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

Armand Mve-Obiang,[1] Richard E. Lee,[2] Edward S. Umstot,[3] Kristin A. Trott,[4] Timothy C. Grammer,[4] John M. Parker,[4] Brian S. Ranger,[1] Robert Grainger,[5] Engu A. Mahrous,[2] and P. L. C. Small[1]*

Department of Microbiology, University of Tennessee, Knoxville, Tennessee 37996-0845[1]; Department of Pharmaceutical Sciences, University of Tennessee Medical Health Center, Memphis, Tennessee 38163[2]; Stout Neuroscience Laboratory, University of Tennessee, Memphis, Tennessee 38163[3]; Department of Molecular and Cell Biology, University of California, Berkeley, California 94720-3004[4]; and Department of Biology, University of Virginia, Charlottesville, Virginia 22904-4328[5]

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 monooxygenase. 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 cytopathic 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, *esxA* (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 *esxA* 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 monooxygenase 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 tropicalis* in the United States (29) and subsequently spread to *Xenopus laevis* 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, IS*2404* and IS*2606*, whereas *M. marinum* lacks these insertion elements, as well as genes for mycolactone biosynthesis (26, 27, 30). *M. liflandii* contains both IS*2404* and IS*2606* 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
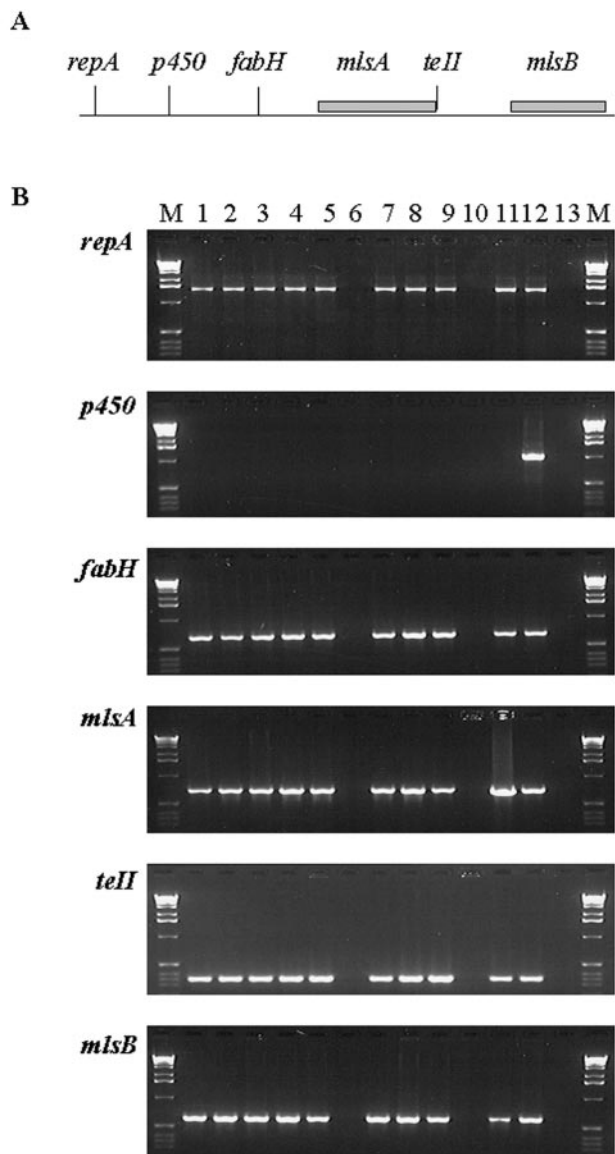
A

B

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 monooxygenase); MUP045, FabH-like ketosynthase; *mlsA*, Pks (lactone core); MUP037, thioesterase (TE II); *mlsB*, 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* from *X. tropicalis*; lanes 5 and 8, *M. liflandii* from *X. laevis*; lane 6, 6F; lane 10, *M. marinum* 1218; lane 11, *M. ulcerans* 1327; lane 12, *M. ulcerans* 1615; lane 13, water. Lane M contained a 1-kb DNA ladder (Invitrogen).

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 *esxA* and *esxB* genes, which encode two small highly antigenic proteins, ESAT-6 and CFP-10, respectively (16, 19, 21, 24). The lack of *esxA* and *esxB* 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 confirmed 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 *esxA* and *esxB*.

MATERIALS AND METHODS

Bacterial strains. *M. liflandii* xt1, xt2, xt3, xt4, xt7, xt128, and xt6808 were isolated from diseased *X. tropicalis*. *M. liflandii* xl5, xl8, and xl7281 were isolated from diseased *X. laevis*. Strains xt6808 and xl7281 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*-contaminated colony. The *M. ulcerans* isolates included South American isolates Valente, Gaillon, 01G897, 842, and 7922; Mexican isolates 5114 and 5143; 98-912 from China; 8756 from Japan; 1615 from Malaysia; Agy99 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% CO_2.

PCR and Southern blot analysis. The following primer sets were used for PCR: for *mlsA*, 5′-GAGATCGGTCCCGACGTCTAC-3′ and 5′-GGCTTGAC TCATGTCACGTAAGG-3′; for *repA*, 5′-CTTGTGCGCAAACCGTGACC-3′ and 5′-CACCAAGCTTCGAGAGCTCG-3′; for MUP045 (FabH-like KS), 5′-GGTGTTCCGAATGATCATACTATCGT-3′ and 5′-CTGTGAGACTTGGA ATTCCTATACCG-3′; for the CYP140A2 gene, 5′-ACCCACCTCGTCGTTA GTCATG-3′ and 5′-CGCAGAGTTCGAGTATCACGTCTAT-3′; for MUP037 (TE II), 5′-GTGTTCATAATGGGCCATTCAAAC-3′ and 5′-GTTGGGCGA TGCTGAAATGTA-3′; for *mlsB*, 5′-TGGAAGGCGTAGGTGGGCAGGTTA ACTGTC-3′ and 5′-ATCTGCGGTTGCCGCTACGCCAAGTTATG-3′; for *esxA* (ESAT-6), 5′-GACAGAACAGCAGTGGAATTTCG-3′ and 5′-CTTCTG CTGCACACCCTGGTA-3′; and for *esxB* (CFP-10), 5′-TTTTGAAGAACGAT GCCGCTAC-3′ and 5′-TGACGGATGTTCGTCGAAATC-3′. DNA from *M. marinum* and strain 6F were used as positive controls for *esxA* and *esxB*. 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) ($r^2 = 0.995$).

Sequence data. PCR 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 (12, 13). Specific lipid bands were eluted in chloroform: methanol (90:10, vol/vol), dried down, and stored at 4°C under N_2.

Cytopathicity assays. Bacteria, culture filtrate, and purified lipid species were tested for cytopathicity using L929 murine fibroblasts as described previously (12, 13, 25). 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, 25).

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; *esxB*, AY736850; *esxA*, AY736851; *mlsA* (enoyl reductase), AY611633; MUP045, AY611634; IS*110*, 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), *mlsB* (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*, *esxB*, *esxA*, *mlsA* [enoyl reductase], MUP045, IS*110*, MUP037, and *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, IS*2404* and IS*2606* were present in all *M. liflandii* isolates tested, as previously reported (29), although Southern blot analysis suggested that there are very few copies of IS*2606* (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, pMUM001 (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
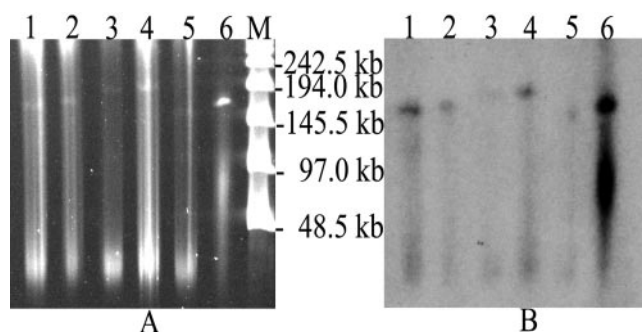

FIG. 2. Mycolactone is plasmid encoded in *M. liflandii*. (A) Pulsed-field electrophoresis; (B) Southern hybridization analysis. Lane 1, *M. ulcerans* 1615; lane 2, *M. ulcerans* Agy99; lane 3, *M. liflandii* xt128; lane 4, *M. liflandii* xl5; lane 5, *M. ulcerans* V2; lane 6, pMUMP-1, showing the presence of a large plasmid hybridized to a combination probe derived from *M. ulcerans* 1615 plasmid-specific enoyl reductase (*mlsA*) and the origin of replication (*repA*). Lane M contained the lambda PFG size ladder (New England Biolabs).

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 737.7, 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 congener (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 spectroscopy provided a molecular weight of 714.4995 and a formula of $C_{43}H_{70}O_8$ 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
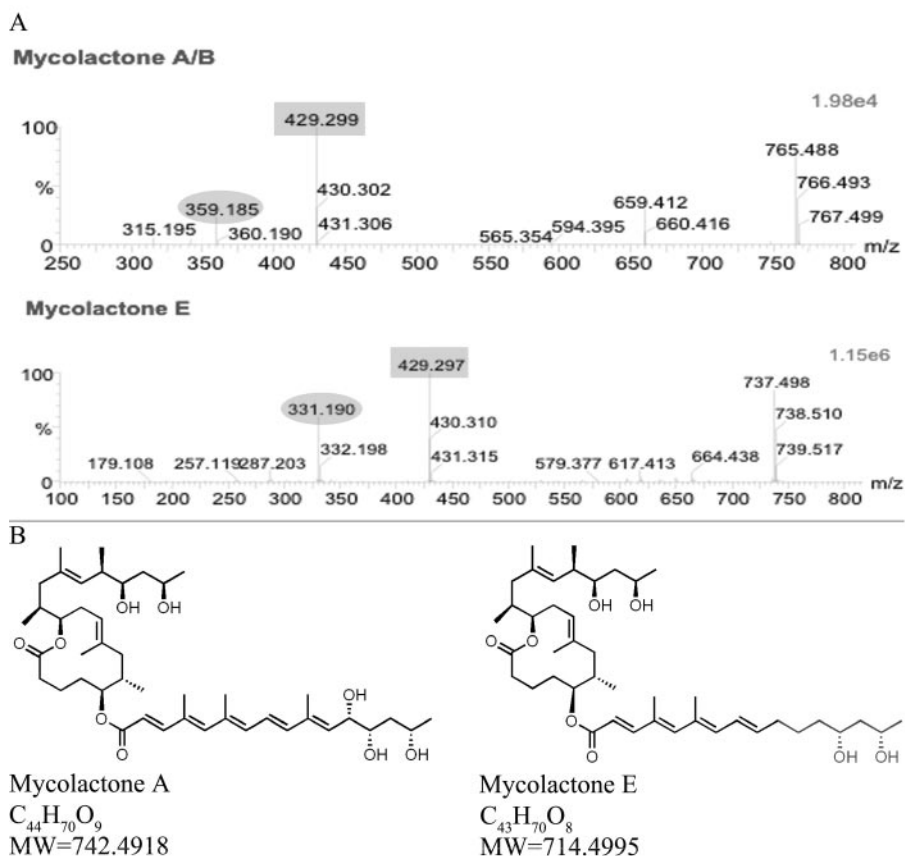
FIG. 3. Comparison of mycolactone E with mycolactone A/B. (A) MS-MS of mycolactones A/B (*m/z* 765.488) (*M. ulcerans*) and mycolactone E (*m/z* 737.498) (*M. liflandii*), showing the presence of core lactone at *m/z* 429 and a side chain at *m/z* 359.4 (mycolactone A/B) and *m/z* 331.4 (mycolactone E). (B) Structure of mycolactone A and mycolactone E. MW, molecular weight.
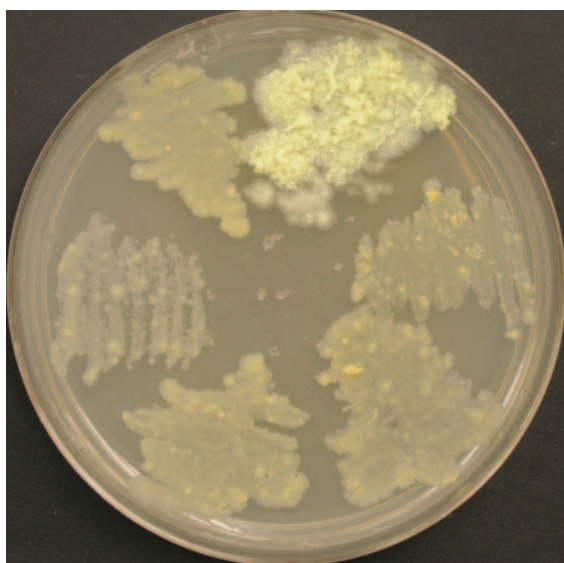

FIG. 4. *M. ulcerans* and *M. liflandii* on Middlebrook 7H10 medium with oleic acid-albumin-dextrose supplement, showing the characteristic pigmentation. Clockwise from the top: *M. ulcerans* 1615 and *M. liflandii* xt3, xt4, xl5, xt6808, and xl7281 (xt, *X. tropicalis;* xl, *X. laevis*).

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
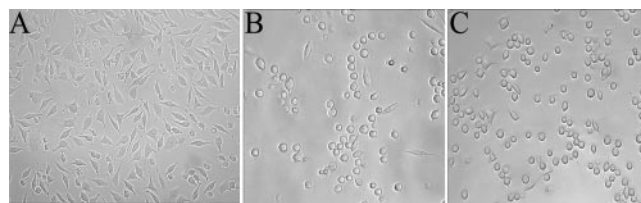

FIG. 5. Cytopathic activity of 100 ng mycolactone on L929 fibroblasts after 36 h of incubation. (A) Untreated cells; (B) mycolactone A/B (100 ng); (C) mycolactone E (100 ng). Total magnification, ×200.
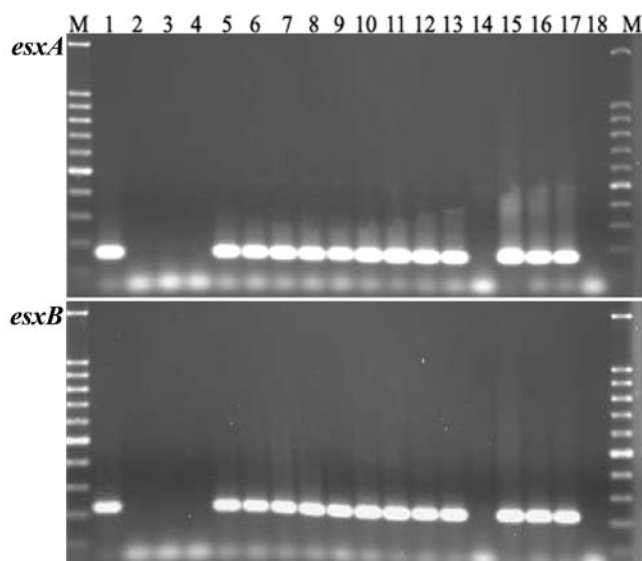
FIG. 6. PCR amplification of *esxA* (ESAT-6) and *esxB* (CFP-10) in *M. liflandii* and geographically diverse isolates of *M. ulcerans*. Positive samples: lane 1, *M. marinum* 1218 (positive control); lane 5, *M. ulcerans* Valente; lane 6, *M. ulcerans* Gaillon; lane 7, *M. ulcerans* 01G897; lane 8, *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* xl7281. 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 IS*2404* and IS*2606* (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 IS*2404* and IS*2606* 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 IS*2404* 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 *esxA* and *esxB*.

This work raises many questions. What is the role of mycolactone E in the virulence of *M. liflandii*? What role, if any, do *esxA* and *esxB* play in the virulence of *M. liflandii* 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. liflandii*. 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. liflandii*.

REFERENCES

1. **Asiedu, K., R. Scherpbier, and M. Raviglione (ed.).** 2000. Buruli ulcer: *Mycobacterium ulcerans* infection. World Health Organization, Geneva, Switzerland.
2. **Barker, D. J.** 1973. Epidemiology of *Mycobacterium ulcerans* infection. Trans R. Soc. Trop. Med. Hyg. **67**:43–50.
3. **Brosch, R., S. V. Gordon, A. Billault, T. Garnier, K. Eiglmeier, C. Soravito, B. G. Barrell, and S. Cole.** 1998. Use of a *Mycobacterium tuberculosis* H37Rv bacterial artificial chromosome library for genome mapping, sequencing, and comparative genomics. Infect. Immun. **66**:2221–2229.
4. **Cadapan, L. D., R. L. Arslanian, J. R. Carney, S. M. Zavala, P. L. Small, and P. Licari.** 2001. Suspension cultivation of *Mycobacterium ulcerans* for the production of mycolactones. FEMS Microbiol. Lett. **205**:385–389.
5. **Chemlal, K., G. Huys, P. A. Fonteyne, V. Vincent, A. G. Lopez, L. Rigouts, J. Swings, W. M. Meyers, and F. Portaels.** 2001. Evaluation of PCR-restriction profile analysis and IS*2404* restriction fragment length polymorphism and amplified fragment length polymorphism fingerprinting for identification and typing of *Mycobacterium ulcerans* and *M. marinum*. J. Clin. Microbiol. **39**:3272–3278.
6. **Connor, D. H., and H. F. Lunn.** 1965. *Mycobacterium ulcerans* infection. Int. J. Lepr. **33**(Suppl.):698–709.
7. **Connor, D. H., C. M. Washington, and H. F. Lunn.** 1966. Buruli ulceration. A clinicopathologic study of 38 Ugandans with *Mycobacterium ulcerans* infection. Arch. Pathol. **81**:183–199.
8. **Fidanze, S., F. Song, M. Szlosek-Penaud, P. L. C. Small, and Y. Kishi.** 2001. Complete structure of the mycolactones. J. Am. Chem. Soc. **123**:10117–10118.
9. **Flint, J. L., J. C. Kowalski, P. K. Karnati, and K. M. Derbyshire.** 2004. The RD1 virulence locus of *Mycobacterium tuberculosis* regulates DNA transfer in *Mycobacterium smegmatis*. Proc. Natl. Acad. Sci. USA **101**:12598–12603.
10. **Gao, L. Y., S. Guo, B. McLaughlin, H. Morisaki, J. N. Engel, and E. J. Brown.** 2004. A mycobacterial virulence gene cluster extending RD1 is required for cytolysis, bacterial spreading and ESAT-6 secretion. Mol. Microbiol. **53**:1677–1693.
11. **George, K. M., L. Barker, D. Welty, and P. L. C. Small.** 1998. Partial purification and characterization of biological effects of a lipid toxin produced by *Mycobacterium ulcerans*. Infect. Immun. **66**:587–593.
12. **George, K. M., D. Chatterjee, G. Gunawardana, D. Welty, R. E. Lee, and P. L. C. Small.** 1999. Mycolactone: a polyketide toxin from *Mycobacterium ulcerans* required for virulence. Science **283**:854–857.
13. **George, K. M., D. M. Welty, L. Pascopella, and P. L. C. Small.** 2000. A *Mycobacterium ulcerans* toxin, mycolactone, causes apoptosis in guinea pig ulcers and tissue culture cells. Infect. Immun. **68**:877–883.
14. **Gunawardana, G., D. Chatterjee, K. M. George, P. Brennan, D. Whittern, and P. L. C. Small.** 1999. Characterization of novel macrolide toxins, mycolactone A and B, from a human pathogen. J. Am. Chem. Soc. **121**:6092–6093.
15. **Hong, H., P. J. Gates, J. Staunton, T. Stinear, S. T. Cole, P. F. Leadlay, and J. B. Spencer.** 2003. Identification using LC-MSn of cometabolites in the biosynthesis of the polyketide toxin mycolactone by a clinical isolate of *Mycobacterium ulcerans*. Chem. Commun. **22**:2822–2823.
16. **Hsu, T., S. M. Hingley-Wilson, B. Chen, M. Chen, A. Z. Dai, P. M. Morin, C. B. Marks, J. Padiyar, C. Goulding, M. Gingery, D. Eisenberg, R. G. Russell, S. C. Derrick, F. M. Collins, S. L. Morris, C. H. King, and W. R. Jacobs, Jr.** 2003. The primary mechanism of attenuation of bacillus Calmette-Guerin is a loss of secreted lytic function required for invasion of lung interstitial tissue. Proc. Natl. Acad. Sci. USA **100**:12420–12425.
17. **Jenkin, G. A., T. P. Stinear, P. D. Johnson, and J. K. Davies.** 2003. Subtractive hybridization reveals a type I polyketide synthase locus specific to *Mycobacterium ulcerans*. J. Bacteriol. **185**:6870–6882.
18. **Kazumi, Y., K. Ohtomo, M. Takahashi, S. Mitarai, I. Sugawara, J. Izumi, A. Andoh, and H. Hasegawa.** 2004. *Mycobacterium shinshuense* isolated from cutaneous ulcer lesion of right lower extremity in a 37-year-old woman. Kekkaku **79**:437–441.
19. **Lewis, K. N., K. M. Guinn, M. J. Hickey, S. Smith, M. A. Behr, and D. R. Sherman.** 2003. Deletion of RD1 from *Mycobacterium tuberculosis* mimics bacille Calmette-Guerin attenuation. J. Infect. Dis. **187**:117–123.
20. **MacCallum, P., J. C. Tolhurst, G. Buckle, and H. A. Sissons.** 1948. A new mycocobacterial infection in man. J. Pathol. Bacteriol. **60**:93–122.
21. **Marmiesse, M., P. Brodin, C. Buchrieser, C. Gutierrez, N. Simoes, V. Vincent, P. Glaser, S. T. Cole, and R. Brosch.** 2004. Macro-array and bioinformatic analyses reveal mycobacterial 'core' genes, variation in the ESAT-6 gene family and new phylogenetic markers for the *Mycobacterium tuberculosis* complex. Microbiology **150**:483–486.
22. **Marsollier, L., R. Robert, J. Aubry, J. P. Saint Andre, H. Kouakou, P. Legras, A. L. Manceau, C. Mahaza, and B. Carbonnelle.** 2002. Aquatic insects as a vector for *Mycobacterium ulcerans*. Appl. Environ. Microbiol. **68**:4623–4628.
23. **Mve-Obiang, A., R. E. Lee, F. Portaels, and P. L. C. Small.** 2003. Heterogeneity of mycolactones produced by clinical isolates of *Mycobacterium ulcerans*: implications for virulence. Infect. Immun. **71**:774–783.
24. **Pym, A. S., P. Brodin, L. Majlessi, R. Brosch, C. Demangel, A. Williams, K. E. Griffiths, G. Marchal, C. Leclerc, and S. T. Cole.** 2003. Recombinant BCG exporting ESAT-6 confers enhanced protection against tuberculosis. Nat. Med. **9**:533–539.
25. **Snyder, D. S., and P. L. C. Small.** 2003. Uptake and cellular actions of mycolactone, a virulence determinant for *Mycobacterium ulcerans*. Microb. Pathog. **34**:91–101.
26. **Stinear, T. P., B. C. Ross, J. K. Davies, L. Marino, R. M. Robins-Browne, F. Oppedisano, A. Sievers, and P. D. R. Johnson.** 1999. Identification and characterization of IS*2404* and IS*2606*: two distinct repeated sequences for detection of *Mycobacterium ulcerans* by PCR. J. Clin. Microbiol. **37**:1018–1023.
27. **Stinear, T. P., G. A. Jenkin, P. D. R. Johnson, and J. K. Davies.** 2000. Comparative genetic analysis of *Mycobacterium ulcerans* and *Mycobacterium marinum* reveals evidence of recent divergence. J. Bacteriol. **182**:6322–6330.
28. **Stinear, T. P., A. Mve-Obiang, P. L. Small, W. Frigui, M. J. Pryor, R. Brosch, G. A. Jenkin, P. D. Johnson, J. K. Davies, R. E. Lee, S. Adusumilli, T. Garnier, S. F. Haydock, P. E. Leadlay, and S. T. Cole.** 2004. Giant plasmid-encoded polyketide synthases produce the macrolide toxin of *Mycobacterium ulcerans*. Proc. Natl. Acad. Sci. USA **101**:1345–1349.
29. **Trott, K. A., B. A. Stacy, B. D. Lifland, H. E. Diggs, R. M. Harland, M. K. Khokha, T. A. Grammer, and J. M. Parker.** 2004. Characterization of a *Mycobacterium ulcerans*-like infection in a colony of African tropical clawed frogs (*Xenopus tropicalis*). Comp. Med. **54**:309–317.
30. **van der Werf, T. S., W. T. van der Graaf, J. W. Tappero, and K. Asiedu.** 1999. *Mycobacterium ulcerans* infection. Lancet **354**:1013–1018.
31. **van der Werf, T. S., T. P. Stinear, W. T. van der Graaf, and P. L. C. Small.** 2003. Mycolactones and *Mycobacterium ulcerans* disease. Lancet **362**:1062–1064.
32. **Volkman, H. E., H. Clay, D. Beery, J. C. Chang, D. R. Sherman, and L. Ramakrishnan.** 2004. Tuberculous granuloma formation is enhanced by a mycobacterium virulence determinant. Public Library Sci. **2**:e367.