APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 2002, p. 5281–5287 Vol. 68, No. 11
0099-2240/02/$04.00+0 DOI: 10.1128/AEM.68.11.5281–5287.2002

Strain Variation in *Mycobacterium marinum* Fish Isolates

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Received 24 June 2002/Accepted 7 August 2002

A molecular characterization of two *Mycobacterium marinum* genes, 16S rRNA and *hsp65*, was carried out with a total of 21 isolates from various species of fish from both marine and freshwater environments of Israel, Europe, and the Far East. The nucleotide sequences of both genes revealed that all *M. marinum* isolates from fish in Israel belonged to two different strains, one infecting marine (cultured and wild) fish and the other infecting freshwater (cultured) fish. A restriction enzyme map based on the nucleotide sequences of both genes confirmed the divergence of the Israeli marine isolates from the freshwater isolates and differentiated the Israeli isolates from the foreign isolates, with the exception of one of three Greek isolates from marine fish which was identical to the Israeli marine isolates. The second isolate from Greece exhibited a single base alteration in the 16S rRNA sequence, whereas the third isolate was most likely a new *Mycobacterium* species. Isolates from Denmark and Thailand shared high sequence homology to complete identity with reference strain ATCC 927. Combined analysis of the two gene sequences increased the detection of intraspecific variations and was thus of importance in studying the taxonomy and epidemiology of this aquatic pathogen. Whether the Israeli *M. marinum* strain infecting marine fish is endemic to the Red Sea and found extremely susceptible hosts in the exotic species imported for aquaculture or rather was accidentally introduced with occasional imports of fingerlings from the Mediterranean Sea could not be determined.

Fish mycobacteriosis has been one of the most devastating diseases in Israeli aquaculture in recent years. The disease was first diagnosed in Eilat (Red Sea) in 1990 in cultured sea bass, *Dicentrarchus labrax* (7), and the causative agent, *Mycobacterium marinum*, has been isolated from several additional marine fish species since then (9). *M. marinum* is an acid-fast rod that causes chronic systemic infections in fish (1, 11) and other cold-blooded animals (32) and is capable of causing skin lesions in humans (13, 28).

Traditional diagnosis of this pathogen requires that it be recovered on culture medium and identified by means of a battery of differential biochemical tests (16). This classical method, however, often fails to identify *M. marinum* conclusively. Not only may the morphology and biosynthetic capabilities of mycobacteria vary depending on culture conditions (20), but also not uncommon are strain varieties that do not quite fit the typical biochemical profile for the species. In fact, the Red Sea *M. marinum* isolates, by being scotochromogenic and unable to hydrolyze Tween 80 and deamidate pyrazinamide, were atypical (7) and could not be positively identified to the species level until a molecular approach was adopted (17).

Over the last two decades, the 16S rRNA gene has emerged as a good standard for determining phylogenetic relationships of bacteria (35, 36). Knibb et al. (17) identified *M. marinum* directly from infected fish by using PCR amplification and direct sequencing of 16S rRNA products, allowing at the same time proper taxonomic assignment and opening the way to molecular epidemiologic analysis. This gene is still considered a key standard for bacterial identification due to the wealth of data available from public sequence databases (14, 34). However, as more sequence information accumulated over time, it became evident that the resolution power of 16S rRNA sequences alone is often insufficient when closely related organisms are compared (19, 27). According to Palys et al. (19), protein-encoding genes may be more discriminative than those encoding rRNA, while the analysis of two or more unlinked loci would prevent bacterial misclassification due to possible homologous recombination with other taxa.

The *hsp65* gene has been highly conserved during evolution (18), is present in all known *Mycobacterium* species, contains species-specific variations (21) but, on average, is more variable within the genus than the gene encoding 16S rRNA (15).

In the present work, a molecular characterization of 16S rRNA and *hsp65* genes was carried out with *M. marinum* fish isolates from various geographically distant sources to determine whether combined analysis of the two genes increases the detection of intraspecific variations. We also hoped to shed some light on the sources and epidemiology of the Israeli isolates.

MATERIALS AND METHODS

Fish and bacterial sources. The origins of all *Mycobacterium* isolates used in this study and their respective hosts are listed in Table 1. Sixteen isolates originated from cultured and wild fish in Israel; one was isolated from a captive hawksbill sea turtle in Eilat (Red Sea). Six were from Europe and two were from the Far East. Reference strain ATCC 927 was isolated from a marine fish in the United States and was obtained directly from the American Type Culture Collection, Manassas, Va.

Bacteriologic analysis. Mycobacteria were isolated on either Löwenstein-Jensen egg medium or Middlebrook 7H-10 agar medium (Difco) and incubated

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TABLE 1. *Mycobacterium* isolates and their hosts

Isolate[a]	Host	Origin[b]	Group[c]		Identity to GenBank accession no. for:	
			16S rRNA	*hsp65*	16S rRNA	*hsp65*
ATCC 927	Unspecified fish	United States, marine, captive	C	CII	AF456240	AF456470
DL240490	European sea bass (*Dicentrarchus labrax*)	Israel, marine (RS), cultured	A	A	AF456238	AF456468
DL150991	European sea bass (*D. labrax*)	Israel, marine (MS), cultured	A	A	AF456238	AF456468
DL045[d]	European sea bass (*D. labrax*)	Greece, marine (MS), cultured	A	A	AF456241	AF456471
DL049[d]	European sea bass (*D. labrax*)	Greece, marine (MS), cultured	F	F	AF456242	AF456472
CF030494	Butterfly fish (*Chaetodon fasciatus*)	Israel, marine (RS), captive	A	A	AF456238	AF456468
PP241194[e]	Sea bream (*Puntazzo puntazzo*)	Israel, marine (RS), cultured				
SO020195[e]	Red drum (*Sciaenops ocellatus*)	Israel, marine (RS), cultured				
SR250195	Rabbit fish (*Siganus rivulatus*)	Israel, marine (RS), cultured	A	A	AF456238	AF456468
DLDK[f]	European sea bass (*D. labrax*)	Denmark, marine, cultured	C	CII	AF456240	AF456470
MC110595[e]	Grey mullet (*Mugil cephalus*)	Israel, marine (RS), cultured				
SA200695[e]	Gilthead sea bream (*Sparus aurata*)	Israel, marine (RS), cultured				
Hybrid 270995[g]	Hybrid red sea bream (*Pagrus major* [female] × *S. aurata* [male])	Israel, marine (RS), cultured	A		AF456238	
EA040995[g]	White grouper (*Epinephelus aeneus*)	Israel, marine (RS), cultured	A		AF456238	
SR300397[g]	Rabbit fish (*S. rivulatus*)	Israel, marine (RS), wild	A		AF456238	
SR030597	Rabbit fish (*S. rivulatus*)	Israel, marine (RS), wild	A	A	AF456238	AF456468
EI100398[g]	Hawksbill sea turtle (*Eretmochelys imbricata*)	Israel, marine (RS), captive	A		AF456238	
CC240299	Koi (*Cyprinus carpio*)	Israel, freshwater, cultured	B	B	AF456239	AF456469
EA110499[g]	White grouper (*E. aeneus*)	Israel, marine (RS), cultured	A		AF456238	
BB170200	Silver perch (*Bidyanus bidyanus*)	Israel, freshwater, cultured	B	B	AF456239	AF456469
SV300500	Lizard fish (*Synodus variegatus*)	Israel, marine (RS), wild	A	A	AF456238	AF456468
DL041200	European sea bass (*D. labrax*)	Greece, marine (MS), cultured	A	A	AF456238	AF456468
185–408[h]	Golden steaty (*Acipenser baeri*)	Holland, freshwater	G	G	AF152558[j]	AF456473
185–409[h]	Golden steaty (*A. baeri*)	Holland, freshwater	G	G	AF152558[j]	AF456473
S4[i]	Snakehead (*Channa striatus*)	Thailand, freshwater	C	CIII	AF251565[j]	AF456474
S267[i]	Snakehead (*C. striatus*)	Thailand, freshwater	C	CIII	AF251565[j]	AF456474

[a] Unless otherwise indicated, isolates were from Israel Oceanographic and Limnological Research Ltd., National Center for Mariculture, Eilat, Israel (the two first letters indicate the initials of the host genus and species; the digits indicate the date of isolation [daymonthyear]).
[b] RS, Red Sea; MS, Mediterranean Sea.
[c] According to the phylogenetic analysis as it appears in Fig. 1 and 2A.
[d] Kindly provided by H. Nousias, Oceanos, Fish Health Center, Preveza, Greece.
[e] Not included in the 16S rRNA and *hsp65* gene sequence study.
[f] Kindly provided by I. Dalsgaard, Fish Disease Laboratory, Danish Institute for Fisheries and Marine Research, Frederiksberg, Denmark.
[g] Not included in the *hsp65* gene sequence study.
[h] Kindly provided by O. L. M. Haenen and D. Bakker, Laboratory of Fish Diseases, Institute for Animal Science and Health, Lelystad, Holland.
[i] Kindly provided by A. Adams, Institute of Aquaculture, University of Stirling, Stirling, Scotland.
[j] Accession numbers are already available from GenBank.

at 24 ± 0.5°C. Subcultures were made every 3 months by using the same media. Presumptive identification was reached by using standard biochemical tests (16).

DNA extraction. *M. marinum* cells from fresh or lyophilized cultures were used. The cells from the cultures were first washed in 10 mM Tris (pH 8.0)–1 mM EDTA (pH 8.0) buffer. The resulting pellet was ground with 300 μl of grinding buffer (100 mM Tris-HCl [pH 9], 100 mM EDTA, 1% sodium dodecyl sulfate). The homogenate was incubated for 30 min at 70°C. Forty-two microliters of 8 M potassium acetate was added, and the mixture was placed on ice for 30 min and then centrifuged for 15 min at 4°C and 12 000 × *g*. In order to avoid traces of the pellet, the supernatant was transferred to a fresh tube and centrifuged again for 5 min. DNA was precipitated with 1 volume of isopropanol and allowed to stand for 15 min at room temperature. Pelleted DNA was washed in 70% ethanol twice, and the air-dried pellet was dissolved in 50 μl of double-distilled H_2O. DNA quality was assessed by electrophoresis in 0.7% agarose and ethidium bromide staining. DNA quantity and purity were estimated with an RNA/DNA calculator (Gene Quant *pro*; Amersham, Cambridge, England).

PCRs. PCRs were performed with programmable thermal controllers (PTC-100; MJ Research, San Francisco, Calif.) and a final volume of 50 μl containing 1 U of *Taq* DNA polymerase (Promega, Madison, Wis.), PCR buffer (Promega), 1.5 mM $MgCl_2$, deoxynucleotide triphosphates each at a final concentration of 0.2 mM, 12.5 pmol of each primer, and 10 to 100 ng of template DNA. Typical cycling parameters were 1 min of denaturation at 94°C, 1 min of annealing at 50°C, and 1.5 min of extension at 72°C for 30 cycles. The initial denaturation step was extended to 4 min, and the final extension step was extended to 10 min.

The primers used in this study are listed in Table 2. The primers for the 16S rRNA gene were designed by comparing the 16S rRNA gene sequences of *Mycobacterium* species with the *E. coli* 16S rRNA gene sequence (GenBank accession no. J01859). Primers 246, 414, 266, and 1522 were designed by Böddinghaus et al. (2), while primers My1 and 614 were designed by us. The primers for the *hsp65* gene were designed on the basis of the sequence of *M. tuberculosis* published by Shinnick (26). Primers TB11 and TB12 were designed by Telenti et al. (31), primer M2 was designed by Plikaytis et al. (21), and primers HSF1, HS12F, HS4F, and HSR1were designed by us.

TABLE 2. PCR primers

Gene	Primer	Position[a]	Sequence[b]
16S rRNA	246[c]	1–20	F 5′-AGA GTT TGA TCC TGG CTC AG-3′
	My1	61–78	F 5′-GGA AAG GTC TCT TCG GAG-3′
	414[c]	200–216	R 5′-CAT CCC ACA CCG C**W**A AAG-3′
	266[c]	593–612	R 5′-CAC GC**Y** CAC AGT TAA GC**Y** GT-3′
	614	593–614	F 5′-CTT AAC TGT GAG CGT GCG-3′
	1522[c]	1503–1522	R 5′-AAG GAG GTG ATC CAG CCG CA-3′
hsp65	HSF1	1–21	F 5′-GAT CCG GAG GAA TCA CTT CGC-3′
	TB11[d]	167–186	F 5′-ACC AAC GAT GGT GTG TCC AT-3′
	TB12[d]	588–607	R 5′-CTT GTC GAA CCG CAT ACC CT-3′
	HS12F	588–607	F 5′-AGG GTA TGC GGT TCG ACA AG-3′
	HS4F	1079–1099	F 5′-ACA GCG ACT CCG ACT ACG ACC-3′
	M2[e]	1367–1383	R 5′-TTG AAG GCG ATC TGC TT-3′
	HSR1	1628–1648	R 5′-TCA GAA ATC CAT GCC ACC CAT-3′

[a] According to GenBank accession no. AF456238 for the 16S rRNA gene and accession no. AF456468 for the *hsp65* gene.
[b] F, forward, R, reverse. Bold letters in primers sequences represent the following degeneracy: W, A or T; Y, C or T.
[c] Designed by Böddinghaus et al. (2).
[d] Designed by Telenti et al. (31).
[e] Designed by Plikaytis et al. (21).

TABLE 3. *Mycobacterium* sp. sequences available in the GenBank database compared to the sequences of our isolates

Gene study	Isolate	Origin	Cluster[a]	GenBank accession no.
16S rRNA	*M. marinum*	Unknown[b]; identical to strain ATCC 927	C	X52920
	M. marinum	Rainbow fish (*Melanotaenia praecox*); Taiwan	C	AF251565
	Mycobacterium sp. strain M175	Striped bass (*Morone saxatilis*); United States	C	AY005147
	M. ulcerans	Human	C	X88926
	M. bovis	Strain BCG	D	M20940
	M. tuberculosis	Human; strain H37Rv	D	Z83862
	M. avium	Human	E	AF306455
	M. avium subsp. *paratuberculosis*	Strain ATCC 19698	E	X52934
	M. chlorophenolicum	Freshwater lake sediment	F	X79094
	Mycobacterium sp. strain Fuerth 1999	Human	G	AF152558
	N. asteroides[c]	Strain ATCC 3306	H	X57949
hsp65	*M. ulcerans*	Human; Australia (isolate 93147172)	CI	AF456475[d]
	M. avium subsp. *paratuberculosis*	Ruminant	E	X74518
	M. avium	Unknown[b]	E	AF281650
	M. tuberculosis	Human; strain H37Rv	D	AL021932
	M. bovis	Strain BCG P3	D	M17705
	N. asteroides[c]	Strain ATCC 3306	H	AF352019

[a] According to the phylogenetic analysis as it appears in Fig. 1 and 2A.
[b] Unspecified source.
[c] Used as an outgroup.
[d] Isolate was kindly provided by A. Sievers, Mycobacterium Reference Laboratory, Fairfield Hospital, Fairfield, Victoria, Australia. It was sequenced and submitted by us to GenBank.

Restriction mapping. A 614-bp PCR product from the 16S rRNA gene (obtained with primer set 246-266) and a 796-bp PCR product from the *hsp65* gene (obtained with primer set HS12F-M2) were purified by using a QIAquick PCR purification kit (Qiagen, Hilden, Germany) and digested with restriction enzyme *Msl*I or *Dde*I (New England Biolabs, Beverly, Mass.), respectively. The digestion was carried out according to the manufacturer's recommendations, except for the *Msl*I reaction, which was extended overnight at room temperature in order to ensure a complete cut. Israeli and foreign isolates were selected as representative of geographically distant regions (see Fig. 3). A 100-bp molecular ruler (Bio-Rad, Hercules, Calif.) was used as a DNA size marker.

DNA sequencing. Double-stranded PCR products were purified by using a QIAquick PCR purification kit. Double-stranded DNA templates (50 ng) were sequenced by using an automated DNA sequencer (Perkin-Elmer model 377) and the Big Dye Deoxy Terminator cycle sequencing enzyme at the DNA Sequencing Biological Services Unit, Weizmann Institute, Rehovot, Israel. Gene-specific primers (Table 2) yielded the complete sequences of both DNA strands and confirmed reading accuracy.

Twenty-one *Mycobacterium* fish isolates (and the isolate from the sea turtle) were selected for 16S rRNA (1,522 bp) sequencing, and 17 fish isolates were selected for *hsp65* (1,648 bp) sequencing.

Phylogenetic analysis. The sequences of all the isolates used in this study were compared with those of several closely related isolates available in the GenBank database (Table 3).

Sequence alignments were obtained by using the ClustalX program (EMBL, Heidelberg, Germany). Phylogenetic trees were constructed by maximum-parsimony analysis with the computer program PAUP 4 (beta7). Bootstrap proportions were used to assess the robustness of the tree with 100 bootstrap replications. Numbers on the branches (Fig. 1 and 2) indicate bootstrap proportions. The *Nocardia asteroides* 16S rRNA or hsp65 sequences were used as outgroups while constructing the respective phylogenetic trees.

Nucleotide sequence accession numbers. All the sequences obtained from the 16S rRNA gene and the *hsp65* gene were submitted to the GenBank database under the accession numbers listed in Table 1.

RESULTS

16S rRNA phylogenetic analysis. The phylogenetic tree constructed from the 16S rRNA gene sequences (Fig. 1) revealed eight distinct groups (groups A to H). All the Israeli isolates (marine and freshwater) clustered together in groups A and B and were therefore designated the Israeli type.

M. marinum marine isolates from wild Red Sea fish as well as fish farmed along both the Red Sea and the Mediterranean Sea coasts of Israel all shared an identical nucleotide sequence (group A) different from that of reference strain ATCC 927 (group C) by five bases (99.7% homology). The three Greek isolates from cultured Mediterranean Sea fish were different from each other: the first isolate (DL041200) shared an identical nucleotide sequence with the Israeli marine type, while the second isolate (DL045) was found to differ from it by only one base out of 1,522 bp (99.93% homology). Both clustered in group A. The third Greek isolate (DL049) had only 96.8% homology with the other two isolates and belonged to group F.

The two Israeli isolates from farmed freshwater fish species (koi [*Cyprinus carpio*] and silver perch [*Bidyanus bidyanus*]) shared an identical nucleotide sequence (group B), which differed by five bases from the sequence of the Israeli marine isolates in group A (99.7% homology) and by four bases from the sequence of ATCC 927 in group C (99.7% homology).

A sea bass (*Dicentrarchus labrax*) isolate from Denmark (DLDK), two snakehead (*Channa striatus*) isolates from Thailand (S4 and S267), one rainbow fish (*Melanotaenia praecox*) isolate from Taiwan (GenBank accession no. AF251565), one *M. marinum* isolate from an unspecified source in Germany (GenBank accession no. X52920), and an *M. ulcerans* isolate from a human infection (GenBank accession no. X88926) all shared high sequence homology to complete identity (99.8 to 100%) with ATCC 927 and clustered in group C.

Group G includes isolates from golden sturgeon (*Acipenser baeri*) from Holland (isolates 185408 and 185409), which exhibit 100% identity with *Mycobacterium* sp. strain Fuerth 1999 (GenBank accession no. AF152558), a clinical isolate from a human infection from an unspecified location.

***hsp65* phylogenetic analysis.** The tree constructed from the *hsp65* gene sequences (Fig. 2A) revealed 10 distinct groups. The marine and freshwater Israeli type isolates (groups A and

B) showed the same clustering pattern as in the 16S rRNA tree and differed from each other by four bases (99.7% homology). They differed from strain ATCC 927 by nine bases (99.4% homology) and seven bases (99.6% homology), respectively.

The three Greek isolates showed the same clustering pattern as well: isolates DL041200 and DL045 shared an identical nucleotide sequence with the marine Israeli type isolates and clustered in group A, while the third isolate (DL049) belonged to group F.

In contrast to what was seen with the tree constructed from the 16S rRNA gene sequences, group C was further divided into three subgroups, which included *M. ulcerans* (subgroup CI) (99.4% homology with ATCC 927), the isolate from sea bass from Denmark (isolate DLDK) and ATCC 927 (subgroup CII) (100% homology), and the isolates from snakehead from Thailand (subgroup CIII) (99.6% homology with ATCC 927). The isolates from golden sturgeon from Holland (isolates 185408 and 185409) clustered in group G.

A comparison of the deduced sequences of the amino acids encoded by the *Mycobacterium hsp65* gene is shown in Fig. 2B. All Israeli isolates, marine and freshwater, and two of the Greek isolates clustered in one group (group A). *M. ulcerans* belonged to group B, while the isolate from sea bass from Denmark (isolate DLDK), snakehead from Thailand (isolates S4 and S267), and ATCC 927 clustered together in group C. The isolates from golden sturgeon from Holland (isolates 185408 and 185409) clustered in group F, while the Greek isolate from sea bass (isolate DL049) belonged to group G.

The sequences of the Israeli type isolates (marine and freshwater) differed from the sequences of all published *Mycobacterium* spp. in both genes. Consequently, a new name, *M. marinum* strain Eilaticum DL240490, was proposed for the marine isolates (GenBank accession no. AF456238 for the 16S rRNA gene sequence and AF456468 for the *hsp65* gene sequence). The Israeli freshwater isolates were named *M. marinum* strain *Cyprinum* CC240299 (GenBank accession no. AF456239 for the 16S rRNA gene sequence and AF456469 for the *hsp65* gene sequence).

Restriction enzyme mapping. Based on the sequence information obtained from both genes, a two-step restriction map was drawn (Fig. 3B and D). *Msl*I digestion of a 614-bp PCR product from the 16S rRNA gene yielded cleavage products of 80 and 534 bp, which separated the Israeli type isolates from the other selected isolates, as they have a unique site for the enzyme (Fig. 3A, lanes 1 to 4). This group was further characterized by *Dde*I digestion of a 796-bp PCR product from the *hsp65* gene, which yielded cleavage products of 182 and 614 bp. Having a unique site for this enzyme, the freshwater isolates could be differentiated from the marine isolates (Fig. 3C).

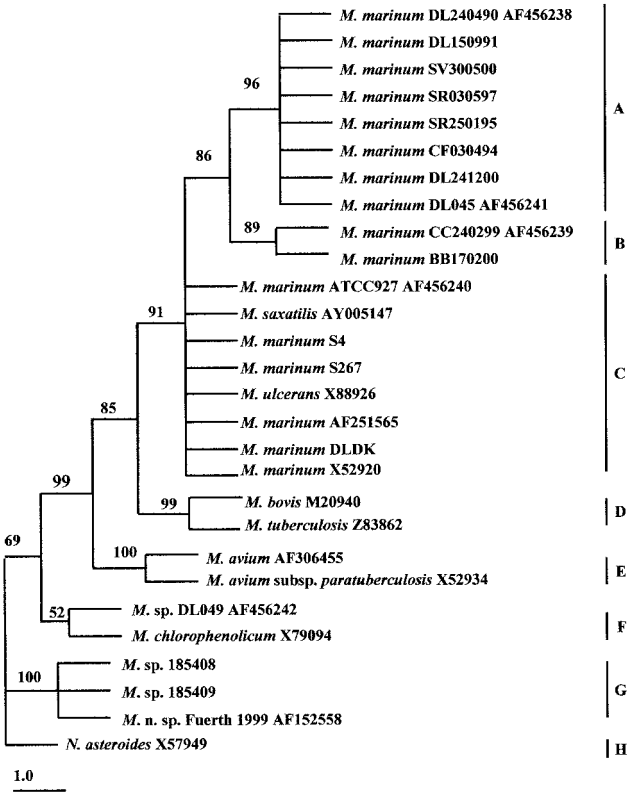

FIG. 1. Phylogenetic relationships among *Mycobacterium* species derived from the 16S rRNA gene sequences, as constructed by maximum-parsimony analysis. The tree was rooted by using *N. asteroides* as the outgroup. Numbers on the branches indicate bootstrap proportions (100 replicates). Bar, 1.0 nucleotide replacement. Available GenBank accession numbers are shown.

DISCUSSION

Our results show that the combined analysis of both the 16S rRNA gene and the protein-encoding *hsp65* gene of *M. marinum* increases the detection of intraspecific variations.

Direct sequencing of the 16S rRNA and *hsp65* genes confirmed the identities of all the Israeli isolates that were characterized in this study as *M. marinum*. Phylogenetic analysis based on the 16S rRNA and *hsp65* gene sequences clustered the Israeli type *Mycobacterium* isolates, discriminating this type from all other *Mycobacterium* isolates found in the literature. Site-specific nucleotide differences revealed that in the last decade, all *M. marinum* isolates in Israel have belonged to two strains, one that has been infecting marine fish (both cultured and wild) and one that has been infecting freshwater fish (cultured).

Bacterial strains have been considered to be within the same species if they have fewer than 5 to 15 base differences in the 16S rRNA gene (10). For mycobacteria, a difference of less than 5 nucleotides within the complete 16S rRNA gene, along with clear phenotypic differences, is supposed to indicate a genetically unique and distinct taxon (34). Accordingly, *M. marinum* and members of the *M. tuberculosis* complex lie on close branches (25, 33). However, *M. marinum* shares over 99.8% homology with *M. ulcerans*, with only two nucleotide differences (22, 33). In fact, Tønjum et al. (33) pointed out that the 16S rRNA gene is not useful for discriminating among *M. marinum*, *M. ulcerans*, and *M. haemophilum*. However, the diseases caused by *M. marinum* and *M. ulcerans* in humans differ greatly in their clinical, histopathologic, and epidemiologic aspects (3, 29).

The sequences of the *hsp65* gene discriminated among *M. ulcerans*, *M. marinum* ATCC 927 (reference strain), and *M. marinum* from the Far East (Thailand; group C; GenBank

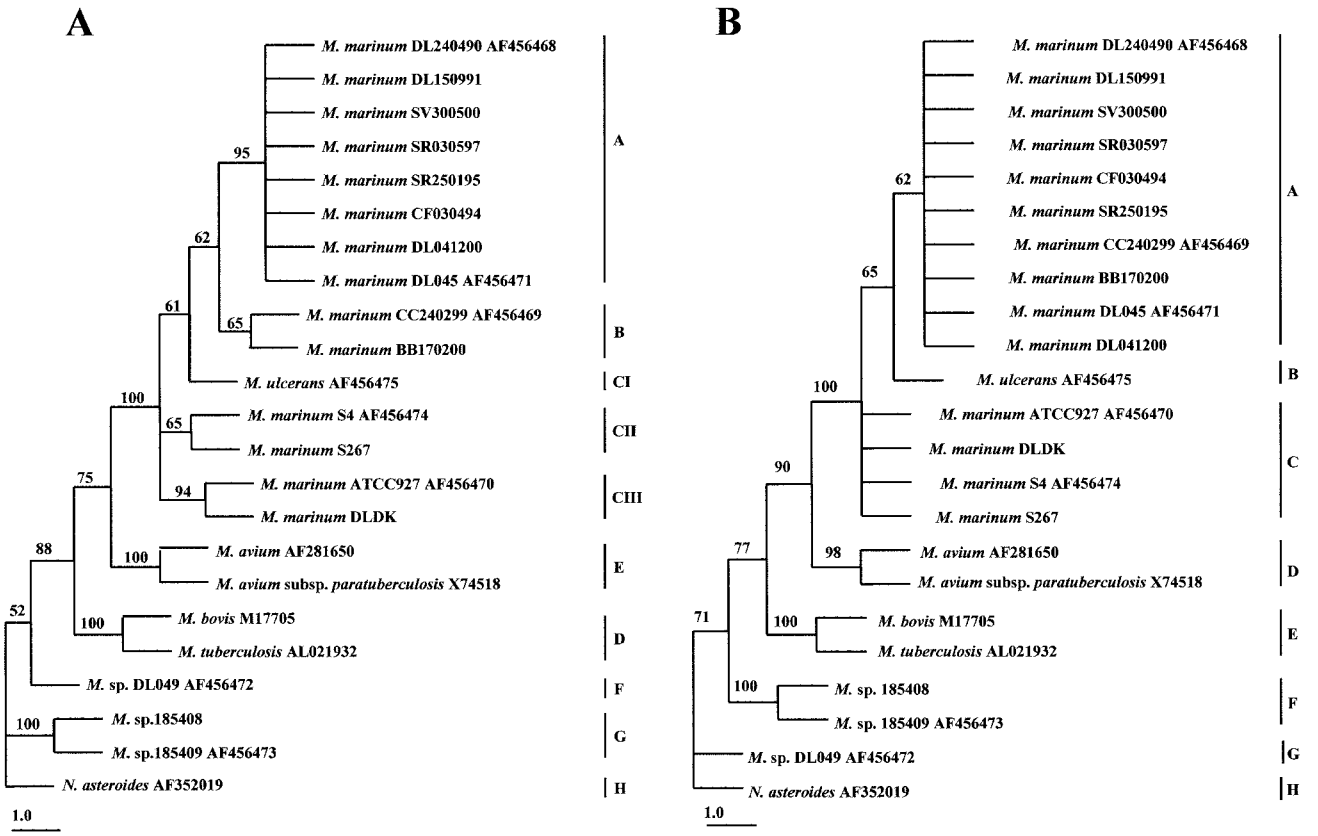

FIG. 2. Phylogenetic relationships among *Mycobacterium* species derived from the *hsp65* gene sequences (A) and the deduced sequences of the amino acids encoded by the *Mycobacterium hsp65* gene (B), as constructed by maximum-parsimony analysis. The tree was rooted by using *N. asteroides* as the outgroup. Numbers on the branches indicate bootstrap proportions (100 replicates). Bar, 1.0 nucleotide or amino acid replacement. Available GenBank accession numbers are shown.

accession no. AF456474), which could not be distinguished with the 16S rRNA gene sequences. Whole-genome techniques, such as IS*2404* restriction fragment length polymorphism analysis and amplified fragment length polymorphism analysis, clustered *M. ulcerans* and *M. marinum* into separate groups as well (5).

In this study, the restriction enzyme map based on the combination of the two gene (16S rRNA and *hsp65*) sequences enabled us to differentiate not only the Israeli type isolates from all other *M. marinum* isolates but also the Israeli marine isolates from the Israeli freshwater isolates. PCR-restriction fragment length polymorphism analysis of the *hsp65* gene (31), although an established discriminating tool used with mycobacteria by many laboratories (4, 8, 24), is not applicable to the *M. marinum* complex, as different strains have the same restriction sites. This problem was thus overcome with our method.

Our sequence of the 16S rRNA gene of *M. marinum* ATCC 927 (reference strain) (GenBank accession no. AF456240) is identical to that published by Rogall et al. (25) (GenBank accession no. X52920) and is in contrast (93 to 98% homology) to the partial sequence published by Talaat et al. (30) (GenBank accession no. U92088). Also, the 16S rRNA gene sequence of the isolates from golden sturgeon (*A. baeri*) from Holland (isolates 185408 and 185409) exhibited 100% identity with that of *Mycobacterium* sp. strain Fuerth 1999 (GenBank accession no. AF152558), whose sequence was in turn found by Turenne et al. (34) to be identical to that of *M. chelonae* ATCC 19237.

Because there were no records of *M. marinum* in the Red Sea before the report by Colorni (7) for sea bass (*D. labrax*) and because of the active fish trade between Israel and other Mediterranean countries in recent years (in particular, for fingerlings hatched in one region to be reared in another), it had been assumed that the pathogen was introduced into Israel via mariculture operations and had then spread to other farmed and native species in the Gulf of Eilat (A. Diamant and A. Colorni, Abstr. Proc. 7th Int. Conf. Eur. Assoc. Fish Pathol., p. 66, 1995). This hypothesis was supported by the facts that acid-fast bacteria, some of which were specifically identified as *M. marinum*, had already been detected in *D. labrax* in Europe (12; W. Verdonck, L. Lambrechts, and F. Ollevier, Abstr. Proc. 2nd Int. Colloq. Pathol. Mar. Aquacult., p. 149, 1986; S. Mellergaard and I. Dalsgaard, Abstr. 6th Int. Conf. Eur. Assoc. Fish Pathol., p. 60, 1993) and that disease in Israel was first detected in offspring of *D. labrax* which originated in France. While our results show that no linkage exists between the Israeli type and the northern European *M. marinum* isolates, the hypothesis that Israeli marine *M. marinum* was nevertheless imported from Europe received support by the identity of one of the Greek isolates to the Israeli marine isolates. On the other hand, the fact that in the last decade, the same strain of

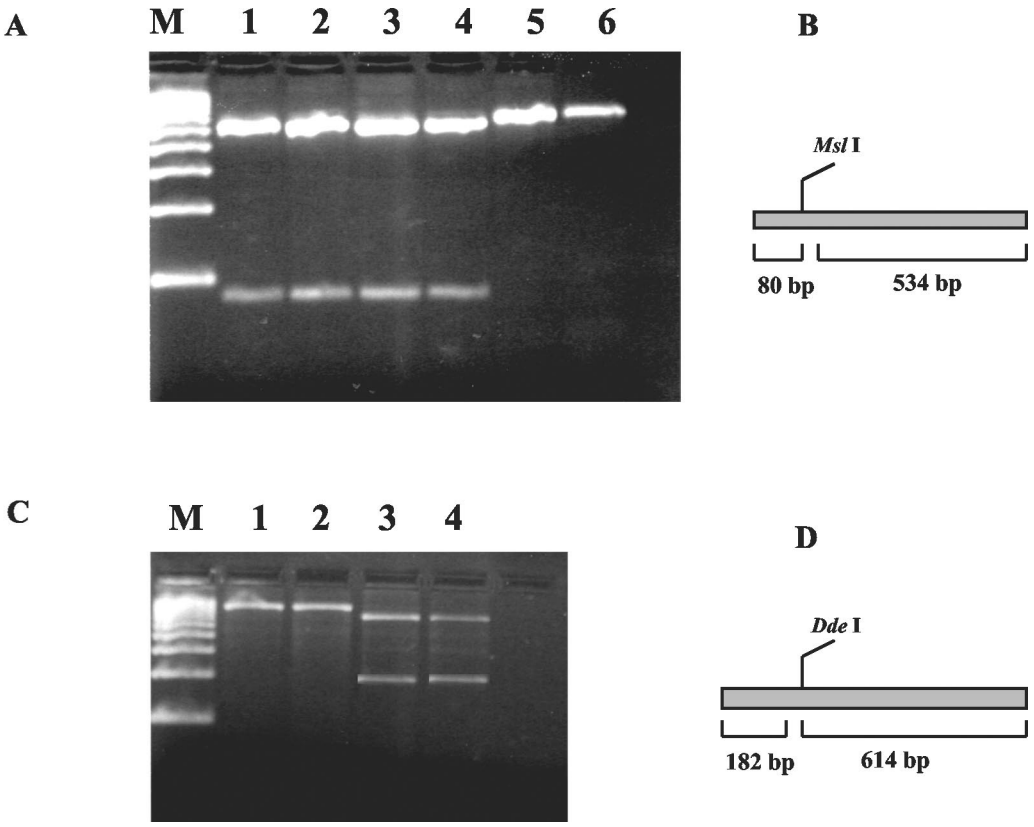

FIG. 3. Enzyme restriction mapping. (A and B) A 614-bp PCR product from the 16S rRNA gene cleaved with restriction enzyme *Msl*I. (A) Gel separation. (B) Restriction map. (C and D) A 796-bp product from the *hsp65* gene cleaved with restriction enzyme *Dde*I. (C) Gel separation. (D) Restriction map. Lanes: M, 100-bp molecular size marker; 1, DL240490; 2, SR030597; 3, CC240299; 4, BB170200; 5, ATCC 927; 6, DLDK.

M. marinum, different from the freshwater isolates, infected all the Israeli marine fish species as well as a captive hawksbill sea turtle in the Red Sea may indicate that a local, Red Sea strain of *M. marinum* exists and found extremely susceptible hosts in the exotic species (mainly sea bass) imported for aquaculture. In fact, our results suggest that every isolate of *M. marinum* examined is endemic, with a unique genotype specific and circumscribed to its geographic region. Interestingly, Portaels et al. (23) and Chemlal et al. (6) have similarly shown for *M. ulcerans* that the polymorphisms in the 3′ end of the 16S rRNA gene sequence and in the IS*2404* restriction fragment length polymorphism pattern, respectively, are related to their geographic origins (Australia, Africa, and Central America). Further characterization of *M. marinum* isolates from additional geographic regions may clarify whether this is indeed the case.

ACKNOWLEDGMENTS

This work was supported by the Israeli Ministry of National Infrastructures (grant 4328-3-96) and Yad haNadiv (grant 5245.12).

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