A Newly Discovered Mycobacterial Pathogen Isolated from Laboratory Colonies of *Xenopus* Species with Lethal Infections Produces a Novel Form of Mycolactone, the *Mycobacterium ulcerans* Macrolide Toxin

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*Mycobacterium ulcerans*, the causative agent of Buruli ulcer, produces a macrolide toxin, mycolactone A/B, which is thought to play a major role in virulence. A disease similar to Buruli ulcer recently appeared in United States frog colonies following importation of the West African frog, *Xenopus tropicalis*. The taxonomic position of the frog pathogen has not been fully elucidated, but this organism, tentatively designated *Mycobacterium liflandii*, is closely related to *M. ulcerans* and *Mycobacterium marinum*, and as further evidence is gathered, it will most likely be considered a subspecies of one of these species. In this paper we show that *M. liflandii* produces a novel plasmid-encoded mycolactone, mycolactone E. *M. liflandii* contains all of the genes in the mycolactone cluster with the exception of that encoding CYP140A2, a putative p450 monoxygenase. Although the core lactone structure is conserved in mycolactone E, the fatty acid side chain differs from that of mycolactone A/B in the number of hydroxyl groups and double bonds. The cytotoxic phenotype of mycolactone E is identical to that of mycolactone A/B, although it is less potent. To further characterize the relationship between *M. liflandii* and *M. ulcerans*, strains were analyzed for the presence of the RD1 region genes, *mlsA* (ESAT-6) and *esxB* (CFP-10). The *M. ulcerans* genome strain has a deletion in RD1 and lacks these genes. The results of these studies show that *M. liflandii* contains both *mlsA* and *esxB*.

Mystery surrounds *Mycobacterium ulcerans* and Buruli ulcer (1, 20, 30). Although the disease occurs globally in rural tropical areas, the mode of transmission is unknown. Exposure to aquatic environments is the only risk factor for infection (2, 6, 30). Only two isolates of *M. ulcerans* have been obtained from the hundreds of environmental samples cultured. Both of these isolates were obtained from aquatic insects (5, 22).

In contrast to other mycobacterial diseases, Buruli ulcer is an extracellular disease, and it is the only mycobacterial disease associated with a secreted toxin (7, 12, 31). The *M. ulcerans* toxins comprise a family of polyketide-derived macrolides, mycolactones, which are formed through condensation of two polyketide chains (8, 14, 23). Each isolate of *M. ulcerans* produces a characteristic mixture of mycolactone congeners (4, 15, 23). *M. ulcerans* strains from different geographic areas produce distinct patterns of mycolactone congeners. The structural heterogeneity in mycolactones is due to variations in the fatty acid side chain. The structure of the core lactone is invariant (15, 23).

Genes for mycolactone biosynthesis form a contiguous 110-kb cluster (Fig. 1A) on a large plasmid (28). The lactone core is encoded by two polyketide synthase (PKs) genes, *mlsA1* and *mlsA2*, and a third polyketide synthase gene, *mlsB*, encodes the fatty acid side chain. Three accessory genes are found in the mycolactone cluster. One of these, MUP053, encodes a p450 monoxygenase that is thought to produce the hydroxyl at C-12 on the fatty acid side chain. The gene encoding a FabH-like, type III ketosynthase (KS), located upstream of *mlsA1*, encodes a putative “joinase” (MUP045), and a small type II thioesterase (TE II) gene (MUP037) is located between *mlsA2* and *mlsB*.

In 2001 a lethal disease caused by a newly discovered mycobacterial pathogen, provisionally designated *Mycobacterium liflandii*, appeared in laboratory colonies of *Xenopus laevis* in the United States (29) and subsequently spread to *Xenopus tropicalis* colonies in the same laboratories. The taxonomic status of *M. liflandii* is uncertain at present, although evidence suggests that it will be eventually designated a variant of *M. ulcerans* or *Mycobacterium marinum*, a common fish pathogen. *M. ulcerans* and *M. marinum* differ by a single base pair in the 16S rRNA gene and can be distinguished from one another by the fact that *M. ulcerans* produces mycolactone and contains over 300 copies of two insertion sequences, IS2404 and IS2606, whereas *M. marinum* lacks these insertion elements, as well as genes for mycolactone biosynthesis (26, 27, 30). *M. liflandii* contains both IS2404 and IS2606 and, like *M. ulcerans*, causes ulcerative, edematous, and plaque forms of skin disease (29). *M. liflandii* causes a fatal systemic disease in frogs, in contrast to *M. ulcerans*, which is primarily limited to cutaneous infections in humans (1, 7, 29, 30). Neither *M. ulcerans* nor *M. liflandii* can grow at temperatures above 35°C. Therefore, the presence of systemic disease in *M. liflandii* cases is likely to be due to the lower core temperature of frogs. *M. ulcerans* has not
been tested in frogs or fish, but it is possible that in these models M. ulcerans may cause systemic disease also.

The close taxonomic relationship of M. liflandii to M. ulcerans, along with the extensive edema found in many diseased frogs, suggested that M. liflandii might make a mycolactone toxin similar to that of M. ulcerans. However, granulomas are a much more prominent feature of M. liflandii frog disease than they are of M. ulcerans human disease. Data from the M. ulcerans genome project suggest that M. ulcerans contains a partial deletion in the Mycobacterium tuberculosis virulence-related region, RD1, and lacks the excA and excB genes, which encode two small highly antigenic proteins, ESAT-6 and CFP-10, respectively (16, 19, 21, 24). The lack of excA and excB in M. ulcerans, along with production of immunosuppressive mycolactones, could contribute to the poor immune response to M. ulcerans infection. M. marinum, like M. tuberculosis, contains an intact RD1 region (10, 21, 32). The widespread occurrence of RD1 in many nonpathogenic mycobacterial species suggests that this region might play a general role in mycobacterial biology. Recent evidence confirms this by showing that ESAT-6 and CFP-10 may provide a barrier to conjugation (9).

The unusual pathology of M. liflandii in frogs, along with the close taxonomic relationship of this organism to M. ulcerans and M. marinum, led us to investigate whether M. liflandii makes a mycolactone and whether this organism contains excA and excB.

FIG. 1. Identification of mycolactone biosynthesis genes in M. liflandii. (A) Schematic arrangement of the mycolactone gene cluster in M. ulcerans. repA, plasmid replication region; p450, CYP140A2 (p450 monoxygenase); MUP037, thioesterase (TE II); mlsA, Pks (fatty acid side chain). (B) PCR evidence for mycolactone and plasmid genes in M. liflandii. Lanes 1 to 4, 7, and 9, M. liflandii; lanes 5 and 8, M. ulcerans; lane 6, 6F; lane 10, M. marinum; lane 11, M. ulcerans; lane 12, M. ulcerans 1615; lane 13, water. Lane M contained a 1-kb DNA ladder (Invitrogen).

MATERIALS AND METHODS

Bacterial strains. M. liflandii x1, x2, x3, x4, x7, x128, and x1680 were isolated from diseased X. laevis. Strains x7608 and x7721 were isolated from infected frogs at the University of Virginia, whereas the other M. liflandii isolates were obtained from the University of California, Berkeley. Strain 6F is a slowly growing mycobacterium isolated from a superficial nose lesion on an X. laevis frog from a M. liflandii-infected colony. The M. ulcerans isolates included South American isolates Valente, Gaillon, 01G097, 842, and 7922; Mexican isolates 5114 and 5143; 98-912 from China; 8756 from Japan; 1615 from Malaysia; Ag99 from Africa; Australian isolates 1327 and V2; and 00-524 from Papua New Guinea. M. marinum 1218 from fish and 00-1026 from a human were used as controls. pMUMP-1 is the mycolactone plasmid from M. ulcerans 1615 cloned into the bacterial artificial chromosome vector pBeloBAC11. Bacteria were cultured on Middlebrook 7H10 medium (Difco) with 10% oleic acid, albumin, dextrose supplement and were incubated at 32°C. All cultures of M. liflandii isolates were supplemented with 5% CO2.

PCR and Southern blot analysis. The following primer sets were used for PCR: for mlsA, 5'-GAGATCGGTCCCGACGCTTAC-3' and 5'-GGCTTGCACCTAAGG-3'; for repA, 5'-CTTGGTCGGCAAAACGTCGACC-3' and 5'-ACCAAGCTTCGAAGCTCG-3'; for MUP045 (FabH-like KS), 5'-GGTGTCCAGAATCTACATCCTG-3' and 5'-CCTGTAAGACGTCGACCTG-3'; for the CYP140A2 gene, 5'-ACCACGCTTCCTCATGTA-3' and 5'-CCGAGATCTTCAGATACCT-3'; for Pks (fatty acid side chain), 5'-GAGATCGGTCCCGACGCTTAC-3' and 5'-GGCTTGCACCTAAGG-3'; for Pks (laconyx core); MUP037, thioesterase (TE II); MUP045, FabH-like ketosynthase; mlsA, Pks (fatty acid side chain); mlsB, Pks (fatty acid side chain). DNA from M. marinum and strain 6F were used as positive controls for excA and excB. Southern blots were prepared from DNA plugs separated by pulsed-field gel electrophoresis as previously described (3). Plasmid size was determined by extrapolation using the linear regressed lambda marker (New England Biolabs) (2). DNA samples were then digested with EcoRI and electrophoresed in 1% agarose gels. Bands were visualized by ethidium bromide staining.

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

Mycolactone isolation. Lipids were extracted from mycobacterial pellets using chloroform:methanol (2:1, vol/vol) and were separated by thin-layer chromatography in a chloroform:methanol:water (90:10:1, vol/vol/vol) solvent system as described previously (11, 23). Specific lipid bands were eluted in chloroform:methanol (2:1, vol/vol) and were separated by thin-layer chromatography. Lipid fractions were then pooled and subjected to saponification and silica gel thin-layer chromatography as previously described (11, 23).

Cytopathicity assays. Bacteria, culture filtrate, and purified lipid species were tested for cytopathicity using L929 murine fibroblasts as described previously (12, 23). Briefly, twofold dilutions of mycolactone in 100% ethanol were added to a semiconfluent monolayer of L929 murine fibroblasts grown in Dulbecco’s modified Eagle’s medium with 5% fetal calf serum in 96-well plates. Assays were read at 36 h and scored for cell rounding and detachment as described previously (12, 23).
Mass spectroscopic analysis. Mass spectrometry (MS) analysis of the mycolactone extracts was performed with a Micromass Q-Tof-2 instrument. Samples were dissolved in methanol (0.8 mM)–NaCl (29%)–water to a final concentration of 1 to 30 pmol/ml of mycolactone and infused using a syringe pump at a rate of 4 ml/min into a nanospray electron spray ionization probe. To ensure accurate mass determinations, the machine was precalibrated using polyalanine to a stable, reproducible accuracy of ±2 ppm. Samples were run alternating with the polyalanine standard. Only sodiated forms of ions were found in the mass spectrometry experiments. The MS-MS experiments were performed with a collision energy of 45 V.

Nucleotide sequence accession numbers. M. liflandii sequences have been deposited in the GenBank database under the following accession numbers: repA, AY736849; essB, AY736850; essA, AY736851; mlsA (enoyl reductase), AY611633; MUP0945, AY611634; IS110, AY611635; MUP037, AY611636; and mlsB (loading module), AY738254.

RESULTS

Analysis of M. liflandii mycolactone genes. To determine whether M. liflandii contained mycolactone biosynthesis genes, PCR primers were used to probe DNA from seven isolates of M. liflandii for mycolactone and plasmid genes. M. marinum 1218 and M. ulcerans 1615 were used as negative and positive controls for the mycolactone gene cluster and plasmid-associated genes, respectively (28). M. ulcerans 1327 (Australia) was used as a negative control for the CYP140A2 gene (23). PCR probes specific for the following genes were used: mlsA1 (core lactone), mlkB (fatty acid side chain), CYP140A2 gene (p450 monooxygenase), MUP045 (FabH-like type III KS), MUP037 (type II TE), and a gene predicted to be involved in plasmid replication (repA).

All seven M. liflandii isolates were PCR positive for all mycolactone and plasmid-associated genes with the exception of the CYP140A2 gene, whereas strain 6F and M. marinum were negative for all probes. M. ulcerans 1327 was also negative for the CYP140A2 gene, as expected (Fig. 1B). The DNA sequences obtained from cloned PCR products showed greater than 99% sequence identity between the M. liflandii genes and the corresponding M. ulcerans genes (see above for the GenBank accession numbers for the M. liflandii repA, essB, essA, mlsA genes and M. ulcerans mlsA, essA, mlsB sequences). Southern blot analysis using labeled CYP140A2 DNA as a probe confirmed the absence of the p450 gene in all seven isolates of M. liflandii (data not shown). Furthermore, IS2404 and IS2606 were present in all M. liflandii isolates tested, as previously reported (29), although Southern blot analysis suggested that there are very few copies of IS2606 (data not shown).

Southern blot analysis of whole-cell DNA separated by pulsed-field gel electrophoresis showed that the mycolactone genes in M. liflandii, like those in M. ulcerans, are carried on a large plasmid (Fig. 2), designated the M. liflandii mycolactone plasmid (pMLMP). pMLMP is slightly larger (180 kb) than the mycolactone plasmid in an African isolate, M. ulcerans Agy99, pUM001 (174 kb), or a Malaysian isolate, M. ulcerans 1615, pMUMP (154 kb) (28).

Purification and structural analysis of M. liflandii mycolactones. Thin-layer chromatography of partially purified M. liflandii lipid extracts revealed the presence of a putative mycolactone with a refractive index of 0.49 in a chloroform:methanol:water (90:10:1) solvent system. The M. liflandii lactones were less polar than mycolactone A/B, which has a refractive index of 0.23 in this system. Mass spectroscopic data for the purified lipid confirmed the presence of a unique mycolactone (Fig. 3A). Whereas analysis of M. ulcerans lipids showed the expected sodium adduct of mycolactone A/B at m/z 765.7, analysis of M. liflandii lipids produced a major peak at m/z 735.7, indicating the presence of a molecule with a molecular mass that is 28 Da less than that of mycolactone A/B. An additional minor peak was identified in M. liflandii extracts at m/z 735.498, indicating the presence of a second mycolactone congenor (data not shown). MS-MS analysis of mycolactone A/B revealed a peak corresponding to the core lactone ion at m/z 429.5 and a peak at m/z 359.4 representing the fatty acid side chain (15). MS-MS of M. liflandii mycolactone E revealed the mycolactone core ion at m/z 429.5, but there was a side chain peak at m/z 331.4 (Fig. 3A). These findings show that the core lactone structure is conserved in mycolactone A/B and mycolactone E and that the structural differences between the two molecules reside in the fatty acid side chain. High-resolution mass spectrometry provided a molecular weight of 714.4995 and a formula of C34H52O4 for mycolactone E. This information, along with a detailed analysis of MS fragmentations, led us to propose a candidate structure for mycolactone (Fig. 3B). The absence of the CYP140A2 gene in M. liflandii is consistent with the lack of a hydroxyl group at C’-12 and accounts for a mass difference of 16 Da between mycolactone, which was confirmed by 1H nuclear magnetic resonance A/B and mycolactone E. The remaining difference in mass could be explained by the addition of an acetate rather than a propionate extender unit and the saturation of one extra double bond in the growing polyketide chain. The presence of four, rather than five, conjugated double bonds is reflected in colony pigmentation. Whereas M. ulcerans forms light yellow colonies, M. liflandii colonies are light orange (Fig. 4).

Mycolactone E has biological activity similar to that of mycolactone A/B and C. Mycolactones produce a distinct phenotype on murine L929 fibroblasts, which is characterized by cell rounding by 12 h, cell cycle arrest at 36 h, and apoptotic cell death by 72 h (11, 13, 25). Addition of either intact M. liflandii or purified mycolactone E to L929 cells produced the typical mycolactone phenotype (Fig. 5). The cytopathic activity of
mycolactone E was 100 ng/ml, compared with 1 ng/ml for mycolactone A/B. The lower potency of mycolactone E than of mycolactone A/B is similar to the potency of mycolactone C (23) and may reflect the absence of the hydroxyl group at C’-12 in the fatty acid side chain.

*M. liflandii* contains genes for ESAT-6 and CFP-10 which are not present in highly virulent *M. ulcerans*. Although Buruli ulcer is globally distributed, the majority of *M. ulcerans* isolates are from African and Australian patients, in which the disease is most prevalent and severe (1, 30). Initial PCR analysis of 11 African and 7 Australian isolates of *M. ulcerans* showed that these isolates, like *M. ulcerans* Agy99, the genome sequencing strain, lack esxA, which encodes ESAT-6, and esxB, which encodes CFP-10. In contrast, esxA and esxB were detected in all isolates of *M. liflandii*.

**FIG. 3.** Comparison of mycolactone E with mycolactone A/B. (A) MS-MS of mycolactones A/B (m/z 765.488) (*M. ulcerans*) and mycolactone E (m/z 737.498) (*M. liflandii*), showing the presence of core lactone at m/z 429 and a side chain at m/z 359.4 (mycolactone A/B) and m/z 331.4 (mycolactone E). (B) Structure of mycolactone A and mycolactone E. MW, molecular weight.

**FIG. 4.** *M. ulcerans* and *M. liflandii* on Middlebrook 7H10 medium with oleic acid-albumin-dextrose supplement, showing the characteristic pigmentation. Clockwise from the top: *M. ulcerans* 1615 and *M. liflandii* xt3, xt4, xt5, xt6808, and xt7281 (xt, *X. tropicalis*; xl, *X. laevis*).

**FIG. 5.** Cytopathic activity of 100 ng mycolactone on L929 fibroblasts after 36 h of incubation. (A) Untreated cells; (B) mycolactone A/B (100 ng); (C) mycolactone E (100 ng). Total magnification, ×200.
FIG. 6. PCR amplification of esxA (ESAT-6) and esxB (CFP-10) in M. liflandii and geographically diverse isolates of M. ulcerans. Positive samples: lane 1, M. marinum 1218 (positive control); lane 5, M. ulcerans Valente; lane 6, M. ulcerans Guillou; lane 7, M. ulcerans 01G897; lane 8, M. ulcerans 842; lane 9, M. ulcerans 7922; lane 10, M. ulcerans 5114; lane 11, M. ulcerans 5143; lane 12, M. ulcerans 98–912; lane 13, M. ulcerans 8756; lane 15, M. marinum 00–1026; lane 16, M. liflandii xt128; and lane 17, M. liflandii xlt281. Negative samples: lane 2, M. ulcerans 1615; lane 3, M. ulcerans Agy99; lane 4, M. ulcerans 1327; lane 14, M. ulcerans 00–524; and lane 18, water (negative control). Lanes M contained a 100-bp DNA ladder (Promega).

seven M. liflandii isolates tested, as well as in an M. marinum control. The DNA sequence of the M. liflandii genes showed 99.6% and 86% nucleotide identity with the corresponding genes from M. marinum and M. tuberculosis, respectively. To extend these findings, experiments were undertaken to compare the presence of these genes in M. liflandii and a geographically diverse group of M. ulcerans strains. The unexpected finding from this study was that whereas African, Malaysian, and Australian M. ulcerans strains lack esxA and esxB, these genes are present in East Asian, Mexican, and South American M. ulcerans isolates (Fig. 6).

DISCUSSION

This is the first report of a mycolactone-producing mycobacterium in the United States. The fact that the disease did not appear in frog colonies prior to importation of X. tropicalis, along with the fact that X. tropicalis was collected in the wild from areas where Buruli ulcer is endemic, suggests that M. liflandii may have been introduced into the United States through importation of X. tropicalis. It is important to note that M. liflandii has never been isolated from a human infection in West Africa. An alternative explanation for the emergence of M. liflandii disease is that M. liflandii is indigenous to the United States but that the disease manifested only when a susceptible frog species was introduced. However, we have recently isolated M. liflandii from a diseased X. laevis frog collected in South Africa and imported into a laboratory which had never housed X. tropicalis. This finding strengthens the argument that M. liflandii is of African origin (unpublished data).

The presence of M. liflandii in frogs is a serious problem for the Xenopus research community because of the high frog mortality that it causes, as well as the difficulty of eradicating M. liflandii from infected facilities. The fact that M. liflandii has spread to X. laevis within infected frog facilities is an additional concern since X. laevis has become naturalized in California. However, the most pressing question is whether M. liflandii can cause human disease. M. liflandii appears to be an M. marinum-like organism with the M. ulcerans plasmid. Since both M. marinum and M. ulcerans are pathogenic for humans, it is likely that M. liflandii is as well. Fortunately, the inability of M. liflandii to grow at temperatures above 35°C suggests that human infections would be cutaneous.

The discovery of mycolactone production in M. liflandii also impacts Buruli ulcer research. The identification of M. ulcerans in the environment has relied on detection of IS2404 and IS2606 (5, 26, 27). The inability to culture M. ulcerans from over 99% of the PCR-positive cultures could be explained by the fact that some of the IS2404 and IS2606 strains detected are organisms like M. liflandii, which cannot be cultured by primary isolation on mycobacterial media. There have also been instances where an M. ulcerans culture could not be obtained from an IS2404 PCR-positive patient sample despite the presence of a huge load of acid-fast bacteria. It is possible that some of these patients were infected with M. liflandii.

An unanticipated finding of this work was the heterogeneity of esxA and esxB among M. ulcerans strains. This heterogeneity is particularly interesting because strains from Asia and Mexico which contain esxA and esxB are associated with less severe disease. Although many of these reports are decades old and several of the strains isolated from these cases have lost plasmid genes (unpublished data), evidence from a recent case of Buruli ulcer reported in Japan confirms earlier reports that Asian isolates are less virulent in humans and guinea pigs (18).

M. liflandii infection in frogs is associated with a much greater inflammatory response than that found in African and Australian cases of M. ulcerans disease. Because ESAT-6 and CFP-10 are strong antigens, it is possible that the presence of esxA and esxB may contribute to the granulomatous response of frogs to M. liflandii. Using a guinea pig model of infection to assess the virulence of M. ulcerans from Africa, Australia, Asia, and Mexico, we found that Asian and Mexican isolates are less virulent and provoke a greater granulomatous response than isolates from Africa or Australia (unpublished data). Although strain differences in virulence may be related to the presence or absence of esxA and esxB, the heterogeneity of toxin genes, as well as the plasmid stability of these strains, makes it impossible to say whether the differential pathogenesis observed is related to differences in esxA and esxB distribution.

The identification of a virulence plasmid in M. liflandii raises questions about the biology of conjugation in Mycobacterium species. Work from the Derbyshire laboratory suggests that ESAT-6 and CFP-10 may prevent conjugation, possibly by coating the bacterial surface and thus preventing close bacterial contact or by negatively regulating conjugation in a pheromone-like manner (9). What, if any, effect does the presence or absence of esxA and esxB mean in terms of the ability of the mycolactone plasmid to be transferred to other mycobacteria?
Unpublished data from our laboratory suggest that the mycolactone plasmid is actually least stable in many isolates that contain exsA and exsB.

This work raises many questions. What is the role of mycolactone E in the virulence of M. liliandii? What role, if any, do exsA and exsB play in the virulence of M. liliandii or M. ulcerans?

Answers to these questions depend on the development of genetic techniques for these organisms. Such methods, although well developed for M. tuberculosis, are rudimentary for M. ulcerans and nonexistent for M. liliandii. Although we have successfully used a bacteriophage mariner system to make mutations in M. ulcerans, no one has reported successful complementation of any M. ulcerans gene despite numerous attempts to do so (17). The major barriers to these experiments include the 36- to 80-h doubling time of M. ulcerans, the extremely low electroporation frequency, and the inability of M. ulcerans to grow at temperatures above 34°C. Research is in progress to develop genetic tools for use with M. ulcerans and M. liliandii.

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REFERENCES


