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Globally Distributed Mycobacterial Fish Pathogens Produce a Novel Plasmid-Encoded Toxic Macrolide, Mycolactone F▽

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***Mycobacterium ulcerans* and *Mycobacterium marinum* are closely related pathogens which share an aquatic environment. The pathogenesis of these organisms in humans is limited by their inability to grow above 35°C. *M. marinum* causes systemic disease in fish but produces localized skin infections in humans. *M. ulcerans* causes Buruli ulcer, a severe human skin lesion. At the molecular level, *M. ulcerans* is distinguished from *M. marinum* by the presence of a virulence plasmid which encodes a macrolide toxin, mycolactone, as well as by hundreds of insertion sequences, particularly IS*2404*. There has been a global increase in reports of fish mycobacteriosis. An unusual clade of *M. marinum* has been reported from fish in the Red and Mediterranean Seas and a new mycobacterial species, *Mycobacterium pseudoshottsii*, has been cultured from fish in the Chesapeake Bay, United States. We have discovered that both groups of fish pathogens produce a unique mycolactone toxin, mycolactone F. Mycolactone F is the smallest mycolactone (molecular weight, 700) yet identified. The core lactone structure of mycolactone F is identical to that of *M. ulcerans* mycolactones, but a unique side chain structure is present. Mycolactone F produces apoptosis and necrosis on cultured cells but is less potent than *M. ulcerans* mycolactones. Both groups of fish pathogens contain IS*2404*. In contrast to *M. ulcerans* and conventional *M. marinum*, mycolactone F-producing mycobacteria are incapable of growth at above 30°C. This fact is likely to limit their virulence for humans. However, such isolates may provide a reservoir for horizontal transfer of the mycolactone plasmid in aquatic environments.**

Mycobacterium marinum is a globally distributed pathogen of marine and freshwater fish which also causes skin infections in humans (7, 9). *M. marinum* is phenotypically distinguished from other mycobacteria by its low optimal growth temperature, light-induced carotenoid production, and relatively rapid growth rate compared to other slow-growing *Mycobacterium* species. There is considerable heterogeneity among *M. marinum* isolates, and several subgroups have been described (28, 33–35).

Mycobacteriosis was first diagnosed in fish from the Red Sea in 1990 (5). The infection was initially found in cultured sea bass (*Dicentrarchus labrax*) in Eilat and has since been found in over 20 different fish species and a hawksbill sea turtle. The Red Sea isolates differed phenotypically from other *M. marinum* strains by being scotochromogenic (having constitutive pigment production). Whereas most *M. marinum* strains form colonies on mycobacterial media within 8 days, initial growth was not obtained from these isolates for at least 2 weeks. Similar isolates have also been found in the Mediterranean Sea in Greece and Italy. Molecular characterization of the Israeli isolates from fish confirmed their identity as *M. marinum*, but analysis of the 16S rRNA gene showed that the isolates formed clades within the species (33, 34). Molecular comparison of the fish isolates with human isolates of *M. marinum* from Israel showed that human and fish isolates fell into discrete groups based on 16S rRNA, *hsp65*, and AFLP patterns (34). Human isolates were designated A strains, whereas fish isolates were placed in the B group. Within group B, marine isolates (B1) could be distinguished from freshwater isolates (B2). A growing number of similar *M. marinum* strains have been isolated from marine fish in various locations along the Mediterranean coast (Italy, Israel, and Greece) as well as from fish from other aquatic environments in Israel.

The Red Sea *M. marinum* strain is the only mycobacterial species detected in the marine environment in Israel and is associated with unusually severe pathology. Though cutaneous lesions are not always present, the spleens and kidneys of diseased fish are riddled with granulomas containing massive numbers of acid-fast bacilli (Fig. 1). The Israeli marine strain was first isolated from cultured sea bass (*Dicentrarchus labrax*), but many Red Sea species appear to be susceptible to infection (5, 8, 33).

Newly emerging mycobacterial fish pathogens from the Chesapeake Bay in the United States have also been reported (15, 25). In 2005 a new mycobacterial species, *Mycobacterium pseudoshottsii*, was described in association with epizootic fish disease among striped bass (*Morone saxatilis*) in the Chesapeake Bay, Maryland. Molecular analysis showed that the 16S rRNA gene from *M. pseudoshottsii* was greater than 99% similar to that of an Israeli marine *M. marinum* isolate, DL240490

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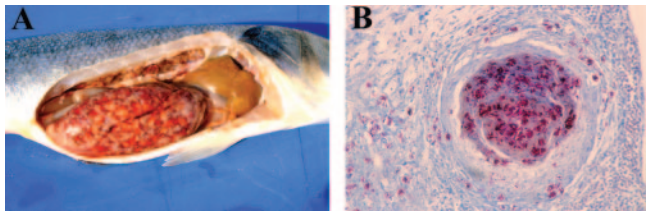

FIG. 1. Pathology showing infection with *M. marinum* DL240490 in sea bass (*Dicentrarchus labrax*) from an outbreak of mycobacterial disease in the Red Sea. (A) Severe splenomegaly and granulomatous spleen and kidney. (B) Massive load of acid-fast bacilli (Ziehl-Neelsen staining) within a splenic granuloma (total magnification, ×100).

(25). *M. pseudoshottsii* was reported to be closely related to *M. marinum*, to the human pathogen *Mycobacterium ulcerans*, and to a newly described frog pathogen, *Mycobacterium liflandii* (24). It was of particular interest that *M. pseudoshottsii*, like *M. liflandii*, contained an insertion sequence, IS*2404*, previously found only in *M. ulcerans* and in an atypical *M. marinum* strain isolated from a human patient (25, 30).

DNA-DNA hybridization and multilocus sequence analysis show that *M. marinum* is extremely closely related to *M. ulcerans* (28, 31). The two species share 99.8% identity within the 16S rRNA gene, and a growing body of evidence suggests that *M. ulcerans* evolved relatively recently from an *M. marinum* ancestor (29). Two major events have been associated with the evolution of *M. ulcerans*. One is acquisition of a plasmid-encoded macrolide toxin, mycolactone, and the second is major genome remodeling associated with insertion of over 300 copies of two insertion sequences, IS*2404* and IS*2606.* Acquisition of these IS elements has created a large number of pseudogenes in *M. ulcerans*, leading to the hypothesis that *M. ulcerans* is adapting to a specialized niche (http://genopole.pasteur.fr/Mulc/BuruList.html).

Despite the close taxonomic relationship, *M. marinum* and *M. ulcerans* produce distinctly different diseases (7, 9). Whereas *M. marinum* causes a granulomatous intracellular infection in fish and humans, *M. ulcerans* produces an extracellular infection, Buruli ulcer, characterized by severe tissue destruction and the presence of few inflammatory cells at the site of infection (2, 6, 20, 36). Genetic evidence with mycolactone-negative mutants suggests that mycolactone is responsible for the unique characteristics of Buruli ulcer (1, 12). Recently a second mycolactone-producing mycobacterial pathogen, *M. liflandii*, was isolated from *Xenopus tropicalis* and *Xenopus laevis* frogs in U.S. laboratories (24, 32). This organism is also closely related to *M. marinum* and *M. ulcerans*.

Mycolactone is a hybrid polyketide composed of a lactone core and a fatty acid side chain (14). All strains of *M. ulcerans* produce a major mycolactone species along with several minor congeners (4, 16, 23). The first mycolactones were isolated from a Malaysian isolate of *M. ulcerans* as a 3:2 mixture of two stereoismers designated mycolactone A/B (14). Since then it has been shown that all African and Malaysian isolates of *M. ulcerans* produce identical molecules. However, there is heterogeneity in the mycolactones produced by *M. ulcerans* from different geographic areas. *M. ulcerans* strains isolated in Australia produce a different mycolactone, mycolactone C, whereas Asian isolates of *M. ulcerans* produce primarily mycolactone D (23). A novel plasmid-encoded mycolactone, mycolactone E, has been recently identified in the frog pathogen *M. liflandii* (23). The large polyketide synthase genes for mycolactone biosynthesis comprise a 110-kb gene cluster on a large (154- to 180-kb) plasmid (29). The lactone core structure, encoded by *mlsA*, is conserved in all mycolactones. Molecular diversity has arisen through modifications of the fatty acid side chain structure encoded by *mlsB*.

Despite the structural heterogeneity, all mycolactone species produce apoptosis and necrosis in cultured cells (1, 13, 26). Mycolactone A/B is the only mycolactone evaluated in vivo. In guinea pig and mouse infection models as well as in human infection, mycolactone A/B causes apoptotic cell death as well as death via necrosis (1). In addition, in humans and guinea pigs, mycolactone is associated with immunosuppression (36, 37).

The close taxonomic relationship of an Israeli *M. marinum* isolate (DL240490) to *M. pseudoshottsii*, the relationship of these species to *M. ulcerans*, and the shared association with aquatic environments led us to investigate further the relationship of *M. pseudoshottsii* and Israeli fish isolates to *M. ulcerans*. Results from these studies show that *M. marinum* strains isolated from fish in several locations in the Mediterranean and Red Seas as well as *M. pseudoshottsii* strains isolated from fish in the Chesapeake Bay produce a plasmid-encoded mycolactone. Despite the wide geographic separation of mycolactone-producing *M. marinum* and *M. pseudoshottsii*, both groups of organisms produce an identical mycolactone, designated mycolactone F.

MATERIALS AND METHODS

Histology. Fish were trapped, euthanatized, and dissected within 48 h. Dissected tissue was fixed with buffered neutral formalin, dehydrated in ethanol, and embedded in paraffin. Paraffin blocks were sectioned and stained with Ziehl-Neelsen stain as described previously (8).

Strains and growth conditions. Strains and their sources are shown in Table 1. All isolates were grown on Middlebrook 7H10 agar medium supplemented with 10% oleic acid, dextrose, and albumin supplement or on Bordet-Gengou medium. *M. ulcerans* strains were incubated at 32°C. All other isolates were grown at room temperature (RT). For determination of optimal growth temperature, mycobacterial isolates were grown at 32°C and RT (23 to 25°C). For determination of pigment production, isolates were grown initially in the dark and exposed to light for 2 h. Plates were subsequently returned to the dark to prevent degradation of mycolactones.

Mycolactone isolation. Lipids were extracted from *M. ulcerans* with chloroform-methanol (2:1, vol/vol), and phospholipids were removed by precipitation with ice-cold acetone to obtain acetone-soluble lipids (ASLs). Purified mycolactone was obtained using centripetal silica chromatography with a chromatotron as previously described (11, 24). ASLs were analyzed by thin-layer silica chromatography using the solvent system chloroform-methanol-water (90:10:1, vol/vol/vol) and visualized by charring with ceric sulfate-ammonium molybdate in 2 M sulfuric acid (24).

Cell culture and cytopathicity assays. L929 mouse fibroblasts (ATCC CCL1) were purchased from the American Type Culture Collection and maintained in the lab in Dulbecco's minimal Eagle medium with 5% fetal calf serum and 1× L-glutamine in tissue culture flasks incubated in 5% CO_2 at 37°C as previously described (1). ASL samples or individual lipid species were diluted in tissue culture medium and added to cells in a 96-well tissue culture plate to determine cytopathicity. In addition, intact bacteria and culture filtrate were directly added to cells in serial dilutions to evaluate the cytopathic capacity of surface-exposed and secreted mycolactone. Cytopathicity was defined as the minimal concentration of ASL or mycolactone necessary to produce cell rounding in 24 h and loss of the monolayer by 48 h (1, 11).

PCR. Fifty-microliter reaction mixtures containing 15.5 μl double-distilled water, 25 μl Failsafe PCR 2× PreMix G (Epicenter) (50 mM Tris-HCl [pH 8.3, 22°C], 50 mM KCl, 200 μM of each deoxynucleoside triphosphate, and proprietary amounts of FailSafe PCR enhancer and $MgCl_2$ per reaction), 1 μM of each

TABLE 1. Mycobacterial strains used for this study, grouped by species

Strain	Species	Host	Geographic origin	Reference or source
Agy99	*M. ulcerans*	Human	Ghana	29
1615	*M. ulcerans*	Human	Malaysia	ATCC[a] 35840
V2	*M. ulcerans*	Human	Australia	23[b]
753	*M. shinshuense*	Human	Japan	18
XL5	*M. liflandii*	Frog (*Xenopus laevis*)	University of Virginia	24
L6	*M. pseudoshottsii*	Striped bass (*Morone saxatilis*)	Chesapeake Bay	25
L15	*M. pseudoshottsii*	Striped bass (*Morone saxatilis*)	Chesapeake Bay	25
L21	*M. pseudoshottsii*	Striped bass (*Morone saxatilis*)	Chesapeake Bay	25
L23	*M. pseudoshottsii*	Striped bass (*Morone saxatilis*)	Chesapeake Bay	25
L50	*M. pseudoshottsii*	Striped bass (*Morone saxatilis*)	Chesapeake Bay	25
1218	*M. marinum*[c]	Salt water fish	Philadelphia, Pa.	ATCC 927
M	*M. marinum*[c]	Human	San Francisco, Calif.	ATCC BAA-535
DL/DK1	*M. marinum*[c]	Sea bass (*Dicentrarchus labrax*)	Denmark	33
DL240490	*M. marinum*[d]	Sea bass (*Dicentrarchus labrax*)	Red Sea, Israel	33
DL150991	*M. marinum*[d]	Sea bass (*Dicentrarchus labrax*)	Mediterranean Sea, Israel	33
DL180892	*M. marinum*[d]	Sea bass (*Dicentrarchus labrax*)	Ein Yahav, Israel	34
045 Thalassa	*M. marinum*[d]	Sea bass (*Dicentrarchus labrax*)	Mediterranean Sea, Greece	33
DL241200	*M. marinum*[d]	Sea bass (*Dicentrarchus labrax*)	Mediterranean Sea, Greece	This work
DL300/04	*M. marinum*[d]	Sea bass (*Dicentrarchus labrax*)	Mediterranean Sea, Italy	This work
DL272/05	*M. marinum*[d]	Sea bass (*Dicentrarchus labrax*)	Mediterranean Sea, Italy	This work
CF030494	*M. marinum*[d]	Butterflyfish (*Chaetodon fasciatus*)	Red Sea, Israel	33
SA200695	*M. marinum*[d]	Sea bream (*Sparus aurata*)	Red Sea, Israel	33
Hybrid270995	*M. marinum*[d]	Red sea bream (*Pagrus major* [female] × *Sparus aurata* [male])	Red Sea, Israel	33
SV300500	*M. marinum*[d]	Lizard fish (*Synodus variegatus*)	Red Sea, Israel	33
SV061004	*M. marinum*[d]	Lizard fish (*Synodus variegatus*)	Red Sea, Israel	This work
SR030597	*M. marinum*[d]	Rabbitfish (*Siganus rivulatus*)	Red Sea, Israel	33
CC240299	*M. marinum*[d]	Koi (*Cyprinus carpio*)	Ma'agan Michael, Israel	33
BB170200	*M. marinum*[d]	Silver perch (*Bidyanus bidyanus*)	Dor-Ma'agan Michael, Israel	32

[a] ATTC, American Type Culture Collection.
[b] The strain is named TS-2 in reference 23.
[c] *M. marinum* isolate displaying light-induced pigment production (photochromogenic).
[d] *M. marinum* isolate displaying constitutive pigment production (scotochromogenic).

primer, 1.25 U Failsafe PCR enzyme mix (Epicenter), and 7 μl template derived from whole-cell boiled preparations were prepared. Cycling was performed in a Mastercycler gradient thermal cycler (Eppendorf) as follows: 98°C for 5 min; 35 cycles of 98°C for 1 min, 55°C for 1 min, and 72°C for 1 min; and 72°C for 10 min. Nine microliters of each reaction mixture was analyzed on 1 to 1.5% agarose gels in 1× Tris-acetate-EDTA stained with 1 μg/ml ethidium bromide.

Gene probing included the following: *repA*, 5′-TGGACCGGTCCTCAGTAACC-3′ and 5′-ATCGACGCTCGCTACTTCTG-3′; *mlsA*, as described previously (23); *mlsB*, 5′-CAGCCAACTGCGCTACTACA-3′ and 5′-AGGAGACACGGTTGGCTATG-3′; MUP045, as described previously (23); MUP053, 5′-ACCCACCTCGTCGTTAGTCATG-3′ and 5′-CGCAGAGTTCGAGTATCACGTCTAT-3′; IS*2404* and IS*2606*, as described previously (26); *uhp-1*, 5′-GCACCGACGACGAGCTTCTT-3′ and 5′-GGCCGAGCATCTCAATCTCC-3′; *uhp-2*, 5′-GCGGAGTTCCACATCACGAC-3′ and 5′-ACCGGCTGAGGATCGAATTG-3′; *chp*, 5′-CGATCCACCACGACTTCACG-3′ and 5′-CAATTCCATGTGCGCCTCTG-3′; *zinc*, 5′-GAGCGGGAAATGGTCACTGG-3′ and 5′-GCGCTGTTCCCAATCCTCCT-3′; *uhp-mem*, 5′-ACCCAGGATGAGCGAACACC-3′ and 5′-TGGCTGAGTTTCGGGTCCAT-3′; and *esxA* and *esxB*, as described previously (24).

DNA sequencing. PCR products were cloned into the pCR2.1-Topo vector (Invitrogen) and sequenced using an ABI 3100 automated genetic analyzer (Applied Biosystems, Inc.) and the ABI Big Dye Terminator 3.1 cycle sequencing kit (Applied Biosystems, Inc.).

LDH release and apoptosis. L929 fibroblasts and peripheral blood neutrophils were assayed for cell death via apoptosis and necrosis. Cytotoxicity/necrosis was measured using a colorimetric kit from Promega as described previously (1). Briefly, cells suspended in Dulbecco's minimal Eagle medium supplemented with 5% (fibroblasts) or 10% (neutrophils) fetal calf serum and 1× L-glutamine were seeded in triplicate at a concentration of 10^4 cells/well in a 96-well plate. The release of cytoplasmic lactate dehydrogenase (LDH) from mycolactone-treated (15 ng, 150 ng, 1.5 μg, and 15 μg), permeabilized cells was measured at 24 h postinfection by using the colorimetric kit from Promega according to the manufacturer's instructions. Background release of LDH was obtained by measurement of ethanol-treated cells, and maximum release of LDH was obtained by lysis of untreated cells according to the manufacturer's protocol. The percent LDH release was then determined using the following calculation: [(release of LDH from mycolactone-treated cells − background release from ethanol-treated cells)/(maximum release of LDH by cell lysis − background release)] × 100.

Apoptosis of mycolactone-treated neutrophils and fibroblasts was measured at 24 h posttreatment by using the Cell Death Detection Plus enzyme-linked immunosorbent assay (Roche, Indianapolis, IN) as described previously (1). Apoptosis was then determined as fold enrichment of nucleosomes [(measurement of DNA-histone complex from treated cells/background measurement of untreated cells)].

HPLC and MS. Methanolic extracts of ASLs were analyzed using Shimadzu LC-20AD analytical high-pressure liquid chromatography (HPLC) with UV detection at 360 nm. Separation of various mycolactones was obtained on a Phenomenex-Luna C_{18} 250- by 4.6-mm column at a flow rate of 2 ml/min with a water-acetonitrile gradient (55% acetonitrile for 3 min [isocratic] followed by a 37-min linear gradient to100% acetonitrile). UV-active fractions were analyzed offline on an ion trap ESI Bruker-Esquire mass spectrometer (MS). MS analysis conditions were as follows: dry temperature of 300°C, gas flow of 5 liters/min, and nebulizer pressure of 15 lb/in^2. For preliminary screening, the methanolic extracts of ASLs were directly analyzed without chromatographic separation.

NMR. Two milligrams of previously purified mycolactone F was dissolved in CD_3COCD_3 (500 μl). ^{1}H nuclear magnetic resonance (NMR) and two-dimensional (2D) spectroscopy (COSY) spectra were recorded on a Varian INOVA spectrometer at 500 MHz. Chemical shifts were reported in ppm relative to the residual acetone peak at 2.09 ppm.

Nucleotide sequence accession numbers. *M. pseudoshottsii* L15 mycolactone gene sequences have been deposited in the GenBank database under the following accession numbers: *mlsA* (enoyl reductase), DQ508258; *mlsB* (loading module), DQ508257; and MUP045 (*fabH*-like "joinase"), DQ508256. *M. marinum* DL240490 mycolactone gene sequences have been deposited in the GenBank database under the following accession numbers: *mlsA*, DQ508261; *mlsB*, DQ508260; and MUP045, DQ508259.

RESULTS

Israeli isolates of *M. marinum* from fish exhibit temperature-restricted growth. The fact that *M. marinum* and *M. ulcerans* grow poorly, if at all, above 35°C is thought to limit infection in humans primarily to cutaneous lesions. In hospital laboratories *M. ulcerans* and *M. marinum* are grown at 30 to 32°C. Furthermore, *M. marinum* strains isolated from fish as well as from humans have been shown to grow readily at 30°C (9, 35). In marine veterinary laboratories, however, including that at Eilat, mycobacteria from fish are routinely grown at 23 to 25°C. Also, the fish pathogen *M. pseudoshottsii* has been reported to be unable to grow at 30°C (25). Since the temperature sensitivity of *M. marinum* isolated from fish in Israel had not been reported, we investigated the ability of these strains to grow above 25°C. *M. ulcerans*, *M. marinum* 1218, *M. marinum* DL/DK1, and *M. marinum* M grew readily at 32°C, a temperature consistent with the growth temperature used in clinical laboratories. The frog pathogen *M. liflandii* also grew at 32°C on M7H10 with oleic acid-albumin-dextrose complex. However, *M. marinum* isolated from fish from the Red and Mediterranean Seas showed more severe temperature-restricted growth. For these isolates, no growth was observed after 3 months of incubation at 32°C. The two freshwater isolates from Israel, CC240299 and BB170200, produced very scant growth at 32°C, suggesting that the growth temperature ranges of these two isolates may be broader than those of the Red Sea isolates. None of the five *M. pseudoshottsii* isolates tested produced growth at 32°C.

The generation time of *M. marinum* is reported to be 4.5 h, and colonies can be detected on solid medium within 7 to 10 days. *M. marinum* 1218 (fish, United States), *M. marinum* DL/DK1 (fish, Denmark), and *M. marinum* M (human, United States) formed colonies by 8 days at 32°C. However, *M. marinum* isolates from the Red and Mediterranean Seas did not produce colonies for at least 14 days at RT. *M. ulcerans*, *M. liflandii*, and *M. pseudoshottsii* required 4 weeks of incubation at their optimal temperatures (32°C, 32°C, and 23 to 25°C, respectively).

Pigment production in Israeli isolates is variable among strains and is constitutive. Most *M. marinum* strains produce large quantities of carotenoid pigments upon exposure to light. Pigment production was variable among Israeli and other Mediterranean strains tested. All of the 11 saltwater isolates tested were deep yellow, consistent with carotenoid production. However, pigment production occurred constitutively without light induction (i.e., these strains were scotochromogenic). Colonies from freshwater isolates CC240299 and BB170200 and two isolates from Italy, DL300/04 and DL272/05, were very pale yellow. Analysis of chloroform-methanol extracts by thin-layer chromatography (TLC) revealed the presence of a major lipid species with a retention factor (R_f) of 0.71 in all strains which produced a deep-yellow pigment consistent with carotenoid production (data not shown).

PCR analysis of *M. marinum* and *M. pseudoshottsii* for the presence of mycolactone- and *M. ulcerans*-specific genes. All Israeli and Mediterranean Sea isolates of *M. marinum* as well as *M. pseudoshottsii* were positive for all mycolactone-associated genes except MUP053, suggesting that mycolactones from these strains would not have a hydroxyl at C-12 on the fatty acid side chain. DNA sequence comparisons of *mlsA*, *mlsB*, and MUP045 from mycolactone-producing *M. marinum* (MPMM) and *M. pseudoshottsii* with those from *M. ulcerans* revealed greater than 99% homology for all three genes. In contrast, these sequences could not be amplified from two *M. marinum* fish isolates, DL/DK1 (Europe) and 1218 (United States) or from the genome sequence strain for *M. marinum* (strain M). *M. marinum* isolates obtained from human patients in Israel (34) were PCR negative for all mycolactone-related genes.

The availability of the *M. ulcerans* and *M. marinum* genome sequence made it possible to further characterize MPMM and *M. pseudoshottsii* isolates with respect to *M. ulcerans*- and *M. marinum*-specific genes (T. P. Stinear et al., unpublished data). A number of *M. ulcerans* genes, including the insertion sequences IS*2404* and IS*2606* as well as a few chromosomally encoded loci, are present in the *M. ulcerans* genome strain, Agy99, but absent in the *M. marinum* genome strain, strain M. A further distinction between *M. ulcerans* and *M. marinum* is that most *M. ulcerans* strains have undergone a 2.8-kb deletion in RD1 (24), a region first defined in *Mycobacterium tuberculosis* (17, 21) which encodes a putative secretion apparatus required for secretion of the small antigenic proteins Esat6 (*esxA*) and Cfp10 (*esxB*). Esat6 and Cfp10 play an important role in the granulomatous response to *M. marinum* and *M. tuberculosis*, and the absence of these genes in most *M. ulcerans* strains may contribute to the diminished granulomatous response to *M. ulcerans* infections.

IS*2404* was detected in all MPMM and *M. pseudoshottsii* isolates tested and was absent from the *M. marinum* 1218 and *M. marinum* DL/DK1 isolates as well as the M strain. In contrast, IS*2606* was not detected in the two MPMM isolates, CC240299 and BB170200, obtained from freshwater fish but was detected in all marine MPMM isolates. The majority of "*M. ulcerans*-specific" genes were detected in MPMM and *M. pseudoshottsii* isolates as well as in *M. liflandii* (Table 2). However, an *M. ulcerans*-specific hypothetical membrane protein gene, *uhp-mem*, was lacking in all isolates of MPMM and *M. pseudoshottsii*, and *uhp-2*, an *M. ulcerans*-specific phage sequence, was absent from *M. liflandii* and MPMM CC240299 and BB170200. Finally, *esxA* and *esxB* were present in MPMM and *M. pseudoshottsii*. The presence of *esxA* and *esxB* in MPMM and *M. pseudoshottsii* might provide insight into the evolutionary relationship between these isolates and *M. ulcerans*.

MPMM and *M. pseudoshottsii* produce a unique mycolactone. ASLs from 2:1 chloroform-methanol extracts of bacterial pellets were analyzed by silica thin-layer chromatography for the presence of polar lipid species typical of mycolactones. In a chloroform-methanol-water (90:10:1, vol/vol/vol) solvent system, *M. ulcerans* mycolactone A/B has an R_f of 0.29, whereas *M. liflandii* mycolactone E has an R_f of 0.53. Silica TLC analysis of lipids from 6 *M. pseudoshottsii* isolates and 15 MPMM isolates showed that ASLs from all strains contained a putative mycolactone species with an R_f of 0.43 (data not shown). Cytopathicity assays using L929 cells showed that this lipid species caused cell rounding after 24 h and detachment at 48 h at a concentration of less than 100 ng/ml, a phenotype consistent with mycolactone activity. Cytopathicity was associated with this lipid species in each strain tested. Addition of approximately 10^4 intact bacteria also produced this cytopathic effect,

TABLE 2. PCR results for other genes common to *M. ulcerans* and/or *M. marinum*

Species or control	Strain	Insertion sequences		Chromosomal genes of *M. ulcerans* but not *M. marinum*[a]					Rd1 genes[b]	
		IS*2404*	IS*2606*	*uhp-1*[c]	*uhp-2*[c]	*chp*[c]	*zinc*[c]	*uhp-mem*[d]	*esxA*	*esxB*
M. ulcerans	Agy99	+	+	+	+	+	+	+	−	−
	1615	+	+	+	+	+	+	+	−	−
	V2	+	+	+	+	+	+	+	−	−
	753	+	+	−	−	+	+	+	+	+
M. liflandii	XL5	+	+	+	−	+	+	−	+	+
M. pseudoshottsii	L15	+	+	+	+	+	+	−	+	+
M. marinum	1218	−	−	−	−	−	−	−	+	+
	DL/DK1	−	−	−	−	−	−	−	+	+
	DL240490[e]	+	+	+	+	+	+	−	+	+
	DL150991[e]	+	+	+	+	+	+	−	+	+
	DL180892[e]	+	+	+	+	+	+	−	+	+
	045 Thalassa[e]	+	+	+	+	+	+	−	+	+
	CF030494[e]	+	+	+	+	+	+	−	+	+
	SA200695[e]	+	+	+	+	+	+	−	+	+
	Hybrid270995[e]	+	+	+	+	+	+	−	+	+
	CC240299[f]	+	−	+	−	+	+	−	+	+
	BB170200[f]	+	−	+	−	+	+	−	+	+
Double-distilled water		−	−	−	−	−	−	−	−	−

[a] Based on comparative genomics of *M. ulcerans* Agy99 and *M. marinum* M strain (*uhp*, unique hypothetical protein gene; *chp*, conserved hypothetical protein gene; *zinc*, putative zinc metalloprotease gene; *uhp-mem*, unique hypothetical membrane protein gene).
[b] Rd1 target genes *esxA* and *esxB* encode Esat6 and Cfp10, respectively.
[c] Genes found on prophage specific to *M. ulcerans*.
[d] Gene determined as *M. ulcerans* specific by subtractive hybridization.
[e] MPMM strain from salt water.
[f] MPMM strain from freshwater.

suggesting that this molecule, like other mycolactones, was present on the cell surface. Finally, addition of sterile culture filtrate elicited cytopathicity, suggesting that this molecule was secreted as well as cell associated.

Identification of mycolactone F. Since all *M. pseudoshottsii* isolates were phenotypically very similar, showed identical lipid profiles by TLC, and were identical by PCR profiling, only L15, the type strain for *M. pseudoshottsii*, was grown in quantity to obtain sufficient mycolactone for detailed chemical analysis. However, both phenotypic heterogeneity and molecular heterogeneity were detected within MPMM isolates. For this reason mycolactones were purified from nine of them, representing each of the major groups of isolates characterized by Ucko and Colorni (34).

Previously identified mycolactones and cometabolites share a conserved core structure, with variation only in the substitution of the side chain. Therefore, mass spectroscopy analysis of ASLs has been a very useful tool in screening for new mycolactones. Mass spectroscopic analysis of *M. pseudoshottsii* L15 and the nine isolates of MPMM revealed a major ion at *m*/*z* 723 (M + Na), suggesting the presence of a novel mycolactone, which was designated mycolactone F (Table 3). The cytopathic activity of this species for L929 cells was 10 ng/ml. This ion was absent from *M. marinum* DL/DK1. Using ion trap MS/MS analysis, the conserved lactone core can be detected as an M + Na ion at *m*/*z* 429 that is produced upon fragmentation of the parent mycolactone. Using this technique, we found that ASLs of all of the MPMM and *M. pseudoshottsii* strains tested showed a major positive ion at *m*/*z* 723 (M + Na) which upon fragmentation produced the conserved core fragment ion at *m*/*z* 429. Moreover, the fragmentation patterns in all samples were identical, suggesting that the mycolactones produced by these strains have the same chemical structure (Fig. 2). Deuterium exchange experiments concluded that mycolactone F has only four hydroxyl groups, two of which are located in the side chain. This is consistent with PCR data suggesting the absence of MUP053, which is thought to produce a third hydroxyl group on carbon 12 of the side chain of mycolactone A/B.

Most mycolactones exist as a mixture of double-bond isomers and are isolated along with minor cometabolites. To investigate this issue, reversed-phase HPLC analysis was performed on the ASL extracts. Under the condition described, the major isomer of mycolactone F was easily identifiable at a

TABLE 3. Mycolactones identified in ASLs from 11 mycobacterial strains isolated from diseased fish

Strain	M + Na ions identified
M. ulcerans 1615	**765**[a], 777, 779
M. pseudoshottsii L15	**723**[b], 739, 755, 763
M. marinum	
DL240490	**723**, 739, 755, 763
DL180892	**723**, 739, 755, 763
DL150991	**723**, 739, 755, 757
DL045 Thalassa	**723**, 739, 763
CF030494	**723**, 739, 755, 757
SA200695	**723**, 739,755
Hybrid270995	**723**, 739, 757
BB170200	**723**, 739, 755
CC240299	721, **723**, 739, 755
DL/DK1	None observed

[a] *m*/*z* 765 (M + Na) represent the major positive ion of mycolactone A/B.
[b] *m*/*z* 723 (M + Na) represents the major positive ion of mycolactone F.

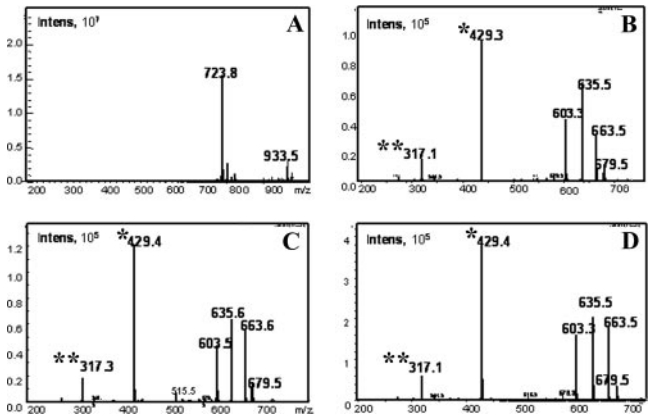

FIG. 2. Mass spectroscopy of ASLs from mycolactone F-producing *M. marinum* strains. (A) MS of ASLs from *M. marinum* DL240490, showing the mycolactone F sodium adduct at *m/z* 723. (B to D) MS/MS of the *m/z* 723 peak showing the core (*) and side chain (**) in *M. marinum* DL180892 from the Red Sea (B), *M. marinum* 045 Thalassa from the Mediterranean Sea (C), and BB170200 from a freshwater pond, Israel (D). The MS/MS peak for *m/z* 723 from DL240490 was identical to that shown for DL180892.

retention time of 20.5 ± 0.05 min (Fig. 3). Other geometric isomers and minor cometabolites were also identified. HPLC and MS/MS analysis confirmed that all strains produce the same molecule. However, differences in stereochemistry, though unlikely, cannot be ruled out by these methods.

Structural characterization of mycolactone F. Although mass spectroscopy is an increasingly valuable tool for molecular analysis, data from NMR analysis provides a more rigorous method for structural assignments. The limited amount of mycolactone produced by mycobacterial species as well as the very low growth rate of mycolactone-producing bacteria has made it extremely difficult to obtain sufficient mycolactone for optimal structural analysis. However, the relatively robust growth yield of lipids from some MPMM isolates made it possible to undertake a more thorough analysis of the mycolactone F structure. For these studies, 1D and 2D NMR experiments were performed on purified mycolactone from *M.*

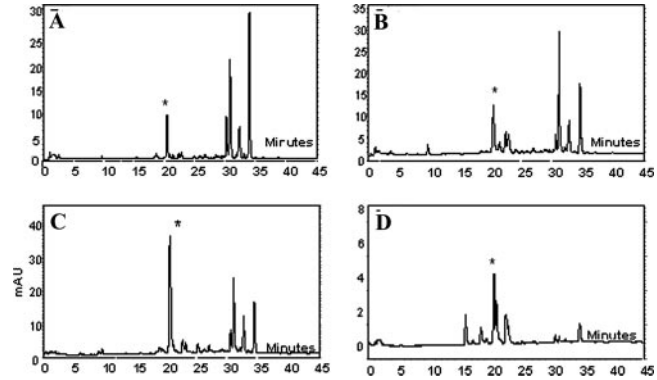

FIG. 3. Reverse-phase HPLC of ASLs derived from mycolactone F-producing strains. (A) *M. marinum* DL240490; (B) *M. pseudoshottsii* L15; (C) *M. marinum* SA200695; (D) *M. marinum* BB170200. The major isomer of mycolactone F at a retention time of 20.5 ± 0.05 min is indicated with an asterisk.

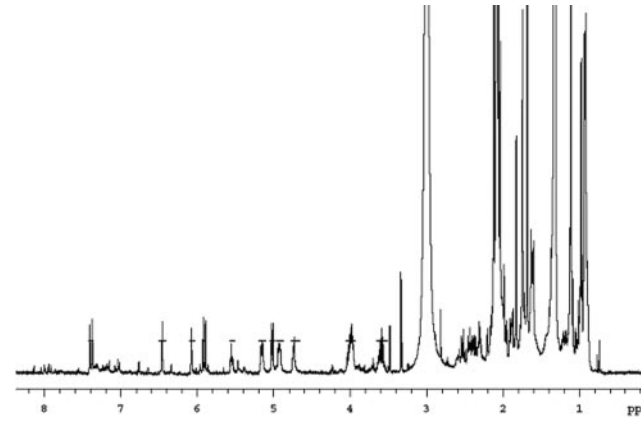

FIG. 4. 1D H^1 NMR spectrum of mycolactone F.

marinum DL240490. ^{1}H NMR revealed a structure very similar to that reported for mycolactone A/B, with the characteristic core signals of protons at C-9, C-15, C-11, and C-5 easily identified at 5.16, 5.03, 4.93, and 4.75 ppm, respectively (Fig. 4; Table 4). In the region corresponding to the side chain, two geometric isomers were distinguished, with an integration ratio of 10 to 1. Both isomers showed five olefinic protons. For the major isomer, the most downfield proton was that of C-3′ which appeared as a doublet at 7.39 ppm and was coupled to the C-2′ proton (doublet at 5.91 ppm). The presence of two singlet peaks at 6.4 and 6.1 ppm suggested methyl substitutions at C-4′, C-6′, and C-8′. This was confirmed by 2D COSY analysis, which showed the long-range coupling of these ole-

TABLE 4. Proton shifts relative to residual acetone for the two major isomers of mycolactone F

Major isomer		Minor isomer		Conserved core	
C	Proton shift (ppm)	C	Proton shift (ppm)	C	Proton shift(s) (ppm)
1′		1′		1	
2′	5.91 (d)	2′	5.94 (d)	2	2.45, 2.04
3′	7.39 (d)	3′	7.94 (d)	3	1.60
4′		4′		4	1.7
5′	6.46 (s)	5′	6.34	5	4.75
6′		6′		6	2.01
7′	6.09 (s)	7′	5.89	7	1.92
8′		8′		8	
9′	5.56 (t)	9′	5.47	9	5.16
10′	2.31 (m)	10′	ND[a]	10	2.51, 2.12
11′	3.99	11′	ND	11	4.93
12′	1.12	12′	ND	12	2.09
13′	3.56	13′	ND	13	2.14, 1.9
14′	1.03	14′	ND	14	
15′	ND	15′	2.05	15	5.03
16′	2.03	16′	1.97	16	2.39
17′	2.06	17′	1.98	17	3.5
				18	1.62, 1.13
				19	3.99
				20	1.13
				21	1.00
				22	1.7
				23	1.03
				24	1.69
				25	0.97

[a] ND, not determined.

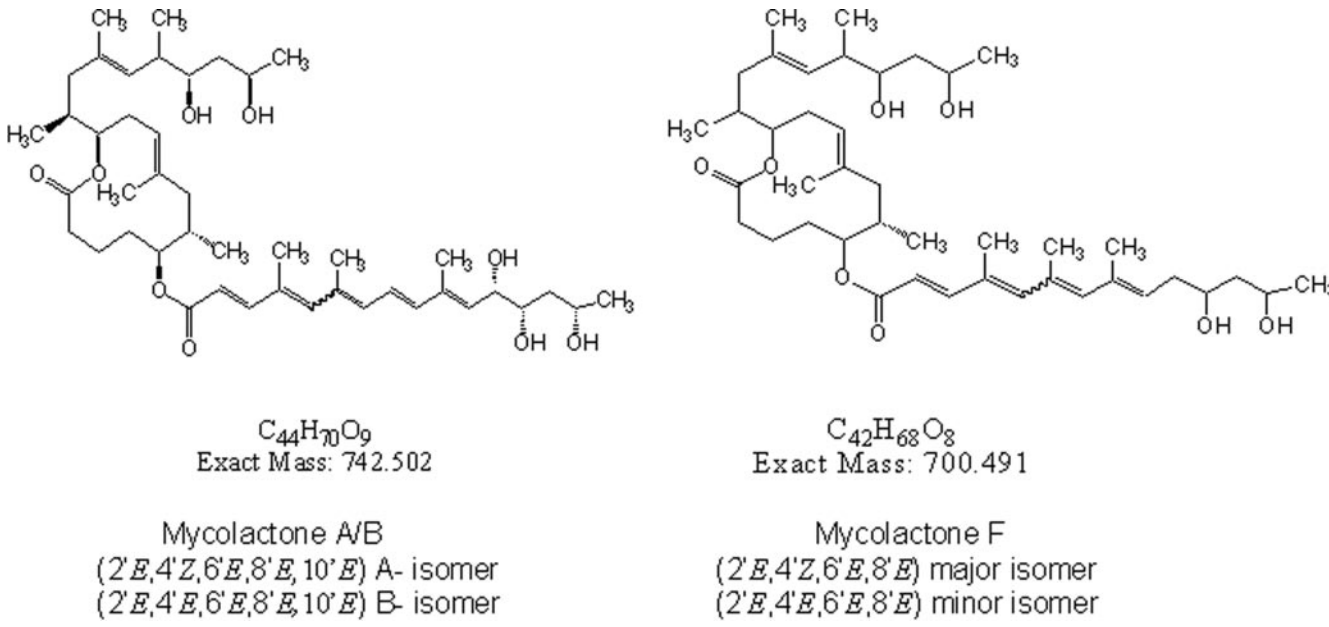

FIG. 5. Structural comparison of mycolactone A/B and mycolactone F.

finic protons to methyl groups at 2.06 and 2.03 ppm. The remaining side chain signals from C-10′ to C-15′ overlapped signals from the mycolactone core in the 1D ^{1}H NMR. Thus, 2D COSY was used to assign these protons (C-9′ to C-14′), using the off-diagonal ^{1}H-^{1}H correlation peaks. Mycolactone F (Fig. 5) is the smallest mycolactone so far identified. As with other mycolactone variants, the core lactone structure is identical to that of other mycolactones, whereas the side chain is unique.

Biological activities of mycolactone F. Mycolactones produce a distinct phenotype on cultured cells, characterized by necrosis at concentrations of above 1 μg/ml within 4 h and delayed apoptosis at concentrations as low as 1 ng/ml within 24 h (1). In order to determine the activity of mycolactone F on cultured cells, purified mycolactone was added to a semiconfluent layer of L929 fibroblasts and tested for the ability to produce necrosis or apoptosis. Although mycolactone F showed typical mycolactone phenotypes, the potency of mycolactone F with regard to apoptosis was significantly less than that of mycolactone A/B (Fig. 6).

DISCUSSION

For 25 years, *M. ulcerans* had the distinction of being the only mycobacterial pathogen to produce a secreted toxin. The finding that mycolactone is plasmid encoded led to the hypothesis that other mycolactone-producing mycobacteria might exist, particularly in aquatic environments. Results from this investigation show that mycolactone-producing *Mycobacterium* species are widely distributed within aquatic environments. It is significant that all mycolactone-producing mycobacteria have been identified as pathogens.

Although the ecology of *M. ulcerans* is poorly understood, the only well-defined risk factor for infection is association with slow-moving water (2, 3, 6, 35). Of particular interest is the fact that human disease caused by *M. ulcerans*, frog disease caused by *M. liflandii*, and fish disease caused by MPMM and *M. pseudoshottsii* have been associated with deteriorating water quality and/or eutrophic enrichment (2, 8, 24, 32). This suggests that similar water quality parameters in different parts of the world may play a role in the ecology of mycolactone-producing bacteria. The geographic heterogeneity of mycolactone structure, along with highly conserved structure within a specific region, has led to the hypothesis that there is strong selection within a specific environment for a particular mycolactone. The discovery of mycolactone F in two mycobacterial species isolated from fish in widely separated geographic areas suggests that mycolactone F plays a particular role in fish disease. Fish may in fact act as "indicator species" in these environments.

Mycolactones are synthesized by large type 1 polyketide synthases. There is a remarkable amount of repeated DNA sequence within the mycolactone *pks* genes, which provides the opportunity for formation of new mycolactones through recombination (29). However, the core lactone structure in mycolactone F is identical to that in mycolactones from *M. ulcerans* and *M. liflandii*. Mycolactone F is the smallest mycolactone identified so far, and it varies from other mycolactones in side chain structure. Our results suggest that side chain structure plays an important role in potency, since the ability of mycolactone F to cause apoptosis is significantly less than that of other mycolactones. In the past five years there has been an intense search for *M. ulcerans* in the environment. Those studies have relied solely on detection of IS*2404* as evidence for *M. ulcerans*, based on a report showing that IS*2404* was absent from 48 species of mycobacteria obtained from a laboratory collection (27). IS*2404* PCR has been used to demonstrate the presence of *M. ulcerans* DNA in fish (10), frogs, many species of insects, snails, and a large range of aquatic samples from Africa (19, 22). The presence of IS*2404* in MPMM, along with its presence in *M. liflandii* and *M. pseudoshottsii*, provides a clear signal that IS*2404* PCR cannot be used as the sole evi-

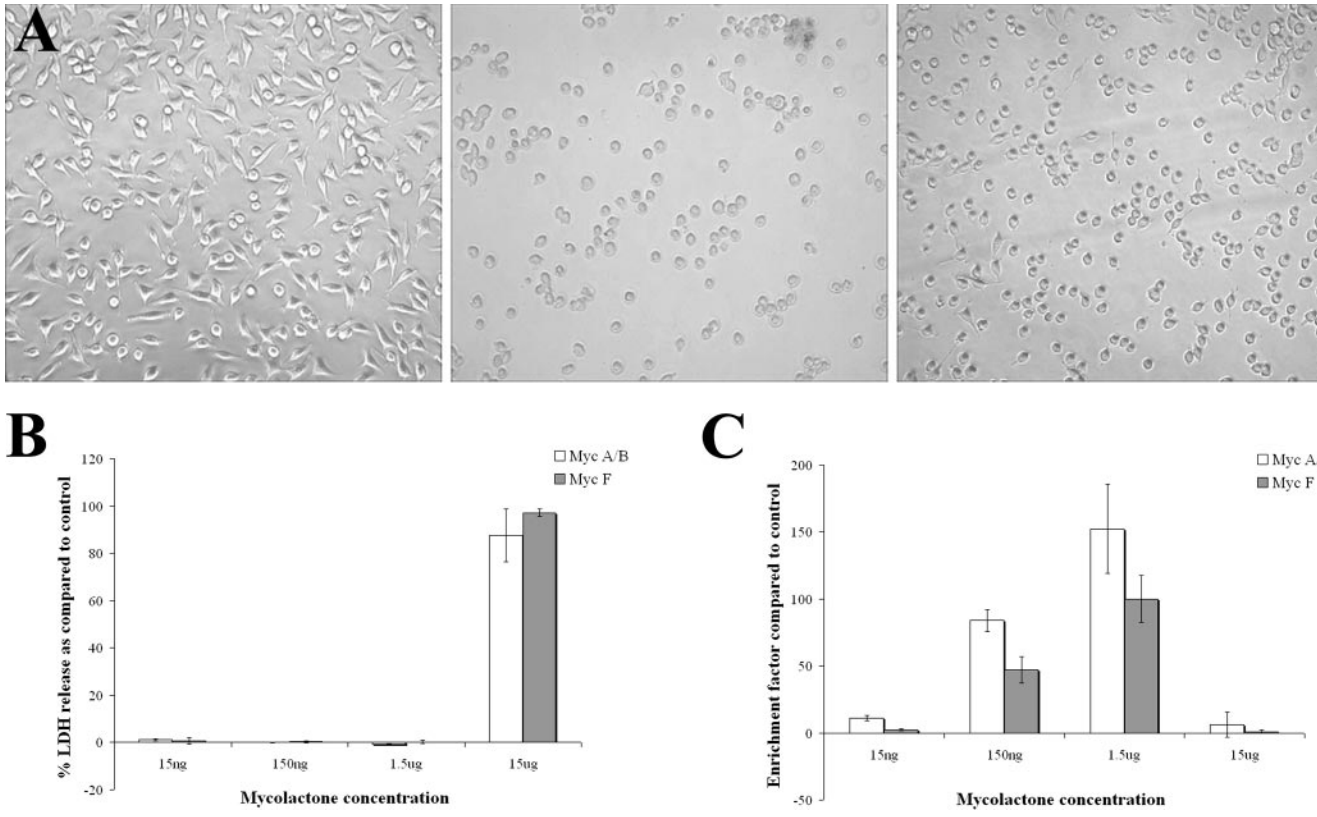

FIG. 6. Analysis of mycolactone-mediated cytopathicity. (A) Cytopathicity on L929 murine fibroblasts, showing untreated cells (left; total magnification, ×200) versus cells treated with 100 ng mycolactone A/B (middle; total magnification, ×200) versus cells treated with 100 ng mycolactone F (right; total magnification, ×200). (B) Cytotoxicity measured by LDH release. Culture supernatants were collected from wells containing L929 cells 4 h after mycolactone treatment, and the amount of LDH was measured using a CytoTox 96 assay kit (Promega). Data are means and standard deviations of the values obtained from triplicate samples; $P > 0.05$ for all concentrations (Student's *t* test). (C) Apoptosis was assessed at 24 h with the cell death detection enzyme-linked immunosorbent assay kit (Roche) and expressed as fold enrichment of nucleosomes. Data are means and standard deviations of the values obtained from triplicate samples; $P < 0.05$ for 15 ng, 150 ng, and 1.5 μg (Student's *t* test).

dence for *M. ulcerans* in environmental samples. Nonetheless, IS*2404* may be a useful marker for the mycolactone plasmid.

There is considerable heterogeneity within *M. marinum* species (31, 35). In the past few years investigators have suggested that *M. marinum* isolates from humans are genetically different than isolates from poikilothermic species. Stinear et al. (28) examined 22 *M. marinum* isolates (19 from humans and 3 from fish) and used multilocus sequence analysis to separate them into five categories. Based on an examination of 11 human isolates and 6 fish isolates, Van der Sar et al. (35) suggested that the human isolates were more pathogenic in zebra fish and better able to grow in the human macrophage THP-1 cell line than isolates from poikilothermic species such as fish. The majority of these strains were from collections, and the country of origin was not provided, making it impossible to compare isolates within a geographic region. The best data for the association of specific *M. marinum* genotypes with human or nonhuman aquatic vertebrate strains comes from a report by Ucko and Colorni (34) comparing 20 human isolates of *M. marinum* with 12 fish isolates from the Red Sea. In that study, human isolates were distinguished phenotypically and genotypically from fish isolates from the Red Sea in Israel. In our study we further distinguish the Red and Mediterranean Sea fish isolates from other groups of *M. marinum* strains by showing that these isolates produce a mycolactone toxin. This work also supports earlier studies describing discrete populations of *M. marinum* strains in Israel (34).

Why is it that the vast majority of *M. marinum* strains do not produce mycolactone? One possibility is that not all strains are capable of acting as recipients for the mycolactone plasmid. This is supported by the fact that MPMM strains form a group distinct from other *M. marinum* isolates in Israel. An interesting parallel may exist between *Shigella* species and mycolactone-producing mycobacteria. Although it is convenient to look at *Shigella* species as *Escherichia coli* with an invasion plasmid, not all *E. coli* strains can act as recipients for the plasmid. In particular, the loss of outer membrane proteins is a prerequisite for conjugal competency. What are the host requirements for horizontal transfer of the mycolactone plasmid? One possibility is that insertion of IS*2404* in *M. ulcerans*, MPMM, *M. liflandii*, and *M. pseudoshottsii* has resulted in loss of membrane-associated genes, thus rendering them competent for horizontal transfer. Alternatively, these strains may have acquired genes enabling horizontal transfer, although this explanation would be inconsistent with molecular evidence suggesting that *M. ulcerans* evolved from an *M. marinum* ancestor. Most of the studies on *M. marinum* and most of the strains in collections are from infected humans. These strains

may not be representative of *M. marinum* in its natural aquatic environment. We cannot rule out the possibility that a more thorough analysis of nonhuman *M. marinum* isolates might show that MPMM are "typical" strains.

Given the importance of the mycolactone plasmid for virulence of *M. ulcerans*, what is the likely virulence of MPMM and *M. pseudoshottsii* for humans? Although mycolactone F is toxic for human cells, this is relevant to human disease only if MPMM or *M. pseudoshottsii* strains are capable of colonizing mammalian species. The temperature-restricted growth of MPMM and *M. pseudoshottsii* may well be a barrier to infection of mammalian species. However, mycolactone-producing aquatic mycobacteria may provide a reservoir for genes important for the evolution of new human pathogens.

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