fallax*.

DNA relatedness. The ratios of absorbance for DNA solutions ranged from 1.9 to 2.1 and from 1.7 to 1.8 for the A_{260}/A_{280} and A_{260}/A_{230} ratios, respectively. The results of DNA-DNA hybridization experiments are shown in Table 4. We selected the type strains of species which produced the same mycolate pattern (only α -mycolates) as *M. brumae* (*M. fallax* and *M. triviale*) for DNA-DNA hybridization experi-

FIG. 3. Thin-layer chromatogram of methylmycolates from *M. tuberculosis* ATCC 27294^T (lane 1), *M. fallax* CIP 1390005^T (lane 2), *M. triviale* ATCC 23292^T (lane 3), *M. brumae* CIP 103465^T (lane 4), *M. fortuitum* ATCC 6841^T (lane 5), and *M. chelonae* NCTC 946^T (lane 6). The mycolate types are indicated as follows: I, α -mycolate; II, α' -mycolate; III, methoxymycolate, IV, ketomycolate; and V, epoxymycolate. The elution system consisted of two runs with petroleum ether-diethyl ether (85:15, vol/vol) as the eluent.

ments, as well as other rapidly growing and slowly growing mycobacterial species. The use of the hybridization and S1 nuclease treatment conditions mentioned above resulted in levels of nonspecific hybridization (in control tubes containing herring DNA and labeled *M. brumae* CIP 103465^T DNA) ranging from 7.6 to 9.4%. Four strains of *M. brumae* were 95 to 100% related to strain CIP 103465^T, with ΔT_m values less than 2.5°C (ΔT_m is the difference between the T_m of the

homologous reaction and the T_m of the heterologous reaction). The percentages of relative binding of strain CIP 103465^T with all other strains were less than 30%. The ΔT_m values determined for the higher levels of DNA complementarity were all more than 12.1°C. The guanine-plus-cytosine (G+C) contents of DNAs were calculated from the melting temperatures by using the equation of Owen and Pitcher (21). The G+C content of strain CIP 103465^T was 70 mol%. This value was an underestimation of the actual G+C content as sonicated DNAs were used to determine the melting temperatures.

Description of type strain CR-270 (= CIP 103465) of *Mycobacterium brumae* sp. nov. *Mycobacterium brumae* (bru' mae. L. gen. n. *brumae*, of winter, referring to the time of year at which the first strains were isolated). The cells of *M. brumae* are gram-positive rods, 2.0 to 2.5 μ m long and 0.3 to 0.5 μ m wide; they are mostly strongly acid fast, except for a small number (less than 10%) of cyanophil forms. Smears prepared from Youmans medium (without Tween 80) and Middlebrook 7H12 medium contain clumps or cords. Colonies on Löwenstein-Jensen medium and on Middlebrook 7H10 agar are eugonic, rough, and nonpigmented in the dark or after exposure to light. Growth occurs within 5 days at 30 and 37°C but not at 45°C. The type strain produces thermostable catalase and is positive for β -glucosidase, nitrate reductase, penicillinase, trehalase, urease, and iron uptake. Tween 80 is hydrolyzed after 10 days; niacin is not produced, and salicylate is not degraded to catechol. No growth occurs on MacConkey agar without crystal violet. Glucose, *meso*-inositol, fructose, and sodium citrate are utilized as sole carbon sources in the presence of ammoniacal nitrogen. Acid is produced from glucose, fructose, *meso*-inositol, and trehalose but not from L-arabinose, dulcitol, mannitol, L-rhamnose, and D-xylose. Growth is inhibited by sodium chloride (5%), hydroxylamine (500 mg/liter), D-cycloserine (30 mg/liter), ethambutol (2 mg/liter), kanamycin (20 mg/liter), capreomycin (40 mg/liter), and isoniazid (10 mg/liter).

FIG. 4. Molecular structures of α -mycolates of *M. brumae* CIP 103465^T. For double bonds, E indicates *trans*, and Z indicates *cis*.

TABLE 4. Levels of DNA relatedness between strain CR 270^T (= CIP 103465^T) and reference strains belonging to selected mycobacterial species

Species	Source of DNA ^a		% of relative binding at 75°C
	Strain	Other designation	
<i>M. brumae</i>	CR-270 ^T		100
	(= CIP 103465 ^T)		
	CR-130		100
	CR-267		100
	CR-269		100
<i>M. agri</i>	CR-142		95
	CIPT 1320001 ^T	ATCC 27406 ^T	20
<i>M. alvei</i>	CIP 103464 ^T		7
<i>M. chelonae</i>	CIPT 0420003 ^T	NCTC 946 ^T	7
	CIPT 801159		4
<i>M. chitae</i>	CIPT 1160001 ^T	ATCC 19627 ^T	12
<i>M. fallax</i>	CIPT 1390005 ^T	CIP 8139 ^T	21
	CIPT 1390007		24
	CIPT 1390014		30
<i>M. fortuitum</i>	CIPT 0410001 ^T	ATCC 6841 ^T	7
	CIPT 0410020	ATCC 14467	11
<i>M. gordonae</i>	CIPT 0210008		6
<i>M. moriokaense</i>	CIPT 1470001 ^T	ATCC 43059 ^T	5
<i>M. phlei</i>	CIPT 1300001 ^T	ATCC 11758 ^T	12
<i>M. porcinum</i>	CIPT 1460001 ^T	ATCC 33776 ^T	10
<i>M. pulveris</i>	CIPT 1480001 ^T	ATCC 35154 ^T	11
<i>M. senegalense</i>	CIPT 1350002 ^T	NCTC 10956 ^T	12
<i>M. smegmatis</i>	CIPT 1330010 ^T	ATCC 19420 ^T	14
<i>M. triviale</i>	CIPT 0330001 ^T	ATCC 23292 ^T	12
<i>M. tuberculosis</i>	CIPT 0010002	ATCC 25177	21

^a CR, Collection of the Department of Tuberculosis, Hospital de la Santa Cruz y San Pablo, Barcelona, Spain; CIP, Collection Institut Pasteur, Paris, France; CIPT, Collection Institut Pasteur Tuberculose, Paris, France; ATCC, American Type Culture Collection, Rockville, Md.; NCTC, National Collection of Type Cultures, London, England.

Tuberculoatearate is detected by gas chromatography; docosanoate is a mycolic acid cleavage product. Secondary alcohols are not detected. *M. brumae* contains only α -mycolates (68 to 76 carbon atoms long), which release only docosanoate after pyrolysis.

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REFERENCES

- Ausina, V., M. Luquin, M. García-Barceló, M. A. Lanéele, V. Lévy-Frébault, F. Belda, and G. Prats. 1992. *Mycobacterium alvei* sp. nov. Int. J. Syst. Bacteriol. 42:529-535.
- Canetti, G., N. Rist, and J. Grosset. 1963. Mesure de la sensibilité du bacille tuberculeux aux drogues antibacillaires par la méthode des proportions. Rev. Tuberc. Pneumol. 27:217-272.
- Crosa, J. H., D. J. Brenner, and S. Falkow. 1973. Use of a single-strand-specific nuclease for analysis of bacterial and plasmid deoxyribonucleic acid homo- and heteroduplexes. J. Bacteriol. 115:904-911.
- 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. Inst. Pasteur Microbiol. 134B:241-256.
- David, H. L., and M. T. Jahan. 1977. β -Glucosidase activity in mycobacteria. J. Clin. Microbiol. 13:6-9.
- David, H. L., I. Traore, and A. Feuillet. 1981. Differential identification of *Mycobacterium fortuitum* and *Mycobacterium chelonae*. J. Clin. Microbiol. 5:383-384.
- Goodfellow, M. E., and D. E. Minnikin. 1984. Circumscription of the genus, p. 1-24. In G. P. Kubica and L. G. Wayne (ed.), The mycobacteria. A sourcebook. Part A. Marcel Dekker, Inc., New York.
- Grimont, P. A. D., M. Y. Popoff, F. Grimont, C. Coynault, and M. Lemelin. 1980. Reproducibility and correlation study of three deoxyribonucleic acid hybridization procedures. Curr. Microbiol. 4:325-330.
- Kubica, G. P., and H. L. David. 1980. The mycobacteria, p. 1693-1730. In A. C. Sonnenwirth and L. Jarett (ed.), Gradwohl's clinical laboratory methods and diagnosis. The C. V. Mosby Co., St. Louis.
- Kubica, G. P., and R. C. Good. 1981. The genus *Mycobacterium*, p. 1962-1984. In M. P. Starr, H. Stolp, H. G. Trüper, A. Balows, and H. G. Schlegel (ed.), The prokaryotes: a handbook on habitats, isolation and identification of bacteria. Springer Verlag, Berlin.
- Kubica, G. P., and G. L. Pool. 1960. Studies on the catalase activity of acid fast bacilli. I. An attempt to subgroup these organisms on the basis of their catalase activities at different temperatures and pH. Am. Rev. Respir. Dis. 81:387-391.
- Lambert, M. A., C. W. Moss, V. A. Silcox, and R. C. Good. 1986. Analysis of mycolic acid cleavage products and cellular fatty acids of *Mycobacterium* species by capillary gas chromatography. J. Clin. Microbiol. 23:731-736.
- Lanéele, M. A., C. Lacave, M. Daffé, and G. Lanéele. 1988. Mycolic acids of *Mycobacterium aurum*. Structure and biogenetic implications. Eur. J. Biochem. 177:631-635.
- Lévy-Frébault, V., F. Grimont, P. A. D. Grimont, and H. L. David. 1984. Deoxyribonucleic acid relatedness study of *Mycobacterium fallax*. Int. J. Syst. Bacteriol. 34:423-425.
- Lévy-Frébault, V., F. Grimont, P. A. D. Grimont, and H. L. David. 1986. Deoxyribonucleic acid relatedness study of the *Mycobacterium fortuitum*-*Mycobacterium chelonae* complex. Int. J. Syst. Bacteriol. 36:458-460.
- Lévy-Frébault, V., J. Rafidinarivo, J. C. Promé, J. Grandry, H. Boisvert, and H. L. David. 1983. *Mycobacterium fallax* sp. nov. Int. J. Syst. Bacteriol. 33:333-346.
- Luquin, M., V. Ausina, F. López-Calahorra, F. Belda, M. García-Barceló, C. Celma, and G. Prats. 1991. Evaluation of practical chromatographic procedures for identification of clinical isolates of mycobacteria. J. Clin. Microbiol. 29:120-130.
- Luquin, M., J. Roussel, F. López-Calahorra, G. Lanéele, V. Ausina, and M. A. Lanéele. 1990. A novel mycolic acid in a *Mycobacterium* sp. from the environment. Eur. J. Biochem. 192:753-759.
- Minnikin, D. E., I. A. Hutchinson, A. B. Caldicott, and M. Goodfellow. 1980. Thin-layer chromatography of methanolsolates of mycolic acid containing bacteria. J. Chromatogr. 188:221-233.
- Moore, W. E. C., and L. V. H. Moore. 1989. Index of the bacterial and yeast nomenclature changes, p. 41. American Society for Microbiology, Washington, D.C.
- Owen, R. J., and D. Pitcher. 1985. Current methods for estimating DNA base composition and levels of DNA-DNA hybridization, p. 67-93. In M. Goodfellow and D. E. Minnikin (ed.), Chemical methods in bacterial systematics. Academic Press, Inc. (London), Ltd., London.
- Rafidinarivo, E., J. C. Promé, and V. Lévy-Frébault. 1985. New kinds of unsaturated mycolic acids from *Mycobacterium fallax* sp. nov. Chem. Phys. Lipids 35:215-228.
- Silcox, V. A., R. C. Good, and M. M. Floyd. 1981. Identification of clinically significant *Mycobacterium fortuitum* complex isolates. J. Clin. Microbiol. 14:686-691.
- Skerman, V. B. D., V. McGowan, and P. H. A. Sneath (ed.). 1980. Approved lists of bacterial names. Int. J. Syst. Bacteriol. 30:225-420.
- Sokal, R. R., and P. H. A. Sneath. 1963. Principles of numerical

- taxonomy. W. H. Freeman and Co., San Francisco.
26. Szabo, I., and E. Vandra. 1963. *Mycobacterium minetti* (Penso et. al., 1952): bacteriological and epidemiological observations. Acta Microbiol. Acad. Sci. Hung. 10:215-223.
27. Tsukamura, M. 1967. Identification of mycobacteria. Tubercle 48:311-338.
28. Vestal, A. 1975. Procedures for the isolation and identification of mycobacteria. Publication (CDC) 76-8230. Centers for Disease Control, Atlanta.
29. Wayne, L. G., H. C. Engbaek, H. W. B. Engel, S. Froman, W. Gross, J. Hawkins, W. Käppler, A. G. Karlson, H. H. Kleeberg, I. Krasnow, G. P. Kubica, C. McDurmont, E. E. Nel, S. R. Pattyn, K. H. Schröder, S. Showalter, I. Tarnok, M. Tsukamura, B. Vergmann, and E. Wolinsky. 1974. Highly reproducible techniques for use in systematic bacteriology in the genus *Mycobacterium*: tests for pigment, urease, resistance to sodium chloride, hydrolysis of Tween 80, and β -galactosidase. Int. J. Syst. Bacteriol. 24:412-419.
30. Wayne, L. G., H. W. B. Engel, C. Grassi, W. Gross, J. Hawkins, P. A. Jenkins, W. Käppler, H. H. Kleeberg, I. Krasnow, E. E. Nel, S. R. Pattyn, P. A. Richards, S. Showalter, M. Slosarek, I. Szabo, I. Tarnok, M. Tsukamura, B. Vergmann, and E. Wolinsky. 1976. Highly reproducible techniques for use in systematic bacteriology in the genus *Mycobacterium*: tests for niacin and catalase and for resistance to isoniazid, thiophene-2-carboxylic acid hydrazide, hydroxylamine, and *p*-nitrobenzoate. Int. J. Syst. Bacteriol. 26:311-318.