Mycobacterium branderi sp. nov., a New Potential Human Pathogen

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A number of mycobacterial strains with similar growth characteristics, metabolic properties, and lipid compositions, which were previously placed in the Helsinki group (E. Brander, E. Jantzen, R. Huttunen, A. Juntunen, and M.-L. Katila, *J. Clin. Microbiol.* 30:1972–1975, 1992), were characterized by performing 16S rRNA gene sequencing. Of the 14 strains studied, 9 had a unique, previously undescribed sequence in the variable region of 16S rRNA. These nine strains, all of which were isolated from respiratory tract specimens, were nonpigmented and grew at 25°C to 45°C, reaching full colony size after 2 to 3 weeks. They produced arylsulfatase, nicotinamidase, and pyrazinamidase and were negative for Tween 80 hydrolysis, catalase, urease, and nitrate reductase activities, and niacin. Their glycolipid patterns were identical. A mycolic acid analysis performed by using thin-layer chromatography showed that these organisms contained alpha-mycolates, ketomycolates, and carboxy mycolates. Gas-liquid chromatography revealed that 2-eicosanol was the major alcohol and hexacosanoic acid was the major mycolic acid cleavage product. On the basis of their growth, biochemical, and lipid characteristics and their unique 16S rRNA sequence, we propose that these organisms should be assigned to a new species, *Mycobacterium branderi*. Comparative 16S rRNA sequencing revealed that this new species is closely related to *Mycobacterium celatum*, *Mycobacterium cookii*, and *Mycobacterium xenopi*. Strains 52157^T (T = type strain) and 43548 have been deposited in the American Type Culture Collection as strains ATCC 51789 and ATCC 51788, respectively.

The taxonomic position of a collection of 14 strains of slowly growing mycobacteria that were identified previously on the basis of biochemical activity and lipid composition as members of a potential new species (the "Helsinki group") (3) was determined by analyzing of their 16S rRNA gene sequences. These strains were isolated from clinical samples in Finland between 1972 and 1990 and were stored at –70°C because their taxonomic identities were ambiguous. These organisms

resembled *Mycobacterium xenopi*, members of the *Mycobacterium avium* complex, and *Mycobacterium shimoidei* in growth characteristics (3, 20) but could be distinguished from them by their fatty acid and alcohol compositions and the results of glycolipid analyses. By using gene sequencing, the group was found to comprise two genetic entities, one identical to the recently described species *Mycobacterium celatum* (4) and the other an undescribed taxon. In this paper we describe the

TABLE 1. Characteristics that distinguish *M. branderi* from some important slowly growing species^a

Taxon	Growth at:		Pigment production in the dark	Arylsulfatase activity ^b	Tween 80 hydrolysis	Urease activity	Nitrate reductase activity	Catalase activity ^c
	25°C	45°C						
<i>M. branderi</i> ^d	+	+	–	+	–	–	–	–
<i>M. avium</i>	+	V	V	V	–	–	–	–
<i>M. celatum</i> ^f	+	+	–	+	–	–	–	–
<i>M. cookii</i>	+	–	+	+	–	–	–	+
<i>M. intracellulare</i>	+	V	V	+	–	–	–	–
<i>M. malmoense</i>	+	–	–	–	+	V	–	–
<i>M. shimoidei</i>	V	+	–	–	+	–	–	–
<i>M. scrofulaceum</i>	+	V	+	V	–	+	–	+
<i>M. simiae</i>	+	–	–	–	–	+	–	+
<i>M. szulgai</i>	+	–	+	V	V	+	+	+
<i>M. terrae</i> complex	+	–	–	V	+	–	V	+
<i>M. xenopi</i>	–	+	+	+	–	–	–	–

^a The data for all taxa except *M. branderi* and *M. celatum* are from references 7, 11, 17, 20, and 21.

^b 14-day test.

^c Semiquantitative catalase test (<45 mm was a negative reaction).

^d Results of analyses of nine strains whose sequences were identical. The catalase test was negative (<30 mm) for all strains. In the nicotinamidase and pyrazinamidase tests, the strains exhibited trace to 2+ reactions.

^e Symbols: +, more than 80% of the strains were positive; V, variable reactions; –, 15% or less of the strains were positive.

^f Results of analyses of five strains whose sequences were identical. The strains were negative for catalase (<30 mm). In nicotinamidase and pyrazinamidase tests the strains exhibited trace to 1+ reactions.

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TABLE 2. Fatty acid and alcohol markers used for the initial separation of the Helsinki group (*M. branderi* and *M. celatum*) from some important nonchromogenic and scotochromogenic slowly growing species

Taxon	No. of strains	Mean peak area % of:						
		10Me-18:0	2-OH-20:0alc	2-Me-20:0	2-OH-22:0alc	24:0	2,4,6-triMe-24:0	26:0
<i>M. branderi</i>	9	19	8	ND ^a	ND	4	ND	8
<i>M. celatum</i>	5	20	10	ND	ND	4	ND	6
<i>M. avium</i> complex ^b	14	14	9	ND	ND	6	ND	ND
<i>M. malmoense</i> ^b	14	14	ND	3	ND	2	3	8
<i>M. shimoides</i> ^b	4	10	5	ND	2	6	ND	ND
<i>M. terrae</i>	7	10	11	ND	ND	7	ND	ND
<i>M. xenopi</i> ^b	14	24	2	ND	11	11	ND	7

^a ND, not detected in significant amount.^b See reference 3.

characteristics of the latter taxon and its phylogenetic position in the genus *Mycobacterium*, and we propose that this taxon is a new species, *Mycobacterium branderi*.

MATERIALS AND METHODS

Bacterial strains. Fourteen strains belonging to a previously described collection, the Helsinki group (3), were obtained from storage at -70°C for 16S rRNA sequence determinations. Three nonpigmented strains (strains 52157^T [T = type strain], 38426, and J249) and three strains that produced a yellow pigment (strains 9220, 17410, and 28970) were subcultured for three to five subpopulations; each subculture was grown from a single colony. The subcultures were analyzed to determine their fatty acid and alcohol compositions as described previously (3), and when subcultures were found to be identical, one subculture of each strain was randomly selected for 16S rRNA gene sequencing. The sequences of the other strains were determined by using the original bacterial populations.

Preliminary identification. The growth and biochemical characteristics used initially to separate the Helsinki group from other grossly similar slowly growing species are shown in Table 1. The methods which we used have been described previously (3).

Lipid analyses. Lipid analyses were carried out by using three complementary techniques. Glycolipids were analyzed by one-dimensional thin-layer chromatography (3, 10), mycolic acids were analyzed by two-dimensional thin-layer chromatography (3), and fatty acid methyl esters, alcohols, and mycolic acid cleavage products were analyzed by capillary gas-liquid chromatography (GLC) as described previously (3) by using a Perkin-Elmer Autosystem chromatograph (Perkin-Elmer, Norwalk, Conn.).

rRNA gene sequencing and phylogenetic analysis. Nucleic acids were extracted as described previously (12, 16). The 16S rRNA gene was amplified by using the PCR technique, and the sequence was determined as described previously (1, 12, 14) by using the solid-phase method (8). Sequencing reactions were performed either manually or with an automated A.L.F. DNA sequencer (Pharmacia, Uppsala, Sweden). In case of automated sequencing, the sequences were assembled by using the Staden Package program (5) and a Sun workstation. In the sequencing strategy which we used, the nucleotides of each isolate were

determined at 1,416 contiguous positions. The sequence obtained was aligned with selected 16S rRNA sequences (6, 12) as previously described (15). Regions of alignment uncertainty were omitted in the phylogenetic analysis. Pairwise distances were calculated by weighting nucleotide differences and insertions-deletions (Hamming distances). A phylogenetic tree was constructed by using a neighborliness method (18) as previously described.

Nucleotide sequence accession number. The 16S rRNA sequence of *M. branderi* has been deposited in the EMBL database under accession number X82234.

RESULTS AND DISCUSSION

The key characteristics used for the initial separation of the Helsinki group strains from other slowly growing species were as follows: wide temperature range for growth (from room temperature to 45°C), strongly positive 14-day arylsulfatase test, negative Tween 80 hydrolysis reaction (Table 1), susceptibility to ethambutol combined with resistance to isoniazid

CAAGTCGAAC GGAAAGGTTT TTTCGGGAAC ACTCGAGTGG CGAACGGGTG AGTAACACGT
GGGTGATCTG CCCTGCACCT CGGGATAAGC TTGGGAAACT GGGTCTAATA CCGGATAGGA
CCACATGCTG CATGGTGTGT GGTGGAAAGC TTTTTCGCGT TGTGGATAGG GCCCGCGGCC
TATCAGCTTG TTGGTGGGGT GATGGCCCTAC CAAGCGGACG ACGGGTATGC GGCCCTGAGAG
GGTGTCCGGC CACACTGGGA CTGAGATACG GCCCAGACTC CTACGGGAGG CAGCAGTGGG
GAATATTGCA CAATGGGCGC AAGCCTGATG CAGCGACGCG GCGTGGGGGA TGACGGCCCTT
CGGGTTGTAA ACCTCTTTC AATCGACGAG AGCTTCAGCG TGTGTGTGGG GTGACGGGTAG
GTGGAGAAGA AGCACCGGCC AACTACGTGC CAGCAGCGCG GGTAAATACGT AGGGTCCGAG
CGTGTCTCGG AATTAACGGG CGTAAAGAGC TCGTAGGTGG TTTGTGCGGT TGTTCGTGAA
ATTCTCTGGC TTAACCTGGG GCGTCCGGGC GATACGGGCA GACTGGAGTA CTGCAGGGGA
GACTGGAAAT CTGGGTGTAG CCGTGGGAATG CGCAGATATC AGGAGGAACA CCGGTGGCGA
AGCGGGGTCT CTGGGACGTA ACTGACGCTG AGGAGCGGAA GCGTGGGGAG CGAACAGGAT
TAGATACCTT GGTAGTCCAC GCCGTAAAGC GTGGGTACTA GGTGTGGGTT TCCTTCTCTG
GGATCCGTCG CGTAGCTAAC GCATTAAGTA CCCCCCTGG GGAAGTACGG CGCAAGGCTA
AAACTCAAAG GAATTGACGG GGGCCCGCAC AAGCGCGGGA GCATGTGGAT TAATTCTGAT
CAACCGGAAG AAGCTTACCT GGGTTTGACA TGCACAGGAC GGCATCAGAG ATGGTCTGTC
CCTTGTGGTC TGTGTGACGG TGGTGCATGG CTGTCTGTAG CTCGTGTCTG GAGATGTTGG
GTTAATGCCC GCAACGAGCG CAACCTTGTG CTCATGTTGC CAGCGCGTGA TGCGGGGNAC
TCGTGAGAGA CTCCGGGGT CAACCTGGAG GAAGGTGGGG ATGACGTCAA GTCATCATGC
CCCTTATGTC CAGGGCTTCA CACATGCTAC AATGGCCGGT ACAGAGGGCT GCGATCCGCG
GAGGTATTAG GAATCTTGTG AAGCCGGGTC TCAGTTCGGA TCGGGGTCTG CAACCTCGAC
CCGTGAAAGT GAGGTCTGTA ATAATCGCAG ATCAGCAATG CTGCGGTGAA TACGTTCGCG
GGCCTTGTAC ACACCGCCCG TCACGTCTATG AAGTCTGGTA ACACCGGAAG CCCATGGCCT
AACCCGGAAG GGAGGAGGTG GTCGAAGGTG GGATCGG

FIG. 1. Partial 16S rRNA gene sequence of strain 52157^T (total length, 1,416 bp), which contains 301 adenosine residues, 332 cytidine residues, 492 guanosine residues, and 291 thymidine residues.

FIG. 2. Phylogenetic tree based on 16S rRNA sequences showing the position of *M. branderi* sp. nov. (Helsinki 52157) among members of the genus *Mycobacterium*. Bar = 10 nucleotide differences.

<i>M.fortuitum</i>	<i>M.smegmatis</i>	<i>M.flavescens</i>	<i>M.simiae</i>	<i>M.genavense</i>	<i>M.terrae</i>	<i>M.nonchromogenicum</i>	<i>M.xenopi</i>	<i>M.gordonae</i>	<i>M.bovis</i>	<i>M.leprae</i>	<i>M.marinum</i>	<i>M.scrofulaceum</i>	<i>M.gastri/M.kansasii</i>	<i>M.szulgai</i>	<i>M.malmoeense</i>	<i>M.intracellulare</i>	<i>M.paratuberculosis</i>	<i>M.avium</i>	<i>M.intermedium</i>	<i>M.interjectum</i>	<i>M.asiaticum</i>	<i>M.Helsinki 52157</i>	<i>M.celatum I</i>	<i>M.celatum II</i>	<i>M.cookii</i>	<i>M.hiberniae</i>	<i>M.ulcerans</i>			
76	89	83	81	80	103	109	125	103	114	117	110	110	112	110	112	111	110	112	88	84	108	112	108	111	109	108	111	<i>N.asteroides</i>		
	30	26	46	46	47	51	79	65	63	76	57	65	69	67	67	72	70	71	47	44	62	68	60	62	66	51	58	<i>M.fortuitum</i>		
		12	45	49	51	57	80	63	66	75	65	65	69	68	68	71	70	71	48	44	64	75	67	67	68	59	66	<i>M.smegmatis</i>		
			44	47	52	56	83	68	72	79	71	71	75	72	72	76	76	77	49	43	68	79	70	71	69	60	72	<i>M.flavescens</i>		
				11	57	64	78	48	59	62	58	43	47	46	46	52	52	53	28	15	44	61	56	58	62	63	59	<i>M.simiae</i>		
					56	68	82	52	62	69	61	54	58	54	54	60	55	56	38	23	47	67	57	60	61	67	62	<i>M.genavense</i>		
						23	56	37	38	55	35	53	46	45	47	47	46	47	61	55	34	52	39	40	42	20	36	<i>M.terrae</i>		
							64	49	50	60	46	56	55	51	51	53	50	51	68	63	47	52	53	52	42	16	47	<i>M.nonchromogenicum</i>		
								66	57	68	59	68	64	61	63	63	65	66	76	78	65	67	55	58	69	63	60	<i>M.xenopi</i>		
									34	42	30	34	27	26	26	30	28	29	51	43	17	52	42	41	49	46	31	<i>M.gordonae</i>		
										28	11	31	20	19	21	25	22	23	48	53	26	50	41	40	51	43	12	<i>M.bovis</i>		
												28	36	29	23	23	27	25	26	53	54	37	59	52	46	61	57	29	<i>M.leprae</i>	
													26	19	18	20	22	19	20	46	52	21	50	40	38	48	37	1	<i>M.marinum</i>	
														17	19	19	19	21	22	35	38	33	47	49	49	55	27	<i>M.scrofulaceum</i>		
															10	12	14	14	15	33	44	24	50	48	46	56	48	20	<i>M.gastri/M.kansasii</i>	
																2	12	12	13	35	38	19	50	43	36	51	46	19	<i>M.szulgai</i>	
																	12	12	13	37	38	21	52	45	38	53	48	21	<i>M.malmoeense</i>	
																		7	8	39	45	27	56	47	44	58	47	23	<i>M.intracellulare</i>	
																				1	41	44	24	55	46	42	56	45	20	<i>M.paratuberculosis</i>
																				42	45	25	56	47	43	57	46	21	<i>M.avium</i>	
																					22	50	67	65	65	70	61	47	<i>M.intermedium</i>	
																						38	63	56	62	60	53	<i>M.interjectum</i>		
																							50	38	37	44	42	22	<i>M.asiaticum</i>	
																								36	36	45	59	51	<i>M.Helsinki 52157</i>	
																									10	40	51	41	<i>M.celatum I</i>	
																										40	48	39	<i>M.celatum II</i>	
																										44	49	<i>M.cookii</i>		
																											38	<i>M.hiberniae</i>		

FIG. 3. Hamming distances derived from 16S rRNA sequences. *M. Helsinki 52157* = *M. branderi* 52157^T.

and rifampin, a characteristic glycolipid composition (3), and unique fatty acid and alcohol compositions as determined by GLC (Table 2). Three of the strains differed from the strains in the main cluster by producing a yellow pigment whose intensity varied, and two differed by dysgonic growth.

An analysis of the hypervariable region in the 16S rRNA gene sequence, which corresponded to positions known to be specific to mycobacteria at the species level (12), revealed that the strains in the original collection could be placed in three subgroups. Nine strains had an identical, previously undescribed sequence; four strains, including the three pigmented strains, had a sequence identical to that of the recently described taxon *M. celatum* cluster I (4); and the sequence of one strain, which originally was isolated from a Somali patient, was identical to that found in the other *M. celatum* cluster (cluster II) (4).

The 16S rRNA gene sequence of strain 52157^T (total length, 1,416 nucleotides) is shown in Fig. 1. This sequence was found in all nine *M. branderi* strains, and it was different from all previously published mycobacterial sequences in the EMBL nucleotide sequence database. The most distinct differences from the *M. xenopi*, *Mycobacterium cookii*, *Mycobacterium malmoeense*, *Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycobacterium scrofulaceum*, and *M. shimoidei* sequences were detected at *Escherichia coli* positions 125 to 185 and 430 to 500.

The presence of one adenosine at position 458 was another unique property of the sequence. This region corresponds to helix 18, where rapidly and slowly growing mycobacteria have a short helix and a long helix, respectively. In members of the *Mycobacterium terrae*-*Mycobacterium nonchromogenicum* complex, this helix structure is extended by two nucleotides, in comparison with other slowly growing mycobacteria such as *M. avium* (2).

Compared with the sequence of the most similar taxa as determined by biochemical and lipid analyses, *M. celatum* clusters I and II, the *M. branderi* 16S rRNA sequence differed by 36 nucleotides after the regions of alignment uncertainty were omitted. This corresponds to a level of homology of 97.5%. As Stackebrandt and Goebel (19) state, it is unlikely that at a level of sequence homology of less than 98% two organisms exhibit more than 60 to 70% DNA similarity and hence that they are related at the species level. A phylogenetic tree showing the relationship of *M. branderi* to the other mycobacteria (Fig. 2) was constructed by using equally weighted Hamming distances (Fig. 3).

The strains of *M. branderi* which we analyzed were isolated from samples obtained from nine patients, some of whom had cavitary mycobacteriosis of the lungs that was resistant to available drugs. In most cases, repeat samples obtained from each patient were positive for acid-fast bacilli as determined by

FIG. 4. *M. branderi* colonies on Middlebrook 7H11 agar. Magnification, $\times 8$.

microscopy, and the only cultivable species was *M. branderi*. Hence, *M. branderi* has to be considered a potential human pathogen.

Taxonomic description of *Mycobacterium branderi* sp. nov. *Mycobacterium branderi* (bran' de.ri. L. gen. n. *branderi*, of Brander, referring to Eljas Brander, the former head of the Tuberculosis Laboratory of the National Public Health Institute, Finland, who collected the strains). The strains that have the newly described sequence are nonchromogenic and produce smooth, often umbonate, off-white to grayish colonies on Middlebrook 7H11 agar (Fig. 4). Growth is equally good at 37 and 45°C and is only slightly delayed at 25°C. In acid-fast staining preparations the bacterial cells, which are 1.2 to 3 μ m long, are delicate slender rods that are often slightly curved. The strains are negative for Tween 80 hydrolysis, catalase, urease, and nitrate reductase activities (Table 1), and niacin. They are strongly positive for arylsulfatase activity in 14-day tests and moderately to weakly positive for nicotinamidase and pyrazinamidase activities. In susceptibility tests they are resistant to isoniazid, rifampin, pyrazinamide, and cycloserine but susceptible to ethambutol, streptomycin, ethionamide, and capreomycin.

All strains have a glycolipid pattern unlike the patterns of previously described species (3). As determined by thin-layer chromatography, they contain alpha mycolates, ketomycolates, and carboxy mycolates (3). When the fatty acid methyl esters, alcohols, and mycolic acid cleavage products are analyzed by GLC, hexacosanoic acid (26:0) is the major mycolic acid cleavage product and 2-eicosanol (2-OH-20:0alc) is the major alcohol. A high level (15 to 22%) of tuberculostearic acid (10-Me-18:0) is also present. The type strain is strain 52157, which has been deposited in the American Type Culture Collection as strain ATCC 51789.

Characteristics which differentiate *M. branderi* from related mycobacteria. When conventional and lipid techniques are used, *M. branderi* can be distinguished from *M. celatum* only on the basis of pigment production (Tables 1 and 2). Nonpigmented strains of *M. celatum* can be confused with *M. branderi* unless gene sequencing (12) is used; this occurred in the initial classification of the Helsinki group. *M. branderi* can be differentiated from *M. xenopi*, another closely related species, on the basis of the good growth of *M. branderi* that occurs at room temperature, the lack of pigment production by *M. branderi*, and the combination of a high relative amount of 2-eicosanol

and the presence of no significant amount of 2-docosanol in *M. branderi* when fatty acid and alcohol compositions are analyzed. Since *M. cookii* is scotochromogenic and does not grow at 37°C (11), it poses no difficulty for differentiation. The key characteristic which distinguishes *M. branderi* from *M. avium* and *M. intracellulare* is the presence of a high level of hexacosanoic acid in *M. branderi* as determined by GLC fatty acid analysis, compared with no or insignificant amounts in both *M. avium* and *M. intracellulare* (9). The useful conventional biochemical features for differentiation are the characteristic colony morphology, including the off-white to grayish colony color, the high level of arylsulfatase activity, and susceptibility to ethambutol and streptomycin combined with resistance to cycloserine. Other nonpigmented slowly growing species, including members of the *M. terrae* complex, *M. malmoeense*, and *M. shimoidei*, are easily distinguished from *M. branderi* by their inability to grow at 45°C and by the results of arylsulfatase and Tween 80 hydrolysis tests (Table 1). GLC analysis of fatty acid compositions provides an excellent way to distinguish these species (Table 2) (3, 9, 13).

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