Mycobacterium madagascariense sp. nov.

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Strains of a new type of rapidly growing, scotochromogenic mycobacterium were isolated from sphagnum vegetation in Madagascar. These strains grew at 31 and 22°C but not at 37°C, possessed catalase, acid phosphatase, and arylsulfatase activities, split urea and pyrazinamid, hydrolyzed Tween, and produced acid from glucose, L-arabinose, fructose, mannitol, rhamnose, sorbitol, xylose, and trehalose. Furthermore, they metabolized iron and possessed putrescine oxidase activity but did not reduce nitrate. The internal similarity level of the strains, as determined by taxonomic methods, was 92.50%. The phylogenetic relationships of strain P2^T (T = type strain) with members of the genus *Mycobacterium*, as determined by comparing the 16S rRNA primary structure of this strain with the 16S rRNA primary structure of this stain with the 16S rRNA primary structures of 41 other mycobacterial species, indicated that strain P2^T belongs to a separate line of descent within a cluster that includes *Mycobacterium phlei*, *Mycobacterium smegmatis*, *Mycobacterium confluentis*, *Mycobacterium flavescens*, and *Mycobacterium thermoresistibile*. Hence, the new strains are considered members of a new species of nonpathogenic, rapidly growing mycobacteria, for which we propose the name *Mycobacterium madagascariense*. Strain P2 is the type strain; a culture of this strain has been deposited in the American Type Culture Collection as strain ATCC 49865.

In August 1988, 18 samples of sphagnum vegetation were collected in the southeast coastal region of Madagascar and were examined for mycobacteria; 83.3% of these samples contained mycobacteria (16). A group of four strains that were isolated from three different biotopes exhibited high levels of internal similarity. The properties of these strains differed significantly from the properties of other species of rapidly growing mycobacteria.

MATERIALS AND METHODS

Bacterial strains. Strains P1, P2^T (T = type strain), P3, and P4 were isolated from three of the five sphagnum biotopes examined between Taolanaro and Sainte-Luce in the southeast coastal region of Madagascar. Strain P1 was isolated from *Sphagnum gradiente* (Warnst.), strain P2^T was isolated from *Sphagnum obtusiusculum* (Lindb.), and strains P3 and P4 were isolated from *Sphagnum balfourianum* (Warnst.).

Methods. Colony morphology and the ability to grow at various temperatures (22, 31, and 37°C) were determined for the strains which we tested during 1 week of incubation on Löwenstein-Jensen medium and Middlebrook 7H10 agar, which were inoculated with approximately 10⁻² mg (wet weight) of mycobacteria per plate. Pigment production in the dark and photoactivity during 1 week of incubation were also determined (6, 24).

The following characteristics were also determined: growth on MacConkey agar (17); resistance to 0.2% *p*-aminosalicylic acid (23), 20 µg of sodium azide per ml, 250 µg of hydroxylamine per ml, 1% sodium deoxycholate, 3% sodium chloride, and 0.01% sodium nitrite (21, 22, 24); iron uptake (20); nitrate reduction (2); arylsulfatase activity in 3 days

(11); acid phosphatase (4 h, 31°C) (9), acetamidase, benzamidase, urease, isonicotinamidase, nicotinamidase, pyrazinamidase, salicylamidase, allantoinase, succinamidase, and malonamidase activities (1); and putrescine oxidase production (3). Acid production from carbohydrates (glucose, L-rhamnose, D-xylose, L-arabinose, D-galactose, D-mannitol, D-sorbitol, inositol, dulcitol, trehalose, and D-fructose) was tested during 3 weeks of incubation (7). The utilization of fumarate, succinate, citrate, malonate, oxalate, benzoate, hippurate, and 1-propanol as sole carbon sources and the utilization of acetamide, benzamide, and trimethylene diamine as sole nitrogen and carbon sources were determined by using the methods described by Tsukamura (25). A test to determine the degradation of salicylate was also performed (23).

Phylogenetic analysis. A phylogenetic analysis of strain P2^T (= *madagascariense*^T) was performed recently (18). After this analysis was performed, *Mycobacterium confluentis* was described, and the 16S rRNA sequence was published (10). Since *M. confluentis* and strain P2^T are both members of the same phylogenetic cluster, the position of strain P2^T was defined. Pairwise evolutionary distances (expressed as the estimated numbers of changes per 100 nucleotides) were computed from percentages of similarity by using the correction of Jukes and Cantor (8). A phylogenetic tree was constructed from the distance matrix by using the algorithm of De Soete (5). Sequence alignment and data analysis were done by using a SUN Sparc workstation.

Lipid analyses. Lipid analyses were carried out for all strains by using cells that were scraped from Löwenstein-Jensen slants. Free lipids were extracted from cells with CHCl₃-CH₃OH (1:1, vol/vol) and were analyzed by thin-layer chromatography, using CHCl₃-CH₃OH-H₂O (30:8:1, 30:12:1, and 65:25:4, vol/vol/vol) and CHCl₃-CH₃OH (99:1 and 90:10, vol/vol) as eluents. The defatted cell residues

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were saponified and converted to methyl esters, and mycolate patterns were determined as previously described (12, 13). Alternatively, scraped cells were saponified and converted to methyl esters, and nonhydroxylated esters were examined by gas chromatography, using a Perkin-Elmer model 8310B apparatus, a 1-m column packed with 3% OV-1, and nitrogen as the carrier gas. To determine the compositions of the chains in position 2 of the mycolates, pyrolytic conditions were used, with the injector temperature raised from 300 to 400°C (4).

For the pathogenicity tests, a 4-day-old culture of strain P2^T was used. Four rabbits were injected intravenously with 10 mg (semiwet weight) of bacteria, five white mice were injected with 1 mg intravenously, and four guinea pigs were injected with 10 mg subcutaneously. After 15 weeks, the animals were sacrificed and autopsied (14).

Numerical analysis. On the basis of the results of a comparison of 51 properties, the level of internal similarity of strains P1, P2^T, P3, and P4 was determined (19). A total of 43 properties of 22 species of rapidly growing mycobacteria were determined and compared with the properties of strain P2^T. All of the data were converted to the simple binary form (i.e., 1 or 0) for analysis; both positive and negative matches were used (19).

RESULTS AND DISCUSSION

The cells of strains P1, P2^T, P3, and P4 grown on Löwenstein-Jensen medium and on Middlebrook 7H10 agar were rod shaped (0.6 by 1.2 to 1.5 µm), often polymorphic, gram positive, and acid-alcohol fast and formed clumps but not cords or cross bands. Spores, capsules, true branching, and aerial hyphae were not observed. On Löwenstein-Jensen medium, all of the strains were eugonic and smooth, and yellow- or orange-pigmented colonies were produced when cultures were incubated at temperatures of 22 and 31°C. At 31°C growth was observed after 3 days; at 22°C growth was observed after 7 days. No growth occurred at 37, 42, or 45°C. Smooth, glistening, yellow or orange colonies that were 1 to 2 mm in diameter and had entire margins developed on Middlebrook 7H10 agar from dilute inocula after 3 days of incubation at 31°C. Properties of the strains are shown in Table 1. The strains created a homogeneous group that had a level of internal similarity of 92.50%.

The whole-cell mycolate patterns (Table 2) confirmed the homogeneity of the taxon, but the same mycolate types are also present in several rapidly growing mycobacterial species (4, 12, 15). However, on the basis of the nature of the major pyrolysis esters (docosanoate), only three species (*Mycobacterium aurum*, *Mycobacterium neoaurum*, and *Mycobacterium gadium*) had to be compared with the members of the new taxon. Moreover, when the nonhydroxylated fatty acid methyl esters were considered, *M. aurum*, *M. neoaurum*, and *M. gadium* accumulated significant amounts of 2,4-dimethyl docosanoate, as did strains P1, P2^T, P3, and P4 (26).

When testing for pathogenicity, we found that strain P2^T produced neither organic nor local lesions in infected rabbits, guinea pigs, or mice.

On the basis of a comparison of the 16S rRNA of strain P2^T and 57 reference sequences from 41 previously described species of the genus *Mycobacterium*, we concluded that strain P2^T (listed as strain madagascar in reference 18) is a nonthermotolerant member of a group of mycobacteria that exhibit increased thermotolerance (18). Recently, *M. confluentis* has been shown to be a member of this group,

TABLE 1. Characteristics of strains P1, P2^T, P3, and P4

Characteristic	% of strains giving positive reaction
Pigment production in the dark	100
Photoactivity after 1 wk	0
Growth at 22°C in 7 days	100
Growth at 31°C in 7 days	100
Growth at 37°C in 7 days	0
Enzymatic activities	
Acid phosphatase (31°C, 4 h)	100
Acid phosphatase (70°C, 30 min)	25 ^a
Arylsulfatase (3 days)	100
Putrescine oxidase (24 h)	75 ^b
Nitrate reductase	0
Tween hydrolysis (10 days)	100
Acetamidase	0
Benzamidase	0
Urease	100
Isonicotinamidase	0
Nicotinamidase	50 ^c
Pyrazinamidase	100
Salicylamidase	0
Allantoinase	50 ^c
Succinamidase	0
Malonamidase	0
Growth on the following compounds as single carbon sources:	
Sodium benzoate	0
Sodium fumarate	0
Sodium succinate	0
Sodium citrate	0
Sodium malonate	0
Sodium oxalate	0
Sodium hippurate	0
1-Propanol	0
Growth on the following compounds as single nitrogen and carbon sources:	
Acetamide	0
Benzamide	0
Trimethylene diamine	0
Growth in presence of:	
Hydroxylamine (250 µg/ml)	0
Sodium azide (20 µg/ml)	75 ^d
NaCl (3%)	0
NaNO ₂ (0.1%)	0
Growth on MacConkey agar	0
Degradation of salicylate	0
Iron uptake	100
Acid production from:	
D-Glucose	100
L-Arabinose	100
Dulcitol	100
D-Fructose	100
D-Galactose	50 ^e
Inositol	50 ^f
D-Mannitol	100
L-Rhamnose	100
D-Sorbitol	75 ^g
Trehalose	100
D-Xylose	100

^a Strain P1 was positive.

^b Strain P4 was negative.

^c Strains P1 and P2^T were positive.

^d Strain P3 was negative.

^e Strains P1 and P3 were positive.

^f Strains P3 and P4 were positive.

^g Strain P1 was negative.

TABLE 2. Mycolate types, major pyrolysis esters, and alcohol compositions of strains P1, P2^T, P3, and P4^a

Strain	Mycolate types						2-OH composition		Major pyrolysis esters	
	I	II	III	IV	V	VI	C ₁₈	C ₂₀	C ₂₂	2,4-Dimethyl C ₂₀
P1	+	—	—	(+)	—	+	+	(+)	+	+
P2 ^T	+	—	—	(+)	—	+	+	(+)	+	+
P3	+	—	—	(+)	—	+	+	(+)	+	+
P4	+	—	—	(+)	—	+	+	(+)	+	+

^a The mycolate types have been described previously (4). +, detected; —, not detected; (+), minor compound.

which includes, in addition to strain P2^T, *Mycobacterium phlei*, *Mycobacterium smegmatis*, *Mycobacterium flavescens*, *Mycobacterium thermoresistibile*, and the undescribed organism "*Mycobacterium chromogen*." A phylogenetic analysis of all of the members of this group and certain strains that were also included in the numerical phenetic study indicated that strain P2^T and *M. confluentis* are closely related (Table 3 and Fig. 1). The low level of nucleotide differences in the 16S rRNAs of these two organisms (as little as 1.9%) is not unusual for fast-growing mycobacteria; similar low values have been found for *Mycobacterium diernhoferi*, *Mycobacterium obuense*, and *Mycobacterium gilvum* and for *M. flavescens* and *M. smegmatis* (Table 3).

Organisms that exhibit high levels of phenetic similarity to strain P2^T are less closely related phylogenetically (18) (Fig. 1). *M. aurum* appears to be more closely related to *Mycobacterium vaccae*, while the *M. obuense*-*Mycobacterium chubuense*-*M. gilvum* line and the *M. neoaurum*-*M. diernhoferi* line are two additional separate lines of descent that are equidistantly related to the group of thermotolerant species and their nonthermotolerant relatives. Apparently, the phenotypic similarities of the characteristics used in the numerical study do not reflect the levels of genetic relatedness of the organisms. *M. gadium*, which produced the same major pyrolysis esters as strain P2^T, *M. aurum*, and *M. neoaurum*, is not phylogenetically related to members of the strain P2^T taxon. As shown elsewhere (18), *M. gadium* represents a separate line of descent within the radiation of

mycobacteria and is closely related to the 21 slow-growing species which we investigated.

Taxonomic description of *Mycobacterium madagascariense* sp. nov. *Mycobacterium madagascariense* (mad'a.gas.car.i.en.se. M.L. adj. *madagascariense*, belonging to the island of Madagascar, the source of the strains). The description below is based on a study of four strains which share the characteristics shown in Table 1. Cells are gram-positive, acid-alcohol-fast, polymorphic rods (0.6 by 1.2 to 1.5 µm) which often form clumps but not cords or cross bands. Spores, capsules, and aerial hyphae do not occur. Colonies on Löwenstein-Jensen medium and on Middlebrook 7H10 agar are smooth, glistening with yellow or orange pigmentation, and 1 to 2 mm in diameter. The optimum growth temperature is 31°C; no growth occurs at 37°C or above. Biochemical characteristics are shown in Table 1. Positive for pigment production, acid phosphatase activity (31°C, 4 h), arylsulfatase activity, Tween hydrolysis, and urease and nicotinamidase activities. The strains metabolize iron and form acid from glucose, arabinose, dulcitol, fructose, mannitol, rhamnose, trehalose, and xylose (Table 1). They do not utilize certain substrates as single carbon or single nitrogen and carbon sources and do not tolerate hydroxylamine (250 mg/ml), NaCl (3%), and NaNO₂ (0.1%). Their internal similarity level is 92.50%. The strains possess mycolate types I, IV, and VI (Table 2).

The phylogenetic position of this organism, based on an evaluation of partial 16S rRNA sequences, is within the rapidly growing species of the genus *Mycobacterium*. The

TABLE 3. Evolutionary distances for the 16S rRNAs of *M. madagascariense* P2^T (= ATCC 49865^T) and various representatives of the fast-growing mycobacteria^a

Organism	Evolutionary distance from:													
	<i>M. madagascariense</i>	<i>M. confluentis</i>	<i>M. phlei</i>	<i>M. flavescens</i>	<i>M. smegmatis</i>	" <i>M. chromogen</i> "	<i>M. thermoresistibile</i>	<i>M. neoaurum</i>	<i>M. diernhoferi</i>	<i>M. chubuense</i>	<i>M. obuense</i>	<i>M. gilvum</i>	<i>M. aurum</i>	<i>M. vaccae</i>
<i>M. confluentis</i>	1.9													
<i>M. phlei</i>	3.0	2.9												
<i>M. flavescens</i>	3.2	3.5	2.2											
<i>M. smegmatis</i>	1.6	1.7	2.6	1.2										
" <i>M. chromogen</i> "	3.0	3.0	2.1	2.1	2.0									
<i>M. thermoresistibile</i>	3.2	3.3	3.2	3.0	2.2	2.0								
<i>M. neoaurum</i>	3.2	3.6	3.8	3.7	3.3	3.5	3.7							
<i>M. diernhoferi</i>	3.1	3.9	4.8	4.4	3.2	4.4	4.0	1.8						
<i>M. chubuense</i>	3.2	3.5	3.5	3.6	2.8	4.0	4.4	4.0	3.9					
<i>M. obuense</i>	2.9	3.5	4.0	3.6	2.0	4.5	4.6	3.1	2.9	1.7				
<i>M. gilvum</i>	3.1	3.6	3.4	2.9	2.8	3.8	3.9	3.3	2.8	1.9	2.0			
<i>M. aurum</i>	4.3	5.0	4.5	4.0	4.0	4.7	5.2	3.8	3.9	2.5	3.1	3.0		
<i>M. vaccae</i>	2.9	2.8	4.1	3.6	3.2	4.3	3.7	3.6	3.8	3.0	3.1	2.7	3.0	
<i>M. komossense</i>	3.0	3.2	3.6	3.6	2.8	4.4	4.3	3.3	3.3	2.6	2.2	2.7	3.2	3.5

^a For the accession numbers of the 16S rRNAs of the reference strains, see reference 18.

FIG. 1. Phylogenetic position of *M. madagascariense* P2^T (= ATCC 49865^T) within the radiation of the fast-growing mycobacteria. A more detailed branching pattern for the slow- and fast-growing mycobacteria is given in reference 18. The horizontal components of the branch lengths are proportional to evolutionary distances. Bar = 1% nucleotide differences.

strains are not pathogenic for rabbits, guinea pigs, and mice. The type strain of *M. madagascariense* strain is P2; a culture of this strain has been deposited in the American Type Culture Collection as strain ATCC 49865. Strains of *M. madagascariense* have been isolated from three different sphagnum biotopes in Madagascar.

Characteristics which differentiate *M. madagascariense* from other related mycobacteria. *M. madagascariense* can be easily differentiated from *M. aurum* by its failure to grow at 37°C, by its failure to grow on media containing sodium fumarate, sodium succinate, or sodium citrate as a single carbon source or trimethylene diamine as a single nitrogen and carbon source, by its failure to grow in the presence of 0.1% NaNO₂, and by its failure to split sodium salicylate. Furthermore, *M. madagascariense* possesses acid phosphatase and arylsulfatase activities and produces acid from dulcitol and rhamnose, properties which are negative in *M. aurum*. The 16S rRNA sequences of the two organisms are also markedly different. *M. madagascariense* can be differentiated from *M. obuense* by its failure to grow at 37°C, by its failure to grow in media containing sodium fumarate, sodium succinate, or propanol as a single carbon source, by its failure to grow in the presence of 3% NaCl and 0.1% NaNO₂, and by its failure to produce acid from galactose. *M. madagascariense* possesses acid phosphatase and arylsulfatase activities, hydrolyzes Tween, and produces acid from dulcitol and rhamnose, properties which are negative in *M. obuense*. Furthermore, there are important differences in the 16S rRNA sequences of these two species.

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