SEE COMMENTARY

Giant plasmid-encoded polyketide synthases produce the macrolide toxin of *Mycobacterium ulcerans*

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Mycobacterium ulcerans **(MU), an emerging human pathogen harbored by aquatic insects, is the causative agent of Buruli ulcer, a devastating skin disease rife throughout Central and West Africa. Mycolactone, an unusual macrolide with cytotoxic and immunosuppressive properties, is responsible for the massive s.c. tissue destruction seen in Buruli ulcer. Here, we show that MU contains a 174-kb plasmid, pMUM001, bearing a cluster of genes encoding giant polyketide synthases (PKSs), and polyketide-modifying enzymes, and demonstrate that these are necessary and sufficient for mycolactone synthesis. This is a previously uncharacterized example of plasmid-mediated virulence in a *Mycobacterium*, and the emergence of MU as a pathogen most likely reflects the acquisition of pMUM001 by horizontal transfer. The 12-membered core of mycolactone is produced by two giant, modular PKSs, MLSA1 (1.8 MDa) and MLSA2 (0.26 MDa), whereas its side chain is synthesized by MLSB (1.2 MDa), a third modular PKS highly related to MLSA1. There is an extreme level of sequence identity within the different domains of the MLS cluster (>97% amino acid identity), so much so that the 16 ketosynthase domains seem functionally identical. This is a finding of significant consequence for our understanding of polyketide biochemistry. Such detailed knowledge of mycolactone will further the investigation of its mode of action and the development of urgently needed therapeutic strategies to combat Buruli ulcer.**

A single Buruli ulcer, which can cover >15% of a person's skin surface, contains huge numbers of extracellular bacteria. Despite their abundance and extensive tissue damage, there is a remarkable absence of an acute inflammatory response to the bacteria, and the lesions are often painless (1). This unique pathology is attributed to mycolactone, a macrolide toxin consisting of a polyketide side chain attached to a 12-membered core that seems to have cytotoxic, analgesic, and immunosuppressive activities. Its mode of action is unclear, but, in a guinea pig model of the disease, purified mycolactone injected s.c. reproduces the natural pathology, and mycolactone negative variants are avirulent, implying a key role for the toxin in pathogenesis (2).

Mycobacterium ulcerans (MU) and *Mycobacterium marinum* (MM) share over 98% DNA sequence identity, they occupy aquatic environments, and both cause cutaneous infections (3). However, MM produces a granulomatous intracellular lesion, typical for pathogenic mycobacteria and totally distinct from Buruli ulcer in which MU are mainly found extracellularly. The fact that MM does not produce mycolactone suggested that it might be possible to identify genes for mycolactone synthesis by performing genomic subtraction experiments between MU and MM. Fragments of MU-specific polyketide synthase (PKS) genes were identified from these experiments (4). The subsequent investigation of these sequences led to the discovery of the MU virulence plasmid pMUM001 and the extraordinary PKS locus it encodes.

Methods

Bacterial Strains and Growth Conditions. MU strain Agy99 is a recent clinical isolate from the West African epidemic. MU1615 (ATCC 35840), originally isolated from a Malaysian patient, was obtained from the Trudeau Collection. Strains were cultivated by using Middlebrook 7H9 broth (Difco) and Middlebrook 7H11 agar (Difco) at 32°C.

Plasmid Sequence Determination. A bacterial artificial chromosome (BAC) library was made of *M. ulcerans* strain Agy99 by using the vector pBeloBAC11, and nucleotide end sequences were determined as described (5). This library was then screened by PCR for MU-specific PKS sequences that had been identified in subtractive hybridization experiments between MU and MM (4). The complete sequences of selected BAC clones were obtained by shotgun subcloning and sequencing as described (6). To overcome the difficulties associated with the highly repetitive PKS sequences, two additional BAC subclone libraries were made from (*i*) total *Pst*I digests and (*ii*) partial *Sau*3AI subclones with insert sizes of 6–10 kb. *Sau*3AI subclones that represented a single module (i.e., a single nonrepetitive unit) were then subjected to primer walking. Sequences were assembled by using Gap4 (6, 7). The ARTEMIS tool (www.sanger.ac.uk/Software) was used for the plasmid annotation, with comparisons to public and in-house databases performed by using the BLAST suite and FASTA. The conditions for pulsed-field gel electrophoresis and Southern hybridization were as described (3, 5).

Construction and Analysis of Mycolactone Negative Mutants. Phage MycoMarT7 was propagated in *Mycobacterium smegmatis* mc^{2}155. It consists of a temperature sensitive mutant of phageTM4 containing the mariner transposon C9 *Himar1* and a kanamycin cassette (8). An MU1615 cell suspension, containing $\approx 10^9$ bacteria, was infected with 10^{10} phages for 4 h at 37°C, plated directly onto solid media containing kanamycin, and cultured at 32°C. Nonpigmented colonies were purified, and individual mutants were subcultured in broth and grown for 5

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Abbreviations: MU, *Mycobacterium ulcerans*; MM, *Mycobacterium marinum*; PKS, polyketide synthase; BAC, bacterial artificial chromosome; KS, ketosynthase; TE, thioesterase.

Data deposition: The complete plasmid sequence reported in this paper has been deposited in the EMBL database (accession no. BX649209).

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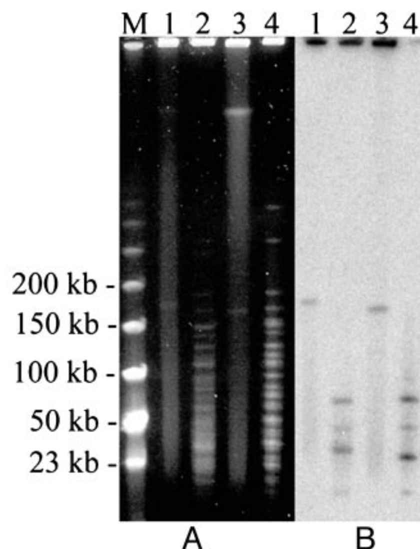

Fig. 1. Demonstration of the mycolactone plasmid. Pulsed-field gel electrophoresis (*A*) and Southern hybridization (*B*) analyses of MU Agy99 (lanes 1 and 2) and MU1615 (lanes 3 and 4), showing the presence of the linearized form of the plasmid in nondigested genomic DNA (lanes 1 and 3) and after digestion with *Xba*I (lanes 2 and 4), hybridized to a combination probe derived from *mlsA*, *mlsB*, *mup038*, and *mup045*. Lane M is the lambda low-range DNA size ladder (NEB).

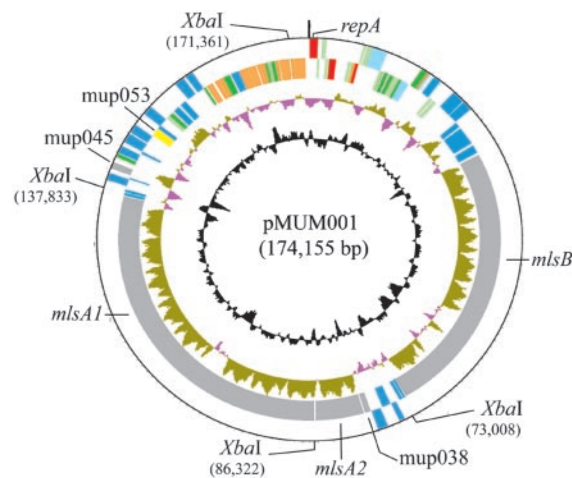

Fig. 2. Circular representation of pMUM001. The scale is shown in kilobases by the outer black circle. Moving in from the outside, the next two circles show forward and reverse strand protein-coding DNA sequences, respectively, with colors representing the functional classification (red, replication; light blue, regulation; light green, hypothetical protein; dark green, cell wall and cell processes; orange, conserved hypothetical protein; cyan, insertion sequence elements; yellow, intermediate metabolism; gray, lipid metabolism). These circles are followed by the GC skew (G − C)/(G + C) and finally the G + C content by using a 1-kb window. The arrangement of the mycolactone biosynthetic cluster has been highlighted, and the locations of all *Xba*I sites are indicated.

weeks. Bacteria, culture filtrate, and lipid extracts were assayed for cytotoxicity by using L929 murine fibroblasts as described (9). Lipids were further analyzed by mass spectroscopy for the presence or absence of ions characteristic of mycolactone: the molecular ion [M + Na] + (m/z 765.5) and the core ion [M + Na] + m/z 447 (9).

Results and Discussion

Identification of the Plasmid. Genomic subtraction experiments led to the identification of several fragments of MU-specific PKS genes (4). In the present work, when undigested MU genomic DNA was analyzed by pulsed-field gel electrophoresis, a band of ≈170 kb was detected (Fig. 1*A*) that hybridized with the MU-specific PKS probes, suggesting that the PKS genes were plasmid-encoded (Fig. 1*B*). Several positively hybridizing clones were isolated from a BAC library of the epidemic MU strain Agy99 and characterized by BAC end sequencing, insert sizing, and restriction fragment profiling. Three BACs were subsequently shotgun sequenced, with the resultant composite sequence confirming the existence in MU of a circular plasmid, designated pMUM001, comprising 174,155 bp, with a GC content of 62.8% and carrying 81 protein-coding DNA sequences (Fig. 2).

In one sense the plasmid seems very simple, with no identifiable transfer or maintenance genes. Replication seems to be initiated by the predicted product of *repA*, which shares 68.3% amino acid identity with RepA from the cryptic *Mycobacterium fortuitum* plasmid, pJAZ38 (10). Two different direct repeat regions were identified 500–1,000 bp upstream of *repA*, suggesting possible replication origins (*ori*). GC-skew plots [(G − C)/(G + C)], which highlight compositional biases between leading and lagging DNA strands, displayed a random pattern and did not help pinpoint a possible *ori* (Fig. 2). Approximately 2 kb downstream of *repA* is *parA*, a gene encoding a chromosome-partitioning protein, required for plasmid segregation on cell division. In this region, there is also a potential regulatory gene cluster composed of a serine/threonine protein kinase (*mup008*), a gene encoding a protein of unknown function (*mup018*) but containing a phosphopeptide recognition domain, a domain found in many regulatory proteins (11), and a WhiB-like transcriptional regulator (*mup021*). This arrangement shares synteny with a region near *oriC* of the *Mycobacterium tuberculosis* (MTB) H37Rv genome. Further upstream of *repA* is a 5-kb region encoding conserved proteins of unknown function, and again there is synteny with the *oriC* region of MTB. There are six genes with products of unknown function but predicted to have membrane-associated domains. None of these displayed similarity to proteins involved in lipid export such as the MMPLs (12) or to any other export systems. The plasmid is rich in insertion sequences (IS), with 26 examples, including 4 copies of IS*2404* and 8 copies of IS*2606* (13). However the primary function of pMUM001 seems to be toxin production. To our knowledge, no reports of plasmids mediating mycobacterial virulence exist.

Most of pMUM001 (≈105 kb) consists of six genes coding for proteins involved in mycolactone synthesis (Fig. 2). Mycolactone core-producing PKS are encoded by *mlsA1* (50,973 bp) and *mlsA2* (7,233 bp) and the side chain enzyme by *mlsB* (42,393 bp). All three PKS genes are highly related, with stretches of up to 27 kb of near identical nucleotide sequence (99.7%). The entire 105-kb mycolactone locus essentially contains only 9.5 kb of unique, nonrepetitive DNA sequence. The repetitive, recombinant, and recent nature of the MLS locus is highlighted in the GC-skew plot (Fig. 2), as it traces the start and end of each of the 2 loading and 16 extension modules that these genes encode (see Fig. 3 and the following section). Ancestral genes of *mlsA* and *mlsB* apparently underwent duplication, followed by in-frame deletions and limited divergence. There are also three genes coding for potential polyketide-modifying enzymes, including a P450 monooxygenase (*mup053*), probably responsible for hydroxylation at carbon 12 of the side chain, and an enzyme resembling FabH-like type III ketosynthases (KS) (*mup045*). The latter has mutations in each of three amino acids critical for KS activity. Similar changes have been detected in KS-like enzymes that catalyze C—O bond formation (14). The product

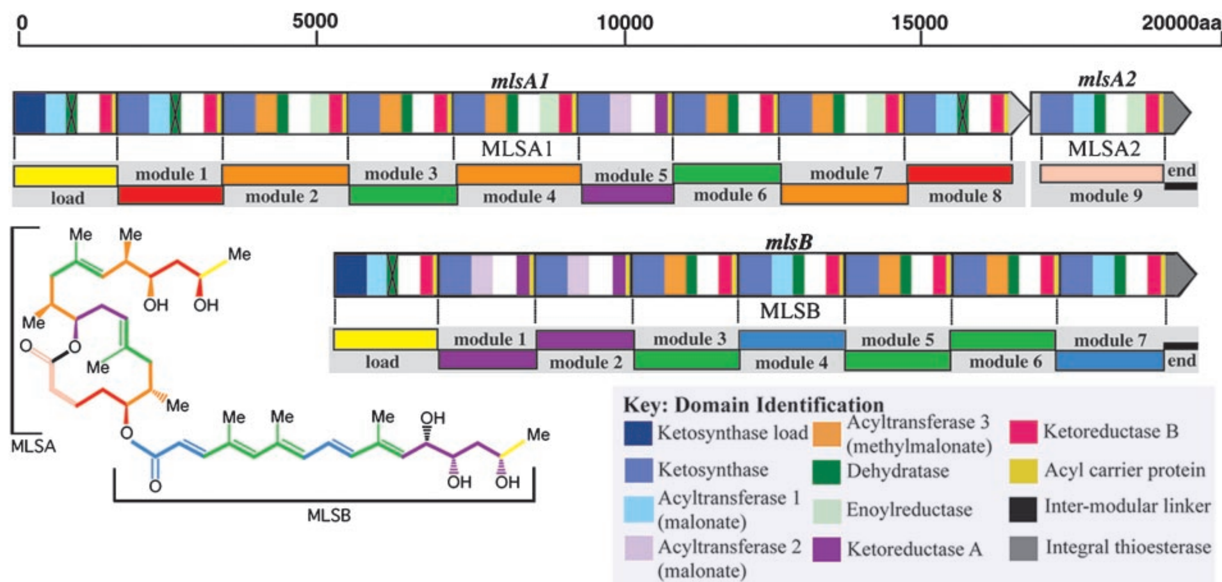

Fig. 3. Domain and module organization of the mycolactone PKS genes. Within each of the three genes (*mlsA1*, *mlsA2*, and *mlsB*), different domains are represented by a colored block. The domain designation is described in the key. White blocks represent interdomain regions of 100% identity. Module arrangements are depicted below each gene, and the modules are color-coded to indicate identity both in function and sequence (>98%). For example, module 5 of MLSA1 is identical to modules 1 and 2 of MLSB. The crosses through four of the dehydratase domains indicate that they are predicted to be inactive based on a point mutation in the active site sequence. The structure of mycolactone has also been color-coded to match the module responsible for a particular chain extension.

of *mup045* may likewise catalyze ester bond formation between the mycolactone core and side chain. Alternatively, attachment of the sidechain may be mediated directly by the C-terminal thioesterase (TE) on MLSB. It is intriguing that the *mup045* gene has a GC content of 52.8%, significantly lower than the rest of the plasmid, suggesting that it has been acquired by recent horizontal transfer. Immediately 3′ of *mlsA2* is *mup037*, a gene encoding a type II TE that may be required for removal of short acyl chains from the PKS loading modules, arising by aberrant decarboxylation (15).

Analysis of the Mycolactone PKS Cluster. The modular arrangement of the mycolactone PKS closely follows the established paradigm for "assembly-line" multienzymes (16, 17). The core of mycolactone is produced by MLSA1 and MLSA2, which contains a decarboxylating loading module (18) and nine extension modules, terminating in an integral C-terminal thioesterase/cyclase (TE) domain that serves to release the product by forming a 12-membered lactone ring (Fig. 3). The pattern of malonate and methylmalonate incorporation predicted by sequence analysis of the acyltransferase domains in each module exactly matches that found in mycolactone (19). Similarly, the oxidation state produced at each stage of chain extension almost wholly corresponds to that predicted on the basis of the mycolactone structure (16, 17). The exception is extension module 2, where dehydratase and enoylreductase domains seem from sequence comparisons to be active although the structure of the product does not require these steps. However, there is a precedent from previously characterized PKS gene clusters for such non-utilization of reductive domains (19). Likewise, the side-chain of mycolactone is produced by MLSB, which contains a decarboxylating loading module and seven extension modules, plus an integral TE domain, and here the pattern of extender unit incorporation, the oxidation state, and the stereochemistry of ketoreductase reduction (20) are exactly as predicted.

On closer inspection, however, the mycolactone PKS presents some highly unusual features that have an important bearing on our view of the structural basis of the specificity of polyketide chain growth on such multienzymes. First, the PKS proteins are of unprecedented size, with MLSA comprising one multienzyme of eight consecutive extension modules (MLSA1) and predicted molecular mass of 1.8 MDa, and a second (MLSA2, 0.26 MDa) harboring the last extension module and the TE. The recognition process between MLSA1 and MLSA2 is mediated in part by specific "docking domains" as in other modular PKSs (21). Meanwhile, MLSB contains all of its seven consecutive extension modules in a single multienzyme (1.2 MDa). These are among the largest proteins predicted to be found in any living cell. The most startling feature of the mycolactone PKS is the extreme mutual sequence similarity between comparable domains in all 16 extension modules (Fig. 3). Whereas modular PKSs routinely show 40–70% sequence identity when domains from the same

Table 1. Shared percentage amino acid identity among the KS domains of four PKS

	MLSA,-B (mycolactone[16]*)	RAPS1,-2,-3 (rapamycin[14])	DEBS1,-2,-3 (erythromycin[6])	PikAI,-II,-III,-IV (pikromycin[6])
MLSA,-B (mycolactone[16])	97			
RAPS1,-2,-3 (rapamycin[14])	66	67		
DEBS1,-2,-3 (erythromycin[6])	38	32	38	
PikAI,-II,-III,-IV (pikromycin[6])	47	39	32	51

*Indicates number of extension modules.

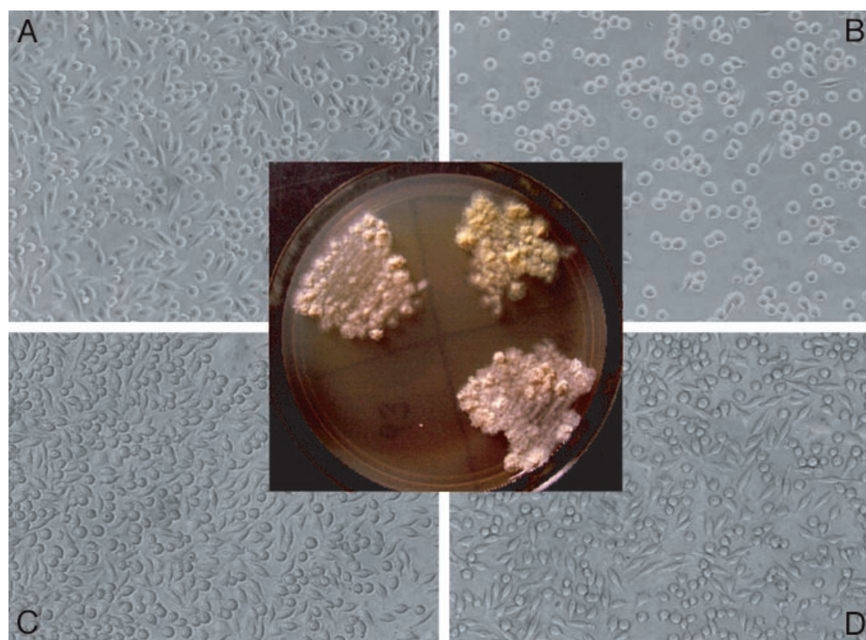

Fig. 4. Mycolactone transposon mutants. Mycolactone negative mutants were identified as nonpigmented colonies (*Inset*). Bacteria (1×10^7) and 50 μl of culture filtrate were added to a semiconfluent monolayer of L929 fibroblasts for detection of cytotoxicity. Treated cells shown at 24 h. (*A*) MU1615::Tn*104* containing an insertion in *mlsB*. (*B*) WT MU1615. (*C*) Untreated control cells. (*D*) MU1615::Tn*141* containing an insertion in *mlsA* (×20).

PKS are compared, and lower identity when domains from different PKS are compared (19), the identity scores for the dehydratase, enoylreductase, and A-type and B-type ketoreductase domains in the mycolactone locus ranged between 98.7 and 100%.

There were three distinct sequence types for the acyltransferase (AT) domains: two with predicted malonate specificity and the third, methylmalonate. Within each of the three AT domain types, identity scores were 100% (Fig. 3) whereas, between the sequence types, the identity was 34%. Interestingly, one of the malonate AT domain types was always linked to the A-type ketoreductase domain. This divergent domain combination was found in module 5 of MLSA1 and modules 1 and 2 of MLSB (Fig. 3), and these were 100% identical for both their

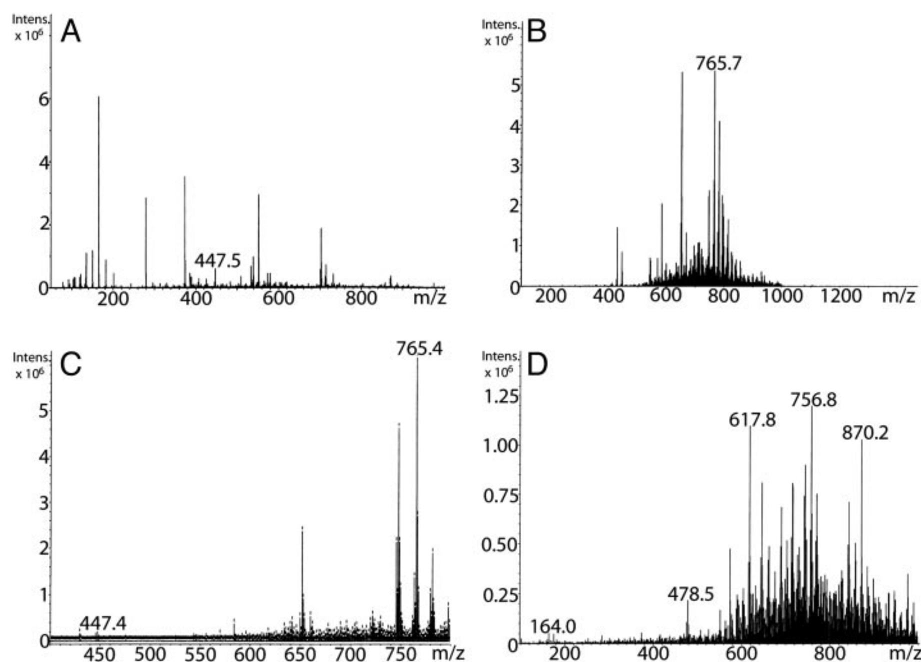

Fig. 5. MS analyses of the mycolactone transposon mutants. (*A*) MU1615::Tn*104* containing an insertion in *mlsB*, showing the absence of the mycolactone ion m/z 765 and the presence of the lactone core ion at m/z 447. (*B*) WT MU1615 showing the presence of the mycolactone ion m/z 765. (*C*) Control mutant MU1615::Tn*99* containing a non-MLS insertion, showing the presence of the mycolactone ion m/z 765. (*D*) MU1615::Tn*141* containing an insertion in *mlsA*, showing the absence of both the mycolactone ion m/z 765 and the lactone core ion at m/z 447.

amino acid and DNA sequences. The most likely explanation is recent acquisition by horizontal transfer followed by duplication. This finding is supported by the significantly lower GC content of this block compared with the surrounding sequences (58% vs. 63%, Fig. 2).

For the KS domains, which catalyze the critical C—C bond-forming steps, the mutual sequence identity within all of the MLS modules is over 97%. Only 11 residues of 420 show variation, and none of this variation seems systematic. Other modular PKSs demonstrate sequence identity between KS domains in the range of 32–67% (Table 1). The synthetic operations catalyzed by various KS domains of the mycolactone PKS involve significant structural variation in both the growing polyketide chain and the incoming extender unit. Liquid chromatography/mass spectrometry (LC-MS) experiments on mycolactone-containing extracts of MU have, however, confirmed that MLSA apparently produces only one product whereas MLSB shows only minor variation in two or three of seven modules (22). These data lead to the unexpected conclusion that the KS domains in this PKS play no significant role in determining the specificity of polyketide chain growth. It remains to be determined how the correct oxidation state is set within each extension unit before the extended chain is passed to the next KS domain. A practical outcome of this finding is that the mycolactone PKS modules might furnish the basis of a set of "universal" extension units in engineered hybrid modular PKSs, with potentially far-reaching implications for combinatorial biosynthesis.

Transposon Mutagenesis. Although the close agreement between the structure-based predictions for the mycolactone genes and the DNA sequence strongly suggested that this was the mycolactone locus, definitive proof was sought by using gene disruption experiments. The genetically tractable MU strain 1615 is highly related to Agy99, and in both strains the mycolactone biosynthesis genes are plasmid encoded and their available DNA sequences are identical. The plasmid from MU1615 is 3–4 kb smaller than MU Agy99. This difference has been mapped to the non-PKS region of pMUM001 (Fig. 2), a region rich in insertion sequences. A transposition library of MU1615 was made by using a mycobacteriophage carrying a mariner transposon (8), and mycolactone-negative mutants were identified by loss of the yellow color conferred by the toxin (2). Putative mutants were characterized by DNA sequencing, and their inability to produce mycolactone was assessed by using cytotoxicity assays and MS of lipid extracts (9) (Fig. 4 and Fig. 5). Nucleotide sequence located the transposon insertion site in MU1615::Tn*141*, a nonpigmented and noncytopathic mutant (Fig. 4), to the dehydratase domain of module 7 in *mlsA*. The side chain produced by MLSB is extremely unstable in the absence of core lactone, and its precursor cannot be detected (9). MS confirmed the absence of both the core lactone as well as intact mycolactone in MU1615::Tn*141* (see Fig. 5). Similarly, MU1615::Tn*104* was mapped to the KS domain of the loading module in *mlsB*. MS analysis confirmed that the insertion was in *mlsB* because the mutant still produced the core lactone as evidenced by the presence of the lactone core ion at m/z 447, and the absence of the mycolactone ion m/z 765.5 (Fig. 5). Characterization of these mutants proves conclusively that MLSA and MLSB are required to produce mycolactone.

Concluding Comments. The singularly high level of DNA sequence homology suggests that the mycolactone system has evolved very recently, arising from multiple recombination and duplication events. It also suggests a high level of genetic instability. Indeed, heterogeneity has been reported both in structure and cytotoxicity of mycolactones produced by MU isolates from different regions (9). High mutability may explain the sudden appearance of Buruli ulcer epidemics as some strains produce mycolactones that confer a fitness advantage for an environmental niche such as the salivary glands of particular aquatic insects (23). This change might be accompanied by an increase in virulence or transmissibility to humans. Loss or gain of pMUM001 may also contribute to these events (24). In any event, the deciphering of the mycolactone biosynthetic pathway should permit new approaches to be used to prevent and combat *M. ulcerans* infection.

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