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Complete DNA Sequence Analysis for 16s Ribosomal RNA Gene of the Leproma-Derived, Cultivable and Nerve-Invading Mycobacterium HI-75[1]

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Although Hansen's disease is now under control in many countries, the causative organism *Mycobacterium leprae* still is regarded as a species of noncultivable bacilli. The aim of the present study is to ascertain the taxonomic characteristics of cultivable mycobacterium HI-75 (M.HI-75), which was isolated as *Mycobacterium leprae* by Skinsnes, *et al.* in 1975 from a lepromatous type of Hansen's disease patient ([17]). By the direct sequencing technique the 16S ribosomal RNA (16SrRNA) gene of the strain was analyzed in full length (1493 bases) and compared with those of other mycobacteria. The bacillus has been maintained in the laboratory of one of the authors since 1984 using Ogawa's medium enriched with glucuronic acid and N-acetyl-D-glucosamine. During that period of time, Sasaki and Hamit have observed both nerve invasion and the growth of inoculated M.HI-75 in either the nude mice or the ^{131}I-treated Swiss mice ([9, 16]).

MATERIALS AND METHODS

Bacterial strain. The leproma-derived and cultivable M.HI-75 has been maintained at 36.5°C utilizing either Ogawa's or Sauton's medium in the beginning and Ogawa's medium enriched with 1% each of glucuronic acid and N-acetyl-D-glucosamine which was recently used for this study.

Extraction of DNA. The M.HI-75 was suspended in 500 µl of TE buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA pH 8.0) to make a final suspension of 10^8 bacilli/ml and heated at 80°C for 20 min. Then, 50 µl of lysozyme (Wako Junyaku, Osaka, Japan) was added to the suspension at a rate of 10 mg/ml and the mixture was incubated at 37°C for 1 hr followed by mechanical disruption with glass beads for 1 min. The DNA was extracted using a Sepa-Gene kit (Sanko Junyaku, Tokyo, Japan) and finally precipitated with isopropanol. The precipitates were then washed with ethanol.

DNA amplification. A polymerase chain reaction (PCR) mixture containing 1 µl of 25 mM $MgCl_2$, 10 µl of 10× PCR buffer, 8µl of 2.5 mM each dNTPs, 60.5 µl of autoclaved ultra-pure water, 0.5 µl of Taq DNA polymerase (2.5 unit) (PCR kit; Takara, Ohtsu, Japan), 5 µl of each sense and antisense primers (20 pmole) (Table 1), and 5 µl of the DNA template mentioned above. The reaction was primed by a template melting step at 94°C for 5 min, followed by 35 cycles of the serial temperature changes consisting of 94°C for 30 sec, 55°C for 1 min and 72°C for 1 min using a Perkin-Elmer Model 480 DNA Thermal Cycler (Perkin-Elmer, Norwalk, Connecticut, U.S.A.).

Direct sequencing technique. The PCR product thus obtained was directly sequenced by the use of an Auto Load Solid Phase Sequence kit (Amersham-Pharmacia Biotec, Uppsala, Sweden) including T7 DNA polymerase, biotinylated primers and Dye Amidite-667 conjugated primers (Amersham-Pharmacia Biotec, Tokyo, Japan) on an A.L.F.red sequencer (Amersham-Pharmacia Biotec, Uppsala, Sweden) at 1500V for 360

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TABLE 1. *Five sets of primers which amplify 16S ribosomal RNA of the bacteria.*

#1	5′-AGA GTT TGA TCC TGG CTC AG-3′
#2	5′-CCA CTG CTG CCT CCC GTA GGA-3′
#3	5′-TGG CCT ACC AAG GCG ACG AC-3
#4	5′-CTA CGA GCT CTT TAC GCC CA-3′
#5	5′-CAG CAG CCG CGG TAA TAC-3′
#6	5′-CCG TCA ATT CCT TTG AGT TT-3′
#7	5′-GTG CCG TAG CTA ACG CAT TA-3′
#8	5′-CGT CAT CCC CAC CTT CCT CC-3′
#9	5′-AAC TGG AGG AAG GTG GGG AT-3′
#10	5′-AGG AGG TGA TCC AAC CGC A-3′

min on a 6% polyacrylamide gel with a 0.55 mm thickness. Both the sequences of sense and antisense strands were analyzed to confirm precision. The sequenced results thus obtained from M.HI-75 16SrRNA were compared with those of other mycobacteria using GenBank data or those by Edwards, *et al.* ([6]) on Rogall, *et al.* ([14, 15]).

RESULTS

Table 2 shows all of the 1493 base sequences for the 16SrRNA gene obtained from the M.HI-75 strain. The sequences were compared with those of the other mycobacterial species shown in Table 3. Most of the sequences were stable in the 17 examined mycobacterial species. There were nine variable regions in the 16SrRNA gene of the bacteria. Several variable regions were also found between mycobacteria species, such as the region from nucleotide No38 to No50, from No134 to No178, and from No411 to No438. Three regions are shown in boxes (1, 2 and 3) in Table 3A. In the first region (No38–50), two substitute nucleotides were observed; however, these sequences are consistent with those of *M. gastri, M. kansasii* or *M. simiae.* But in the second and third regions, M.HI-75 shows no nucleotide difference from that of *M. scrofulaceum.* Other than these variable regions, M.HI-75 has only three nucleotides that are different from that of *M. scrofulaceum,* but *M. leprae* shows 47 nucleotide differences from that of M.HI-75. All in all, the 16SrRNA sequence of M.HI-75 was most similar to that of *M. scrofulaceum* and differed from it in only 5 bases (0.35%) of the complete 1493 bases.

DISCUSSION

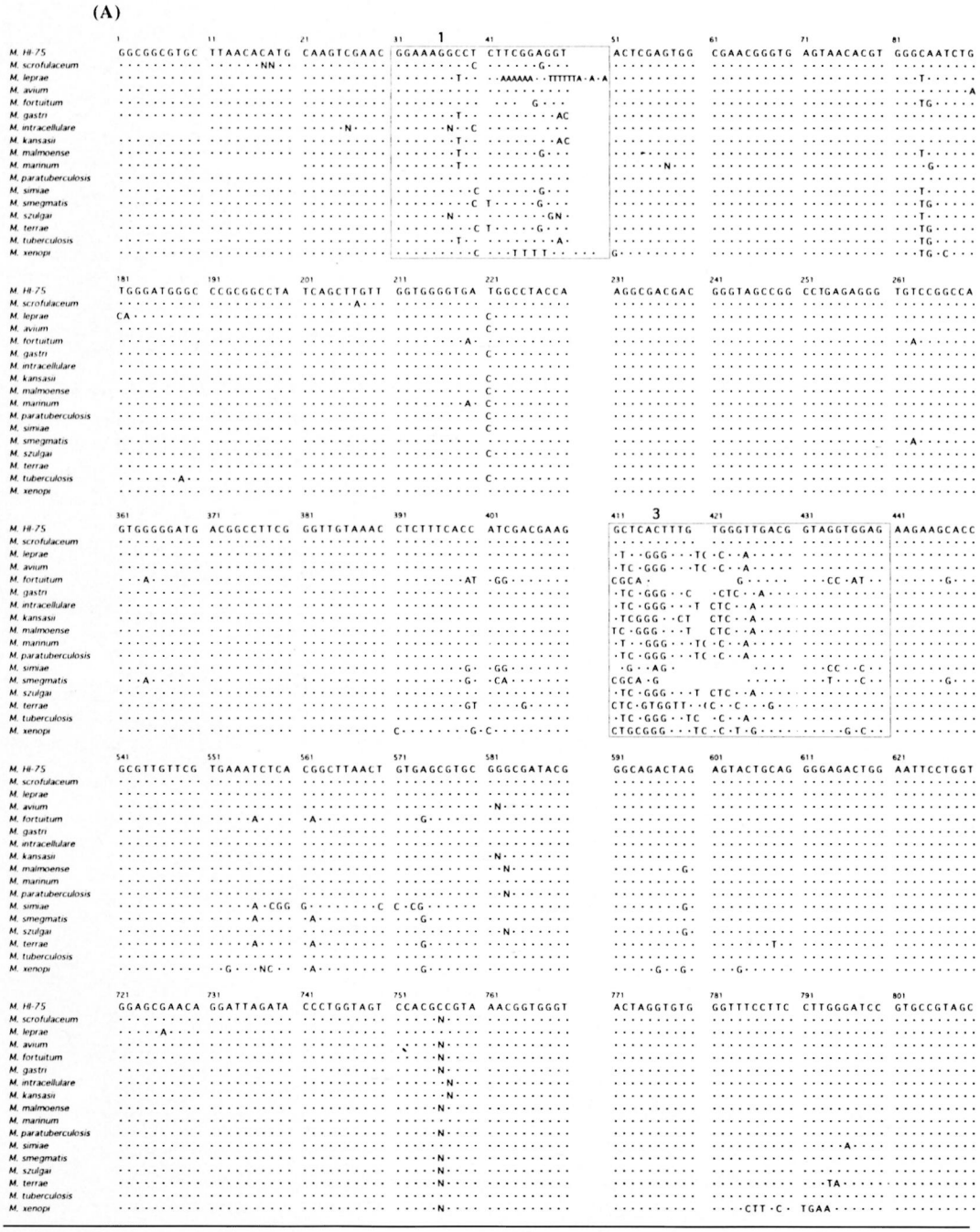
The applicability of 16SrRNA sequences for bacterial classification is well accepted

TABLE 2. *The whole sequence of 1493 bases of 16sRNA of* M.HI-75.[a]

1	11	21	31	41	51	61	71	81	91
GGCGGCGTGC	TTAACACATG	CAAGTCGAAC	GGAAAGGCc	CTTCGGa GGT	ACTCGAGTGG	CGAACGGGTG	AGTAACACGT	GGGCAATCTG	CCCTGCACT
101	111	121	131	141	151	161	171	181	191
CGGGATAAGC	CTGGGAAACT	GGGTCTAATA	CCGGATAGGA	CCACTTGGCG	CATGCCTTGT	GGTGGAAAGC	TTTTGCGGTG	TGGGATGGGC	CCGCGGCCT
201	211	221	231	241	251	261	271	281	291
TCAGCT GTT	GGTGGGGTGA	TGGCCTACCA	AGGCGACGAC	GGGTAGCCGG	CCTGAGAGGG	TGTCCGGCCA	CACTGGGACT	GAGATACGGC	CCAGACTCC
301	311	321	331	341	351	361	371	381	391
ACGGGAGGCA	GCAGTGGGGA	ATATTGCACA	ATGGGCGCAA	GCCTGATGCA	GCGACGCCGC	GTGGGGGATG	ACGGCCTTCG	GGTTGTAAAC	CTCTTTCAC
401	411	421	431	441	451	461	471	481	491
ATCGACGAAG	GCTCACTTTG	TGGGTTGACG	GTAGGTGGAG	AAGAAGCACC	GGCCAACTAC	GTGCCAGCAG	CCGCGGTAAT	ACGTAGGGTG	CGAGCGTTG
501	511	521	531	541	551	561	571	581	591
CCGGAATTAC	TGGGCGTAAA	GAGCTCGTAG	GTGGTTTGTC	GCGTTGTTCG	TGAAATCTCA	CGGCTTAACT	GTGAGCGTGC	GGGCGATACG	GGCAGACTA
601	611	621	631	641	651	661	671	681	691
AGTACTGCAG	GGGAGACTGG	AATTCCTGGT	GTAGCGGTGG	AATGCGCAGA	TATCAGGAGG	AACACCGGTG	GCGAAGGCGG	GTCTCTGGGC	AGTAACTGA
701	711	721	731	741	751	761	771	781	791
GCTGAGGAGC	GAAAGCGTGG	GGAGCGAACA	GGATTAGATA	CCCTGGTAGT	CCACGCCGTA	AACGGTGGGT	ACTAGGTGTG	GGTTTCCTTC	CTTGGGATC
801	811	821	831	841	851	861	871	881	891
GTGCCGTAGC	TAACGCATTA	AGTACCCCGC	CTGGGGAGTA	CGGCCGCAAG	GCTAAAACTC	AAAGGAATTG	ACGGGGGCCC	GCACAAGCGG	CGGAGCATC
901	911	921	931	941	951	961	971	981	991
GGATTAATTC	GATGCAACGC	GAAGAACCTT	ACCTGGGTTT	GACATGCACA	GGACGCGTCT	AGAGATAGGC	GTTCCCTTGT	GGCCTGTGTG	CAGGTGGTC
1001	1011	1021	1031	1041	1051	1061	1071	1081	1091
ATGGCTGTCG	TCAGCTCGTG	TCGTGAGATG	TTGGGTTAAG	TCCCGCAACG	AGCGCAACCC	TTGTCTCATG	TTGCCAGCGG	GTAATGCCGG	GGACTCGTG
1101	1111	1121	1131	1141	1151	1161	1171	1181	1191
GAGACTGCCG	GGGTCAACTC	GGAGGAAGGT	GGGGATGACG	TCAAGTCATC	ATGCCCCTTA	TGTCCAGGGC	TTCACACATG	CTACAATGGC	CGGTACAAA
1201	1211	1221	1231	1241	1251	1261	1271	1281	1291
GGCTGCGATG	CCGCAAGGTT	AAGCGAATCC	TTTTAAAGCC	GGTCTCAGTT	CGGAT GGGG	TCTGCAACTC	GACCCC TGA	AGTCGGAGTC	GCTAGTAAT
1301	1311	1321	1331	1341	1351	1361	1371	1381	1391
GCAGATCAGC	AACGCTGCGG	TGAATACGTT	CCCGGGCCTT	GTACACACCG	CCCGTCACGT	CATGAAAGTC	GGTAACACCC	GAAGCCAGTG	GCCTAACCC
1401	1411	1421	1431	1441	1451	1461	1471	1481	1491
TGGGAGGGAG	CTGTCGAAGG	TGGGATCGGC	GATTGGGACG	AAGTCGTAAC	AAGGTAGCCG	TACCGGAAGG	TGCGGCTGGA	TCACCTCCTT	TCT

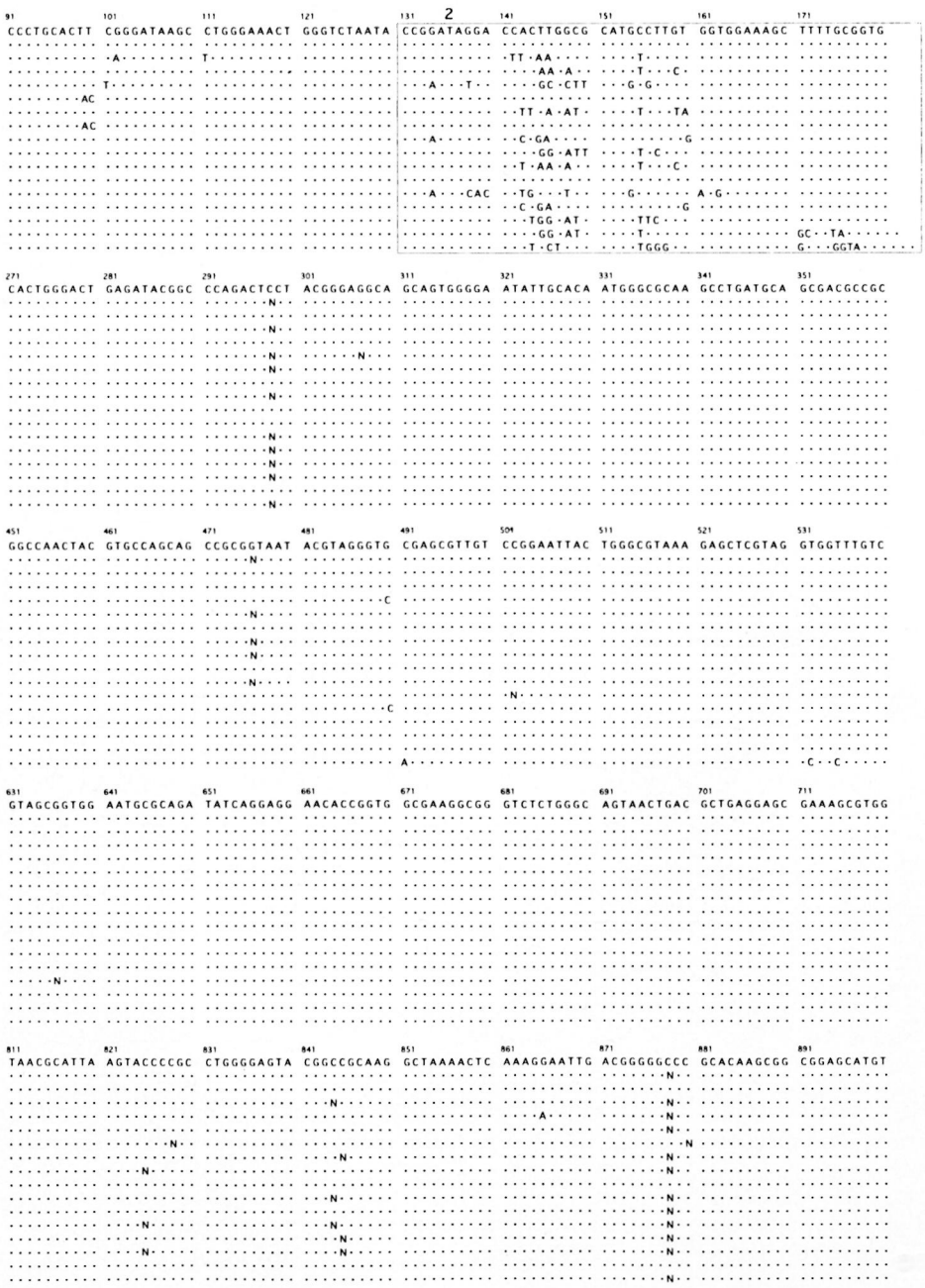
[a] Open letters indicate the sequences different from those of *M. scrofulaceum* and underlined letters indicate the sequences different from those of *M. leprae.*

TABLE 3. *(A) (B) Alignment of DNA sequences in mycobacterial 16SrRNA.*[a]



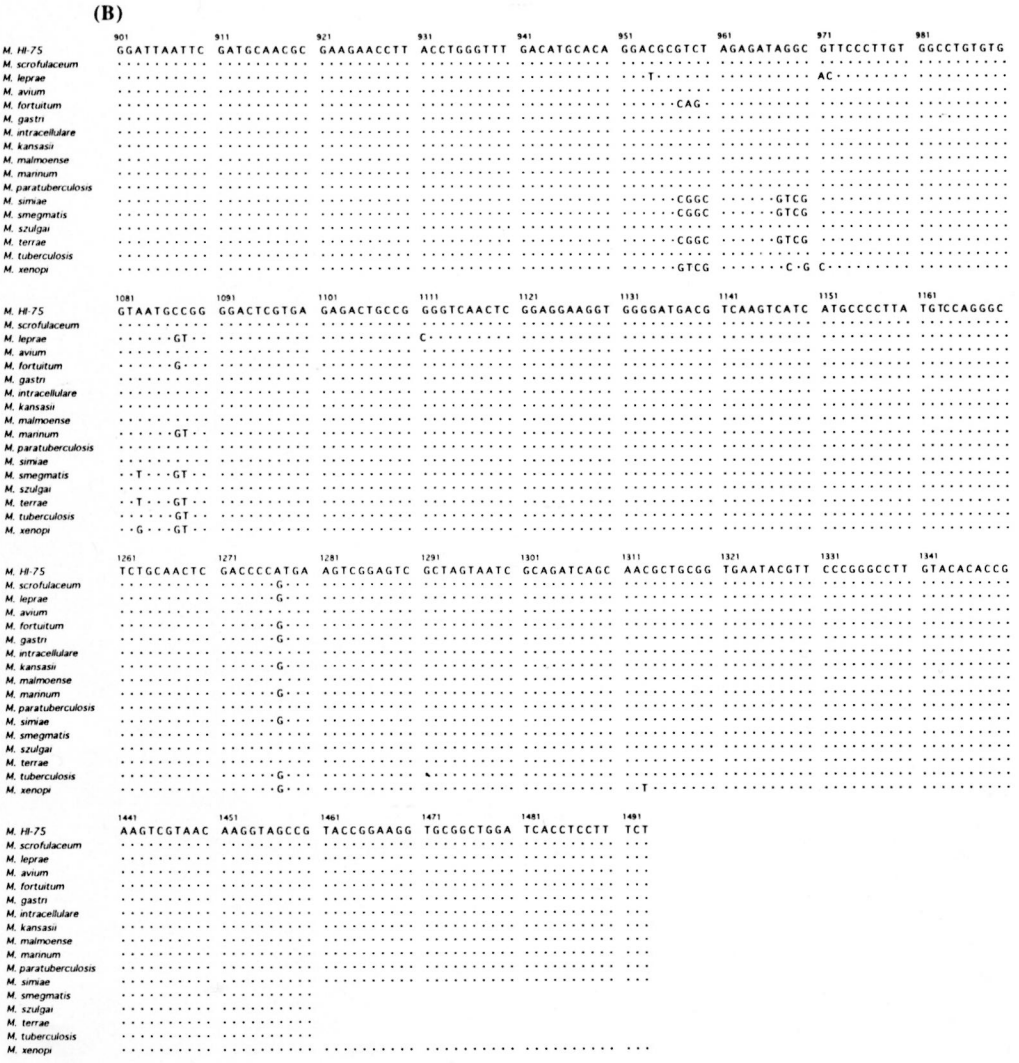
([2, 6–8, 20]). The 16SrRNA gene consists of genomically highly conserved primary sequences and has nine species-specific variable regions ([2, 7, 8]). The mycobacterial species can be distinguished and defined by the nucleotide structure of their 16SrRNA ([1, 14, 15]). PCR-mediated direct sequencing is a rapid and reliable method for obtaining the appropriate species-specific nucleotide sequence information. 16SrRNA sequencing allowed us to resolve the long-standing confusion with respect to the taxonomic

TABLE 3. *Continued*



differentiation within the *M. avium-M. intracellulare* complex. We examined the whole sequence of 16SrRNA of M.HI-75 and found that the sequences of 5 out of 1493 bases (0.35%) deviated from those of *M. scrofulaceum* and it was most similar to *M. scrofulaceum.* This result suggests that M.HI-75 is a variant of *M. scrofulaceum.* The postulated secondary structure of 16SrRNA is aligned to produce maximum base pairing between complementary segments ([12]). The molecule appears to have four major domains of folding. This structure is analogous to the cloverleaf of visualization

TABLE 3. *Continued*



of a tRNA and contains many regions of self-complementarity, capable of forming double-helical segments. Even distantly related 16SrRNA sequences show that the potentially double-strand regions are highly conserved. In this study, bases No. 40 (T) and No. 48 (A), and bases No. 1256 (T) and No. 1277 (A) are compensatory mutations in a secondary structure of 16SrRNA so as to maintain base pairing. Base No. 207 (T), included in a small loop, is independent of base pairing. So nucleotidic changes found in M.HI-75 are thought to be two base pairs and one point mutations from *M. scrofulaceum.*

The mycobacterium examined in this study was originally isolated as *M. leprae* by Skinsnes, *et al.* in 1975 from a leproma of a lepromatous-type Hansen's disease patient and, therefore, named as M.HI-75 by them ([17]). Stanford, *et al.* ([18]) claimed this bacillus to be a variety of *M. marianum* (syn. *scrofulaceum*) in 1977. Sasaki, *et al.* reported the nerve invasion and growth of the inoculated M.HI-75 into nude mice or the ^{131}I-treated immunocompromised Swiss mice ([16]). This episode provoked a doubt that there should be a missing link of knowledge between leprosy and *M. scrofulaceum* since the nerve invasion of the

TABLE 3. *Continued*

[a] The number of base sequences are compared to those of M.HI-75. The partial DNA alignment specific to MS and M.HI-75 are enclosed by open square; i.e., base number from 31 to 50, from 131 to 180, and from 411 to 440, as shown in Table 3(A) 1, 3(A) 2, and 3(A) 3, respectively.

bacilli had been regarded as a characteristic of *M. leprae* and not of *M. scrofulaceum.* In addition to that, the other genomic study on M.HI-75 performed by Williams, *et al.* (1994, personal communication) by restriction fragment length polymorphism of the PCR product suggested the resemblance of the bacilli as either *M. smegmatis* or *M. fortuitum* and not *M. scrofulaceum.* Hamit also reported the peripheral nerve invasion of M.HI-75 on inoculation into nude mice ([9]) besides forming lepromatous lesions with numerous macrophages which were loaded with the mycobacteria. Our present study suggested that *M. scrofulaceum* possibly possesses a pathogenetic activity in the peripheral nerve causing leprous-like lesions.

M. leprae has proved refractory to all attempts at *in vitro* culture. In many of the trials the claimed *M. leprae* have been denied to be so because of the possession of char-

TABLE 4. *DNA differences between* M.HI-75 *and other mycobacteria in sequenced 1493 bases of 16SrRNA.*

Mycobacteria	No. base difference
M. scrofulaceum	5 (0.35%)
M. leprae	47 (3.3%)
M. avium	19 (1.3%)
M. fortuitum	65 (4.5%)
M. gastrii	20 (1.4%)
M. intracellulare	20 (1.4%)
M. kansasii	21 (1.5%)
M. malmoense	22 (1.5%)
M. marinum	28 (1.9%)
M. paratuberculosis	19 (1.3%)
M. simiae	41 (2.9%)
M. smegmatis	61 (4.2%)
M. szulgai	21 (1.5%)
M. terrae	55 (3.8%)
M. tuberculosis	95 (6.6%)
M. xenopi	19 (1.3%)

acteristics which the other cultivable bacilli have or the absence of those which are taken as characteristic for *M. leprae.* And among the isolated and denied candidates for *M. leprae, M. scrofulaceum* received the highest score in frequency ([11]). A number of laboratories have described procedures for the detection and identification of *M. leprae* by PCR ([3–5, 10, 13, 14]). However, these have been applied to purified organisms, *M. leprae* in skin biopsy, or even tested on nerve biopsy to specific identification of *M. leprae* but no attempt has appeared in the literature to identify the whole pathogenetic organisms obtained from the site of the leprous neuropathy by the 16SrRNA sequence. Is there a possibility that *M. scrofulaceum* might cause nerve invasion and, therefore, nerve lesion? To perform a genomic study on the whole bacilli from infected lepromatous lesions of Hansen's disease patients using 16SrRNA might provide the manner with which to answer this question.

SUMMARY

The complete 1493 nucleotide sequence of the 16SrRNA gene of the leproma-derived and cultivable mycobacterium HI-75 strain was analyzed to elucidate the taxonomic characteristics by direct sequencing of the polymerase chain reaction (PCR) products. The results revealed that the sequence of mycobacterium HI-75 was mostly similar to that of *Mycobacterium scrofulaceum* with 5 bases differences in the sequenced 1493 bases (0.35%) of the 16SrRNA gene. *M. leprae* differed from the strain with 47 bases (3.3%). Sasaki and Hamit reported the nerve-invasive activity of the inoculated mycobacterium HI-75 in nude mice or the ^{131}I-treated immunocompromised Swiss mice. The results indicate that mycobacterium HI-75 could be a mutant of *M. scrofulaceum* possessing the ability to invade the peripheral nerve in addition to developing leproma-like lesions.

RESUMEN

Utilizando la técnica de la reacción en cadena de la polimerasa (PCR) se analizó secuencia completa de los 1493 nucleótidos del gene 16SrRNA de la micobacteria HI-75, para dilucidar sus características taxonómicas. Esta bacteria no cultivable se aisló de un leproma de un paciente. Los resultados revelaron que la secuencia de la micobacteria HI-75 fue muy similar a la correspondiente a *Mycobacterium scrofulaceum* con sólo 5 bases diferentes en la secuencia de 1493 bases (0.35%) del gene 16SrRNA. Por el contrario, la diferencia entre *M. leprae* y la micobacteria HI-75 fue de 47 bases (3.3%). Sasaki y Hamit reportaron que la bacteria HI-75 es capaz de invadir los nervios cuando se inocula en los ratones desnudos y en los ratones Suizos tratados con ^{131}I en inmunocomprometidos. Los resultados indican que la micobacteria HI-75 podría ser una mutante de *M. scrofulaceum* capaz de invadir los nervios periféricos y de inducir el desarrollo de lesiones lepromatosas.

RÉSUMÉ

La séquence complète composée de 1493 nucléotides du gène du rARN (ARN ribosomal) de la sous unité 16 S provenant de la souche de mycobactérie HI-75 cultivable et dérivée d'un léprome, fut analysée pour déterminer les caractéristiques taxonomiques, par séquençage direct des produits de la réaction de polymérase en chaîne (PCR). Les résultats ont révélés que la séquence de la mycobactérie HI-75 était la plus proche de celle de *Mycobacterium scrofulaceum,* avec seulement 5 bases de différentes parmi les 1493 bases séquencées (0.35%) du gène 16SrRNA. Quarante sept bases (3.3%) séparaient *M. leprae* de la souche étudiée. Sasaki et Hamit ont rapporté la propriété neuro-invasive de la mycobactérie HI-75 chez les souris nues ou les souris Suisses immuno-déprimées traitées à l'iode 131. Les résultats indiquent que la mycobactérie HI-75 pourrait être un mutant de *M. scrofulaceum* possédant la propriété d'envahir les nerfs periphériques en plus de développer des lésions ressemblant aux lépromes.

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