Mycobacterium hiberniae sp. nov.

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Strains of a new type of slowly growing scotochromogenic, rose-pink-pigmented mycobacterium were isolated repeatedly from sphagnum vegetation, true moss, and soil in Ireland. These strains grew at 22, 31, and 37°C but not at 45°C and possessed acid phosphatase and arylsulfatase activities. They reduced nitrate, tolerated 0.1% NaNO₂, did not split amides, and were resistant to most of the antituberculous drugs tested, except ethambutol. They did not form acid from glucose and mannose. Their internal phenetic similarity was 97.08% ± 2.07%. The whole mycolate pattern confirmed the homogeneity of the taxa sharing similar mycolate types with several other mycobacterial species. However, on the basis of the nature of the major pyrolysis esters, the taxon appeared unique. The phylogenetic analysis based on evolutionary distance values revealed that the strains belong to a new species of slowly growing mycobacteria. The DNA-DNA hybridization values confirmed that these strains differ significantly from *Mycobacterium nonchromogenicum*, *M. terrae*, *M. triviale*, and *M. thermoresistibile*. The strains produced a unique rose-pink pigment and were nonpathogenic for mice, guinea pigs, and rabbits, but they provoked a nonspecific hypersensitivity reaction to bovine tuberculin in guinea pigs and cattle. Hence, they are considered a member of a new species of nonpathogenic slowly growing mycobacteria, for which the name *Mycobacterium hiberniae* is proposed. Strain Hi 11 is the type strain, a culture of which has been deposited in the American Type Culture Collection as strain ATCC 49874.

During 1990 and 1991, 13 strains with homogenous properties belonging to the genus *Mycobacterium* were isolated on culture media for mycobacteria from true moss, sphagnum vegetation, and soil collected in the counties Clare and Donegal in Ireland. These strains were nonpathogenic, produced cross-sensitivity to bovine tuberculin, and are regarded as a source of nonspecific reactions in cattle in some regions of Ireland. The properties of these strains indicate that they belong to a new species of slowly growing mycobacteria. In this paper, the results of a taxonomic and molecular genetic study are described.

MATERIALS AND METHODS

Bacterial strains. The origins of 13 strains of mycobacteria that have not been previously described (referred to below as Hi strains) were sphagnum vegetation (*Sphagnum subnitens*), true moss (*Acrocladium cuspidatum*, *Amblystegium varium*, *Atrichum undulatum*, and *Ctenidium molluscum*), and soil, collected in 1990 and 1991 in the counties Clare and Donegal, Ireland. The type strain Hi 11^T was found in *Ctenidium molluscum* vegetation.

Methods. Colony morphology and the ability to grow at various temperatures (22, 31, 37, 42, and 45°C) were determined after 4 weeks of incubation on Löwenstein-Jensen slants and Middlebrook 7H10 agar inoculated with 10⁻² mg of cells per cultivation test. Pigment production in the dark and photoactivity after 6 weeks of incubation were determined as previously described (13, 22). The following tests were also performed as described previously: nitrate reduction and catalase activity (6, 22); Tween hydrolysis (22); acid

phosphatase production (15); arylsulfatase activity after 3 and 10 days (18); production of acetamidase, benzamidase, urease, isonicotinamidase, nicotinamidase, pyrazinamidase, succinamidase, allantoinase, and malonamidase (5); and resistance to 1 and 5 µg of ethambutol per ml, 1 and 10 µg of isoniazid per ml, 20 µg of rifampin per ml, 5 µg of streptomycin per ml, and 10 and 40 µg of ethionamide per ml (34). In addition, resistance to 0.1% NaNO₂ (33) and to 0.2% picric acid (32) were also tested. Acid production from glucose and mannose was tested in agar medium during 6 weeks of incubation (13, 22).

The lipid analyses were carried out by using cells separated from Löwenstein-Jensen slants. Cells were saponified in 5% (wt/vol) KOH in 2-methoxyethanol (Methylcellosolve) containing 12% (vol/vol) H₂O at 110°C for 4 h to ensure complete hydrolysis of sterically hindered ester groups. After acidification with H₂SO₄, the solution was partitioned between diethyl ether and water. The ether phase was washed five times with distilled water and dried. Free fatty acids in extracts were esterified for 15 min at room temperature by using freshly prepared diethyl ether solution of diazomethane. The solution was then dried under nitrogen, and the lipids were redissolved in diethyl ether and analyzed by thin-layer chromatography. The mycolate patterns were determined as previously described (9). The fatty acids, methyl esters, and alcohols were determined by gas chromatography with a Perkin-Elmer model 8310 B gas chromatograph fitted with a 1-m-high column packed with 3% OV-1. Nitrogen was used as the carrier gas. To determine the composition of the chain in position 2 of the mycolates, pyrolytic conditions were used, with the injection temperature raised from 300 to 400°C. Cultures of strain Hi 11^T and Hi 2 that were 4 weeks old were tested for pathogenicity. For each strain, three rabbits were inoculated intravenously

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with 10 mg (wet weight) of bacteria, five NMRI white mice were injected intravenously with 1 mg of bacteria, and three white guinea pigs were injected subcutaneously with 10 mg of bacteria. After 14 weeks, the animals were necropsied (21).

Phylogenetic analysis. The 16S rRNA gene of strain Hi 11^T (T, type strain) was amplified by the polymerase chain reaction technique and sequenced as described previously (11).

The sequence was aligned manually to the 16S rRNA data base of more than 70 mycobacterial strains (17, 25). For the phylogenetic analysis, only those positions that were unambiguously present in each of the sequences were included. The stretch that was analyzed comprised 1,419 nucleotide positions present in each of the sequences, i.e., positions 50 to 72, 99 to 199, 214 to 451, and 479 to 1376 (according to the method of Weisburg et al. [35]).

Pairwise evolutionary distances (expressed as estimated changes per 100 nucleotides) were computed from the percent similarities by the correction of Jukes and Cantor (14). A phylogenetic tree was constructed from the distance matrix by the algorithm of De Soete (10). The reproducibility of the branching nodes was examined by the neighbor-joining bootstrapping programs PDFIND and NJBOOT (kindly provided by T. S. Whittam, Institute of Molecular Evolution and Genetics, Pennsylvania State University). One thousand trees were generated and examined. Sequence alignment and data analysis were done by using a Sun Sparc Workstation IPC.

In addition, a quantitative evaluation of the DNA-DNA hybridization technique with sulfonated DNA (Sulfo-DNA; Biozym, Hameln, Germany) was included to monitor the difference between the Hi 11^T strain and *M. triviale*, *M. terrae*, *M. nonchromogenicum*, and *M. thermoresistibile* (31).

Numerical analyses. On the basis of a comparison of 32 properties, the level of internal similarity of the 13 Hi strains was determined (28). A total of 33 properties of *M. terrae*, *M. nonchromogenicum*, *M. thermoresistibile*, and *M. triviale* and 32 properties of 17 other species of slowly growing mycobacteria were determined and compared with the properties of strain Hi 11^T. All the data obtained were converted to the simple binary (i.e., 1 or 0) for analysis; both negative and positive matches were used (28).

Nucleotide sequence accession number. The partial 16S rRNA sequence of strain Hi 11^T, consisting of 1,419 nucleotides, has been deposited in GenBank under accession number X67096.

RESULTS AND DISCUSSION

The cells of the 13 Hi strains grown on Löwenstein-Jensen medium and on Middlebrook 7H10 agar were beaded rods (0.9 by 1.2 to 1.5 µm), often polymorphic, gram positive, and acid-alcohol fast, and very often formed clumps but not cords. Spores, capsules, true branching, and aerial hyphae were not observed. On Löwenstein-Jensen medium, the strains were eugonic in the first phase of growth, becoming dysgonic later, and produced rose-pink-pigmented colonies, which assumed a rough, dry appearance after 8 weeks when incubated at temperatures between 22 and 37°C. At 37°C, growth was observed after 20 days; at 31°C, growth was observed after 25 days; at 22°C, growth was observed after 4 weeks. No growth occurred at 42 or 45°C. Smooth, compact, rose-pink colonies, 1 to 1.5 mm in diameter, with entire margins developed on Middlebrook 7H10 agar from dilute

TABLE 1. Characteristics of 13 Hi strains

Characteristic	% of strains giving positive reaction
Pigment production in the dark	100
Photoactivity after 4 weeks	0
Growth after 4 weeks at:	
22°C	100
31°C	100
37°C	100
45°C	0
Enzymatic activity:	
Acid phosphatase (37°C, 20 h)	100
Arylsulfatase (10 days)	100
Nitrate reductase (37°C, 22 h)	100
Tween hydrolysis (10 days)	46 ^a
Acetamidase	0
Benzamidase	0
Urease	0
Isonicotinamidase	0
Nicotinamidase	0
Pyrazinamidase	0
Allantoinase	0
Succinamidase	0
Tolerance to:	
NaNO ₂ (0.1%)	100
Picric acid (0.2%)	0
Acid production from:	
D-(+)-Glucose	0
D-(+)-Mannose	0
Resistance to:	
Ethambutol	
5 µg/ml	0
1 µg/ml	23 ^b
Isonicotinic acid	
10 µg/ml	100
1 µg/ml	100
Rifampin (20 µg/ml)	100
Streptomycin (5 µg/ml)	100
Ethionamide	
10 µg/ml	100
40 µg/ml	100

^a Strains Hi 3, Hi 6, Hi 8, Hi 10, Hi 11, and Hi 13 were positive.

^b Strains Hi 1, Hi 2, and Hi 10 were positive.

inocula at 37°C. A total of 32 properties of the Hi strains are shown in Table 1. These strains constitute a homogeneous group, with a level of internal similarity of 97.08% ± 2.07%.

A comparison of the properties of strain Hi 11^T with the properties of 22 species of slowly growing mycobacteria showed marked differences from other mycobacterial species (Table 2). The whole-cell mycolate pattern of the Hi strains (Table 3) confirmed the homogeneity of the new taxon, sharing similar mycolate types with several other mycobacterial species, notably the *M. avium-M. intracellulare* complex (9, 23). However, on the basis of the nature of the major pyrolysis esters (doco- and tetracosanoate), the taxon appears unique among the group of species that synthesize the same type of mycolates (9, 20).

The 16S rRNA sequence of strain Hi 11^T is unique among mycobacteria, exhibiting the highest degree of variation in the variable regions V1, positions 64 to 104 (26), and V3, positions 437 to 497 (24), compared with more than 70 mycobacterial sequences. A phylogenetic analysis (Fig. 1), based on evolutionary distance values (Table 4), revealed that strain Hi 11^T fell into the confines of slowly growing mycobacteria. This position is supported by the presence of a long helix between positions 451 and 482 (part of region

TABLE 2. Similarity values between strain Hi 11^T and strains of 22 species of slowly growing mycobacteria

Organism and strain	% Similarity
<i>M. terrae</i> TMC 1450.....	84.8
<i>M. gordonae</i> TMC 1324.....	81.8
<i>M. nonchromogenicum</i> TMC 1481.....	78.8
<i>M. triviale</i> TMC 1453.....	78.8
<i>M. asiaticum</i> ATCC 25276.....	78.1
<i>M. intracellulare</i> ATCC 23434.....	75.0
<i>M. szulgai</i> NCTC 10831.....	75.0
<i>M. scrofulaceum</i> TMC 1323.....	71.8
<i>M. flavescens</i> TMC 1541.....	71.8
<i>M. marinum</i> ATCC 927.....	71.8
<i>M. avium</i> TMC 724.....	68.8
<i>M. gastri</i> ATCC 15754.....	68.8
<i>M. ulcerans</i> ATCC 19423.....	68.8
<i>M. xenopi</i> ATCC 19250.....	68.8
<i>M. shimoidei</i> ATCC 27952.....	68.8
<i>M. bovis</i> ATCC 19210.....	65.6
<i>M. kansasii</i> TMC 1204.....	65.6
<i>M. cookii</i> ATCC 49103.....	65.6
<i>M. simiae</i> ATCC 25275.....	62.5
<i>M. africanum</i> ATCC 25420.....	59.4
<i>M. tuberculosis</i> ATCC 25618.....	56.3
<i>M. malmoeense</i> ATCC 29571.....	53.1

V3) of the 16S rRNA, which is a significant signature of all slowly growing members of the genus (25, 26, 29, 30). In particular, strain Hi 11^T exhibits a close relationship to a subcluster of slowly growing mycobacteria consisting of *M. terrae* ATCC 15755^T and *M. nonchromogenicum* ATCC 19530^T (25). This clustering is supported by the presence of a U residue at position 1274, an additional base pair in region V3, and a 9-base loop in region V8 (the last two unique features are also found in *M. cookii* [16]). High bootstrap values of 95% support the membership of *M. hiberniae* in the *M. terrae*-*M. nonchromogenicum* cluster, as well as the clustering of most of the other slowly growing mycobacteria. Interestingly, the branching point of the *M. terrae* cluster (42%), and even more so of *M. cookii* within the radiation of the slowly growing mycobacteria, is of little statistical significance (10%).

Over the complete stretch of 1,419 nucleotides, the degree

TABLE 3. Mycolate types, major pyrolysis esters, and fatty acid and alcohol composition in 13 Hi strains

Composition pattern	Response of all Hi strains
Mycolate type ^a	
I.....	+
II.....	-
III.....	-
IV.....	+
V.....	-
VI.....	+
2-OH	
C-18.....	+
C-20.....	+
C-22.....	-
Major pyrolysis ester	
C-22.....	+
C-24.....	+
C-26.....	-

^a The mycolate types have been described previously (9).

FIG. 1. Phylogenetic position of *M. hiberniae* ATCC 49874^T (strain Hi 11) within the radiation of slowly growing mycobacteria. A more detailed branching pattern of slowly growing and fast-growing mycobacteria has been published in reference 25. The horizontal components of the branch lengths are proportional to evolutionary distances. The bar indicates 1% nucleotide differences.

of similarity between strain Hi 11^T and *M. nonchromogenicum* is substantial (98.7%).

Similarly high values have been found between strains of closely related species, e.g., *Fibrobacter* spp. (1), *Lactococcus lactis* (7), *Streptococcus parasanguis* (36), and *M. intracellulare* (4, 12, 30), and also between closely related species, such as certain slowly growing mycobacteria (25, 27, 29) (Fig. 1), *Streptococcus* spp. (3), *Fusobacterium* spp. (19), *Listeria* spp. (8), and *Enterococcus casseliflavus* and *Enterococcus gallinarum* (37). Consequently, it has been stressed that it is not possible to draw definite conclusions about species differentiation on the basis of 16S rRNA sequences alone (1, 2, 12). The decision of whether to describe a species depends upon the availability of other data, such as DNA hybridization (31) and/or phenotypic differences to neighboring taxa. In the present case, the decision to describe a new species was based on the presence of a unique rose-pink pigment, sensitization to bovine tuberculin, and certain other properties listed below.

The Hi strains are mycobacteria with very limited enzymatic activity. All strains possess acid phosphatase, nitrate reductase, and catalase. Arylsulfatase production was delayed, becoming positive after 10 days of incubation. No amidase activity was found. These strains tolerated 0.1% NaNO₂ but were susceptible to 0.2% picric acid. They did not produce acid from glucose and mannose. All strains were susceptible to 5 µg of ethambutol but resistant to isonicotinic acid, rifampin, streptomycin, and ethionamide in the concentrations tested.

Furthermore, they did not utilize any of the compounds tested. Their optimal growth temperature of 37°C is very unusual for environmental strains originating from a moderate climate. The rose-pink pigmentation is unique within the genus *Mycobacterium*. The strains were not pathogenic for experimental animals, but they provoked a cross-reaction to bovine tuberculin in guinea pigs and cattle. The strains have a mycolate pattern similar to that of the *M. avium*-*M. intracellulare* complex. However, all Hi strains released by

TABLE 4. Evolutionary distances for the 16S rRNAs of *M. hiberniae* ATCC 49874^T and various representatives of slowly growing and fast-growing mycobacteria^a

Organism	<i>M. hiberniae</i>	<i>M. terrae</i>	<i>M. nonchromogenicum</i>	<i>M. cookii</i>	<i>M. asiaticum</i>	<i>M. gordonae</i>	<i>M. marinum</i>	<i>M. scrofulaceum</i>	<i>M. kansasii</i>	<i>M. malmoense</i>	<i>M. intracellulare</i>	<i>M. paratuberculosis</i>	<i>M. bovis</i>
<i>M. terrae</i>	1.9												
<i>M. nonchromogenicum</i>	1.0	1.6											
<i>M. cookii</i>	2.7	2.4	2.4										
<i>M. asiaticum</i>	3.5	2.1	3.5	2.7									
<i>M. gordonae</i>	3.7	2.7	3.3	2.8	1.4								
<i>M. marinum</i>	3.1	2.4	3.0	2.6	1.6	2.0							
<i>M. scrofulaceum</i>	3.7	3.4	3.5	3.0	2.1	1.9	1.1						
<i>M. kansasii</i>	3.8	3.5	3.7	3.3	2.1	2.0	1.2	0.4					
<i>M. malmoense</i>	3.8	3.5	3.5	3.2	2.1	2.0	1.3	0.6	0.8				
<i>M. intracellulare</i>	3.7	3.5	3.5	3.4	2.4	2.2	1.4	0.6	1.0	0.8			
<i>M. paratuberculosis</i>	3.5	3.5	3.3	3.3	2.1	2.0	1.2	0.8	1.0	0.8	0.5		
<i>M. bovis</i>	3.0	2.4	2.9	2.6	1.6	2.0	0.3	1.2	1.2	1.2	1.5	1.2	
<i>M. triviale</i>	3.1	3.0	2.7	2.9	3.8	3.5	3.7	3.6	3.9	4.0	4.2	3.9	3.6

Fast-growing mycobacteria^b

1.7–4.6

^a The origins of strains, 16S rRNA accession numbers, and the phylogenetic structure of fast-growing mycobacteria have been described previously (25).^b The following fast-growing mycobacteria were included in the comparison: *M. aichiense*, *M. neoaurum*, *M. gadium*, *M. komossense*, *M. fallax*, *M. phlei*, *M. smegmatis*, *M. thermoresistibile*, and *M. sphagni* (data not shown).

the pyrolysis of their mycolates, C-22 and C-24 fatty esters, whereas the *M. avium-M. intracellulare* complex releases only C-24 fatty esters. The nature of the pyrolysis esters is regarded as specific and stable for a given species (9).

The DNA-DNA hybridization confirmed the homogeneity of the 13 Hi strains and demonstrated the differences from *M. nonchromogenicum*, *M. terrae*, and *M. thermoresistibile* (Table 5).

Taxonomic description of *Mycobacterium hiberniae* sp. nov.

Mycobacterium hiberniae (hi.ber' ni.ae. L. gen. n. *hiberniae*, of Hibernia, the Latin name for Ireland, the source of these strains). The description of *M. hiberniae* presented below is based on a study of 13 strains which shared the characteristics shown in Table 1. Cells are acid-alcohol fast, polymorphic, beaded rods (0.9 by 1.2 to 1.5 µm) which very often form clumps but not cords. Spores, capsules, true branching, and aerial hyphae do not occur. Colonies on Löwenstein-Jensen and Middlebrook 7H10 agar are smooth and glistening with rose-pink pigmentation but become rough and dry later. The colonies with the unique pigment production are 1 to 1.5 mm in diameter; optimum growth occurs at 37°C. They grow at 22 and 31°C but not at 42 or 45°C. Biochemical characteristics are shown in Table 1. The strains are mycobacteria with limited enzymatic activity.

TABLE 5. Comparison of DNA-DNA hybridization values for Hi 11^T and type strains of *M. terrae*, *M. nonchromogenicum*, *M. thermoresistibile*, and *M. triviale*

Organism	% DNA similarity with Hi 11 ^T
<i>M. terrae</i>	14.29
<i>M. nonchromogenicum</i>	28.57
<i>M. thermoresistibile</i>	28.57
<i>M. triviale</i>	28.75

They possess acid phosphatase, nitrate reductase, and aryl-sulfatase. For all strains, amidase activities and acid production from glucose and mannose are negative. These organisms tolerate 0.1% NaNO₂ but not 0.2% picric acid. The strains are resistant to antituberculous drugs, except to 5 µg of ethambutol (Table 1). The strains have a mycolate pattern similar to that of the *M. avium-M. intracellulare* complex, but they release by pyrolysis their mycolate C-22 and C-24 fatty esters, a unique characteristic for *M. hiberniae*.

The strains which were tested are not pathogenic for rabbits, guinea pigs, or mice. They provoke a nonspecific skin hypersensitivity reaction to bovine tuberculin. The type strain of *M. hiberniae* is Hi 11. A culture of this strain has been deposited in the American Type Culture Collection as strain ATCC 49874. Strains of *M. hiberniae* have been isolated from true moss, sphagnum, and soil in Ireland.

Characteristics which differentiate *M. hiberniae* from other related mycobacteria. *M. hiberniae* can be differentiated from *M. gordonae* by its positive nitrate reductase activity, by its failure to produce acid from glucose, by its production of a rose-pink pigment, and by marked differences in the whole mycolate pattern and the 16S rRNA sequences.

M. hiberniae is easily differentiated from *M. terrae* by its rose-pink pigment formation and its positive arylsulfatase activity. There are a number of differences in the 16S rRNA sequences, major pyrolysis esters, and DNA-DNA hybridization values.

M. hiberniae can be differentiated from *M. triviale* by its production of a rose-pink pigment and its resistance to isonicotinic acid and rifampin. There are also marked differences in the major pyrolysis esters, 16S rRNA sequences, and DNA-DNA hybridization values.

M. hiberniae can be differentiated from *M. nonchromogenicum* by its slow growth on media, by its production of a rose-pink pigment, by its failure to split nicotinamide and pyrazinamide, and by its failure to produce acid from glu-

cose. There are marked differences in the major pyrolysis esters and DNA-DNA hybridization values.

Generally, *M. hiberniae* differs from all other mycobacterial species described thus far by its unusual rose-pink pigmentation, which is unique in the genus *Mycobacterium*.

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