The Cell Wall-Associated Mycolactone Polyketide Synthases Are Necessary but Not Sufficient for Mycolactone Biosynthesis

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Abstract

Mycolactones are polyketide-derived lipid virulence factors made by the slow-growing human pathogen, Mycobacterium ulcerans. Three unusually large and homologous plasmid-borne genes (mlsA1: 51 kb, mlsB: 42 kb and mlsA2: 7 kb) encode the mycolactone type I polyketide synthases (PKS). The extreme size and low sequence diversity of these genes has posed significant barriers for exploration of the genetic and biochemical basis of mycolactone synthesis. Here, we have developed a truncated, more tractable 3-module version of the 18-module mycolactone PKS and we show that this engineered PKS functions as expected in the natural host M. ulcerans to produce an additional polyketide; a triketide lactone (TKL). Fractionation experiments indicated that this 3-module PKS and the putative accessory enzymes encoded by mup045 and mup038 associated with the mycobacterial cell wall, a finding supported by confocal microscopy. We then assessed the capacity of the faster growing, Mycobacterium marinum to harbor and express the 3-module Mls PKS and accessory enzymes encoded by mup045 and mup038. RT-PCR, immunoblotting, and cell fractionation experiments confirmed that the truncated Mls PKS multienzymes were expressed and also partitioned with the cell wall material in M. marinum. However, this heterologous host failed to produce TKL. The systematic deconstruction of the mycolactone PKS presented here suggests that the Mls multienzymes are necessary but not sufficient for mycolactone synthesis and that synthesis is likely to occur (at least in part) within the mycobacterial cell wall. This research is also the first proof-of-principle demonstration of the potential of this enzyme complex to produce tailored small molecules through genetically engineered rearrangements of the Mls modules.

Introduction

The bacterial pathogen Mycobacterium ulcerans causes disease through its ability to produce an immunomodulatory [1–8] macropolyketide called mycolactone [9]. Mycolactone synthesis depends on a highly unusual cluster of plasmid borne, type I modular polyketide synthases (PKS) (Figure 1) [10]. These large, multi-domain enzymatic complexes catalyze polyketide formation by the processive condensation of (usually) acetate and propionate subunits. Different combinations of reductive domains within the extension modules further modify the molecule at each extension step [11].

In the initial description of the mycolactone PKS (Mls) cluster, transposon mutagenesis showed that the mls genes were required for mycolactone synthesis [10]. However, it has never been shown that these genes are sufficient for toxin synthesis. There is only one published report of an experiment to test sufficiency, with the transfer (in two parts) of the 174 kb pMUM001 plasmid that harbours the mls genes to Mycobacterium marinum, a natural producer of many polyketide metabolites and a very close relative of M. ulcerans [12]. This experiment showed that the mls genes were expressed in M. marinum but mycolactones were not detected [12].

The mycolactone PKS modules and their constituent domains are encoded by three large genes (mlsA1: 51 kb, mlsA2: 7.2 kb, mlsB: 42 kb) that possess very high DNA sequence identity (Figure 1). For example, there is less than 3% nucleotide variation among all the ketosynthase (KS) domains of the 16 Mls extension modules [10,13]. The high sequence repetition makes the mls locus prone to recombination-mediated deletion and it is common for laboratory passaged M. ulcerans to lose toxin production by this process [14]. M. ulcerans is a slow growing mycobacterium (>48 h doubling time) that is poorly transformable and for which there are few genetic tools. An additional complication is the host restriction of the pMUM plasmid. Studies of its ori showed plasmid...
replication within *M. marinum* but not within faster growing, but more distantly related, mycobacteria such as *M. smegmatis* and *M. fortuitum* [15]. These issues have made it difficult to explore the biosynthesis of mycolactones in detail.

Despite these barriers, some progress towards understanding mycolactone biosynthesis has been made by studying *M. ulcerans* strains that make different mycolactones. We originally speculated that the modules of the mycolactone PKS might be interchangeable; i.e. because domains are of near-identical sequence, they might be readily exchanged with each other to produce new module combinations and thus new polyketides, without the barriers that have evolved in other PKS where inter-domain identity is less than 80% and where tight specificity has evolved for native incoming precursor polyketides for a given module [10]. Support for this idea comes from the six naturally occurring mycolactone structural variants that have so far been described. These arise among different strains of *M. ulcerans* through in-frame recombination events within *mlsB* that swap, delete or duplicate MlsB modules and domains [16–24]. These genetic changes have resulted in a significant set of chemical modifications, including changes in the length, methylation state, hydroxylation pattern and stereochemistry of mycolactones. These changes also alter the biological activity of the molecule [4]. Such observations support our idea that the mycolactone PKS might be readily reprogrammed by genetically engineered module rearrangements to produce new, complex small molecules.

In the present study, we set out to develop a minimal trimodular version of the mycolactone PKS, reducing its size from 18 modules to only three, composed of a gene encoding the MlsA1 loading module, and module 8 and a second gene encoding module 9 from MlsA2. This trimodular arrangement was expected to produce a triketide lactone (TKL) when the genes were expressed in a compatible host bacterium. With this more tractable system we explored the potential of the Mls PKS for combinatorial polyketide biosynthesis as well as investigating Mls expression in different bacterial hosts and studying the cellular location of these enzymes within different host bacteria.

**Materials and Methods**

**Bacterial Strains**

*Escherichia coli* DH10B was cultured at 37°C in Luria-Bertani (LB) broth or LB agar. *M. marinum* M and *M. ulcerans* 06-3844 (the latter isolate also referred to as *M. marinum* 06-3844) [25] were

**Table 1. Bacterial strains used in the study.**

| Strain   | Description | Reference       |
|----------|-------------|-----------------|
| E. coli  | DH10B       | Invitrogen      |
| Mycobacteria                    |
| TPS8097 | *M. ulcerans* 06-3844 | [25] |
| TPS8164 | *M. ulcerans* 06-3844+ pTPS331 | This study |
| TPS8162 | *M. ulcerans* 06-3844+ pTPS333 | This study |
| TPS8307 | *M. ulcerans* 06-3844+ pTPS438 | This study |
| TPS8024 | *M. marinum* 'M' | [38] |
| TPS8256 | *M. marinum* 'M'+pTPS331 | This study |
| TPS8254 | *M. marinum* 'M'+pTPS333 | This study |
| TPS8313 | *M. marinum* 'M'+pTPS334+ pTPS629 | This study |
| TPS8334 | *M. marinum* 'M'+pTPS334+ pTPS629+ pTPS338 | This study |

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cultured at 30°C in 7H9 Middlebrook broth or 7H10 Middlebrook agar as described [12]. Antibiotics were used at the following final concentrations in mycobacteria: apramycin 50 µg/mL; kanamycin 25 µg/mL; and hygromycin 50 µg/mL. The same concentrations of apramycin and kanamycin were used for *E. coli* and ampicillin was used at 100 µg/mL. All strains used are listed in Table 1.

**General DNA/RNA Methods**

Methods for PCR, Sanger sequencing, ligation and cloning were as previously described [12]. *E. coli* and mycobacterial transformation methods were as previously described [12]. The primers used throughout are listed in Table S1. For RT-PCR, gene-specific primers were used to target mRNA within *mlsA1* LM-M8, *mlsA2*, mup038 and mup045. The *M. marinum* *crtI* gene was used as a positive control. Reverse transcription was performed by combining 1 µg of total RNA, 200 U of Superscript II reverse transcriptase (RT) enzyme (Invitrogen), 4 µl of 5x First

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**Figure 2. Schematic view of the mycobacterial expression vectors developed for this study.** (A) pTPS333, pYUB412-based integrating vector with *mlsA1* LM-M8 under the control of the *mlsA1* promoter; (B) pTPS629, pMUM001-based low-copy number vector with *mlsA1* LM-M8 under the control of the *mlsA1* promoter; (C) pTPS334, pYUB412-based integrating vector with *mlsA2* under the control of the *mlsA1* promoter; (D) pTPS338 pMV261-based vector with mup038 and mup045 in an operon and under the control of the *ermE* promoter.

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Table 2. Plasmids used in this study.

| Name     | Description                               | Source |
|----------|-------------------------------------------|--------|
| pTPS207  | pYUB412                                   | [39]   |
| pTPS331  | pYUB412-LP<sub>min</sub>                  | This study |
| pTPS333  | pYUB412-LP<sub>min</sub>mlsA1 LM-M8<sup>*</sup> | This study |
| pTPS438  | pYUB412-LP<sub>min</sub>mlsA1 LM-M8<sup>*</sup> | This study |
| pTPS334  | pYUB412-LP<sub>min</sub>mlsA2<sup>*</sup>   | This study |
| pTPS628  | pYUB412-APraמצMUR<sub>min</sub>mlsA1 LM-M8 | This study |
| pTPS629  | pYUB412-APraמצMUR<sub>min</sub>mlsA1 LM-M8 | This study |
| pTPS338  | pMV261-LP<sub>min</sub>mlsA1::mup045::mup038 | This study |

*LM-M8 refers to the loading module and module 8 regions encoded within mlsA1; LM<sub>min</sub>-M8 refers to the loading module from M. ulcerans Liflandii mlsB fused with mlsA1 M8 from M. ulcerans Agy99.

Strand buffer (Invitrogen), 10 mM DTT (Invitrogen), 1 mM dNTPs (Promega), 2 pmol of gene-specific primer, made up to 20 μl with RNase-free-dH2O. After incubation at 42°C for 50 minutes, followed by a 15-minute heat inactivation at 70°C, the resulting cDNA was used as template in a standard PCR reaction. Reverse transcriptase reactions were also set up without the addition of RT enzyme to test for the presence genomic DNA.

Plasmid Construction

Two mycobacterial expression plasmids were used. The first was pYUB412, modified to include the mlsA1 promoter region [26] upstream of the unique PacI site in this vector, resulting in pTPS331. The second plasmid was based on replacing the hygromycin resistance gene, the L5 integrase gene, and attP site from pTPS331 with an apramycin resistance gene and pMUM001 ori as follows. The plasmid pTPS331 was digested with SacII to excise the hygromycin resistance gene and replace it with an apramycin resistance marker (pTPS404). A 6383 bp fragment spanning the pMUM001 ori and including repA, parA, and flanking NdeI (5') and XbaI (3') sites was PCR amplified from M. ulcerans Agy99 and cloned into Aavr/Ndel digested pTPS404, replacing the L5 integrase gene and attP site and resulting in the novel mycobacterial expression vector called pTPS628.

A truncated mlsA1 gene comprising the loading module and module 8 (LM-M8) was constructed in E. coli DH10B by separate PCR amplification of each module and then cloning into pCDNA2.1, including the addition of 5' and 3' PacI sites respectively. The two modules were then translationally fused, using the BsrRI site located between the ACP domain of the loading module and the KS domain of both module 1 and module 8 to create pTPS100. An 11 kb LM-M8 fragment was then excised from pTPS100 by PacI digestion and cloned into the unique PacI site of pTPS331 to create pTPS333 (Figure 2A). The same 11 kb fragment was also cloned from pTPS100 into the unique PacI site of the second mycobacterial expression vector pTPS628 to create pTPS629 (Figure 2B) (Table 2).

A second version of the truncated mlsA1 gene was also prepared, comprising the loading module from mlsB from M. ulcerans Liflandii with the M. ulcerans Agy99 mlsA1 module 8 (LM<sub>min</sub>-M8). The mlsB loading module was PCR amplified from M. ulcerans Liflandii and modified to include flanking Nool and BcerI sites. This fragment was then ligated with M. ulcerans Agy99 MlsA1 M8 in pTPS099 using Nool/BcerI to create plasmid pTPS437, producing a translationally fused LM<sub>min</sub>-M8 in the same manner as above. The resulting 11 kb of DNA was excised from pTPS437 using PacI and cloned into the unique PacI site of pTPS331 to create pTPS438.

The full-length mlsA2 gene was also cloned into pTPS331. The mlsA2 gene was PCR amplified from M. ulcerans Agy99, modified to remove an internal Ndel site, and cloned into the Ndel/HindIII site of pET29, creating a C-terminal 5-histidine epitope tag for MlsA2 (MlsA2-His<sub>5</sub>). PacI sites were introduced to the 5' and 3' ends of mlsA2-His<sub>5</sub> to permit excision and subcloning into the unique PacI site pTPS331 and resulting in the plasmid pTPS334 (Figure 2C) (Table 1).

A third expression vector harbouring the putative mycolactone access genes mup045 and mup038 under the p<sub>min</sub> promoter was also prepared by cloning a 2.3 kb fragment harbouring this promoter and these two genes as an operon into the unique Xbal site of pMV261 to create pTPS338 (Figure 2D).

Stability Studies of pTPS629 in M. marinum

Late log-phase cultures of M. marinum M harbouring pTPS629 grown in the presence of apramycin, were diluted 1:100 into three, 50 ml volumes of fresh 7H9 media without apramycin and incubation was continued at 30°C for 12 days. Aliquots of each culture were then removed at successive 3-day time points, appropriate dilutions were made and then plated on solid media with and without apramycin. Colonies were counted after ten days. The total cell number (expressed as colony forming units per ml) and the proportion of the total cell population that had maintained antibiotic resistance at each time point were calculated.

Western Blotting

Mycobacterial whole cell lysates (WCLs) for Western immunoblotting were prepared as described [27]. WCLs were separated by SDS PAGE using NuPage Novex 3–8% Tris-Acetate polyacrylamide gels (Invitrogen). A primary polyclonal antibody was raised in rabbits against a recombinant form of the 34 kDa acyltransferase domain of the MlsA1 loading module [28], then pre-adsorbed against WCL from M. marinum M and used at 1:250 dilution. Primary polyclonal antibodies were raised in rabbits against the synthetic peptide (MRPINDIQVDGVPNC) derived from mup045. Mup045 antibodies were similarly pre-adsorbed against M. marinum WCL and used at a 1:4000 dilution. As a secondary antibody, goat anti-Rabbit-IgG-HRP (Millipore) was used at a 1:5000 dilution. Detection of MlsA2 was also achieved using a HRP conjugated anti-His antibody at a 1:100 dilution (GenScript).

Mass Spectrometry, Peptide Fingerprint Analysis

Bands from Coomassie-stained SDS-PAGE gels representing potential proteins of interest, were excised and treated with destaining solution (50% acetonitrile/50 mM tetra ethyl ammonium bicarbonate [TEAB]) at 37°C for 1 hour. Reduction of the sample was performed using 0.5 M Bondbreaker solution, diluted 1 in 10 in TEAB at 60°C for 1 hour, followed by alkylation using 100 mM iodoacetamide incubated at room temperature for 30 minutes. Iodacetamide was removed and the sample washed with 100 mM of TEAB. Samples were analysed using an Agilent nanoCHIP 3D Ion Trap Mass Spectrometer and peptides identified using the Mascot search engine (www.matrixscience.com).

Analysis of Mycolactone Biosynthesis
Bacterial Cell Fractionation

Cell fractionation was carried out as previously described [29]. Briefly, cells were harvested at 4,400 x g and washed with 0.16 M NaCl. One gram of cells was resuspended in 1 ml of lysis buffer (0.05 M potassium phosphate buffer, 0.022% (v/v) β-mercaptoethanol, pH 6.5) containing 2.4 mg/ml lysozyme and incubated at 37°C for at least two hours. Cells were disrupted using a Precellys24 tissue homogenizer (Bertin Technologies) at speed 6500, twice for 45 sec. Unbroken cells were removed by centrifugation at 1000 x g for 5 mins. Lysates were subjected to ultracentrifugation at 27,000 x g twice for 40 mins at 4°C to isolate the cell wall (P27) fraction. The supernatant was then centrifuged at 100,000 x g for 1 hr at 4°C to separate into the membrane (P100) and cytosolic (SN100) fractions.

Immunofluorescence Assay

Bacteria were fixed on glass slides and stained using the anti-mup045 and anti-AT domain polyclonal sera described above as primary antibodies. These antibodies were applied at 1:1000 dilution in PBS with 0.1% (v/v) Tween 20 for 1 h at 20°C. Alexa Fluor 568 (Invitrogen) conjugated goat anti-rabbit immunoglobulin G was used as secondary antibody at 1:200 dilution for 1 h at 20°C. Cells were mounted in ProLong Gold anti-fade reagent containing 4',6-Diamidino-2-phenylindole (DAPI; Invitrogen).

Images were acquired using a Zeiss LSM700 confocal laser-scanning microscope (x100 oil immersion objective) and images were processed using Zen software (Zeiss, 2009).
TKL Isolation and LC-MS Analysis

Mycobacteria were grown for 4 weeks on solid media and 2 cm² of colony material and surrounding agar were excised and potential triketide lactones (TKLs) were extracted with ethylacetate. Briefly, colony material and surrounding agar was placed into a screw-cap tube containing 500 µl of 100 µm glass beads and 1 mL of an ethylacetate/formic acid mixture (1.2 ml ethylacetate +20 µl of formic acid), samples were placed at 50°C for 15 minutes before bead-beating, 3 × 45 second pulses at speed 5 in a Precellys24 tissue homogenizer, the samples were then briefly centrifuged to collect the liquid phase. The resulting extracts were dried down with N2, the residue was dissolved in 200 µl HPLC grade methanol and 30 µl of the sample was analysed by liquid chromatography-mass spectrometry (LC-MS) using a LTQ mass spectrometer (Thermo Finnigan), with positive mode electrospray ionisation. The mass spectrometer was coupled with a HP1200 HPLC system (Agilent) fitted with a Phenomenex Prodigy C18 column (5 µm, 2.0×250 mm). Samples were eluted with acetonitrile and MilliQ water with 0.1% formic acid using a gradient of 5% to 50% acetonitrile over 25 min at a flow rate of 300 µl/min. Production of triketide lactone was judged by comparison with the standard (5-hydroxyhexanoic acid lactone, Alfa Aesar, UK) using on-line LC-MS/MS analysis on [M+H]+ ion at m/z 115.2 with normalized collision energy of 20%.

Results

Expression of Truncated MlsA1 PKS in M. ulcerans

MlsA1 is encoded by a single 51 kb gene and is composed of a loading module and eight extension modules (Figure 1). The large size of mlsA1 and its significant internal sequence repetition - essentially composed of eight, 6 kb direct repeats - make it difficult to modify and mobilize. We therefore constructed a small, more manageable mlsA1 gene, comprising only the loading module and module 8 (LM-M8) and we cloned it into the Mycobacterium/E. coli shuttle vector pYUB412 that was also modified to include the native mlsA1 promoter upstream of LM-M8 (pTPS333). A second LM-M8 construct called LMp-M8 (pTPS438) was also prepared, taking the loading module from M. ulcerans Lfelandii, where the acyltransferase domain of the loading module confers specificity for methylmalonyl-CoA (propionate instead of acetate starter unit) [13,20]. For each construct, the resulting 11 kb CDS was predicted to encode a 390 kDa protein (Figure 3). When plasmid pTPS333 and pTPS438 were separately transferred to M. ulcerans strain 06-3844, a strain that is more amenable to genetic manipulation than M. ulcerans Agy99 and a natural producer of mycolactone F, we observed by Western immunoblot (using an antibody raised against the acyltransferase domain of the MlsA1 loading module) the presence of a protein with a predicted mass around 400 kDa in each strain that was absent from the same strain harbouring the empty vector (Figure 4). Cell fractionation was also performed for M. ulcerans TPS8162. Interestingly, the LM-M8 Mls protein and a faint band at ~260 kDa, likely representing the native MlsA2, were detected exclusively in the cell wall fraction, suggesting the mycolactone PKS might be cell wall associated. Although the same amounts of protein were loaded (Figure 4A), the ~260 kDa band representing MlsA2 was not detected in TPS8307 (LMp-M8) or TPS8164 (empty vector) (Figure 4B).

Production of the Predicted TKLs in M. ulcerans

We expected that the C-terminal docking domain present in Module 8, expressed from pTPS333, would link with its cognate N-terminal docking domain of the endogenous MlsA2 (Module 9)
enzyme to produce a functional PKS, capable of synthesizing the two predicted TKLs (Figure 3). *M. ulcerans* strain 06-3844 harbouring pTPS333 (TPS8162) or pTPS438 (TPS8307) was cultured for 4 weeks, after which bacterial cells were harvested and subjected to ethylacetate extraction. Analysis of the extracts by HPLC-MS (Figure 5) revealed that TPS8162 produced, in addition to mycolactone F, the expected methyl triketide lactone (5-hydroxyhexanoic acid lactone), albeit in low yield. The yield of mycolactone F was not significantly diminished compared to controls (data not shown). In contrast, the predicted ethyl TKL (5-hydroxyheptanoic acid lactone) was not detected in the second recombinant *M. ulcerans* strain TPS8307 (data not shown).

The Truncated MlsA1 and Full-length MlsA2 PKS are Expressed in *M. marinum*

We next tested the capacity of *M. marinum*, a faster growing and genetically more tractable close relative of *M. ulcerans*, to express LM-M8 and mlsA2 to reconstitute a 3-module PKS, capable of synthesizing TKL as above (Figures 3–5). To attempt this experiment we first cloned the full-length 7.2 kb *mlsA2* gene, under the control of the *mlsA1* promoter into our pYUB412-based expression vector, pTPS331, to create pTPS334 (Figure 2C).

To also introduce the LM-M8 construct into the same host strain, we developed a pTPS331-compatible mycobacterial expression vector called pTPS628 (refer methods, Figure 2B) based on the *M. ulcerans* pMUM001 mycolactone plasmid (Figure 1). This plasmid is maintained at 1–2 copies per cell and has a restricted host range, replicating in *M. marinum* but not in fast-growing mycobacteria such as *M. smegmatis* and *M. fortuitum* [10,15]. We cloned LM-M8 into this vector (pTPS629) and tested the stability of the plasmid in *M. marinum* in the absence of antibiotic selection. Approximately 70% of the population retained pTPS629 in the absence of apramycin selection over the course of the 12-day growth curve experiments (Figure 6).

*M. marinum* was transformed with pTPS334 (*mlsA2*) and pTPS629 (*mlsA1 LM-M8*) and whole cell lysates were screened by Western immunoblot, with the acyltransferase domain antibody. Two proteins with masses around 400 kDa and 260 kDa were observed, corresponding to MlsA2 and LM-M8 respectively (Figure 7A, B). We took advantage of the C-terminal His<sub>5</sub> tag introduced into MlsA2 and also screened the whole cell lysates with an anti-His<sub>5</sub> antibody. As expected, a protein with a
mass of 260 kDa was detected (Figure 7C), that was confirmed by peptide mass fingerprinting, with five high scoring peptides (p<0.05) spanning the 2416 aa of the MlsA2 polypeptide (positions 75–91, 1286–91, 1631–64, 1864–94, 1948–63, 2362–79); Cell fractionation was also performed and the Western blotting repeated, suggesting again that MlsA2 associates with the mycobacterial cell wall (Figure 7D).

The Predicted TKL is not Produced in Recombinant M. marinum

M. marinum with pTPS334 (MlsA2) and pTPS629 (LM-M8) was expected to produce the same TKL previously detected in M. ulcerans 06-3844. However, LC-MS analysis of ethylacetate extracts from this recombinant M. marinum failed to detect the expected metabolite (data not shown). The genes mup045 and mup038 cluster with the mls genes on pMUM001 and are predicted to encode accessory enzymes, important for mycolactone synthesis (Figure 1) [10]. A third expression vector was prepared containing mup045 and mup038 as an operon under the control of the constitutive Streptomyces ermE promoter (pTPS338) and used to transform M. marinum already harbouring LM-M8 and mlsA2. RT-PCR analysis of the triple plasmid recombinant strain (TPS8334) showed that these two additional genes were expressed (Figure 8A). Western blots using a peptide-derived polyclonal antibody against mup038 in M. marinum TPS8334 fractions suggests Mup038 also localizes to the cell wall (Figure 8B). Mup038 immunoblots with M. ulcerans 06-3844 wild type showed very faint reactivity to the antibody, suggesting low expression of Mup038 in the natural host (data not shown). Immunoblotting with polyclonal antibodies raised against a Mup045-specific peptide confirmed Mup045 protein expression in wild type M.
ulcerans 06-3844 (Figure 8C). Fractionation experiments with wild type and recombinant M. ulcerans 06-3844 showed endogenous Mup045 partitioning with the cell wall fraction (Figure 8D). The same fractions from M. marinum were also screened to confirm the presence and location of the MlsA1 LM-M8 PKS and MlsA2. Immunoblotting with the α-AT domain antibody showed again that LM-M8 and MlsA2 associate with the mycobacterial cell wall (Figure 9). Together with the other fractionation experiments for recombinant M. marinum and M. ulcerans (Figures 4 & 7), these data suggest that mycolactone biosynthesis occurs in association with the mycobacterial cell wall. Ethylacetate extracts were then prepared from the recombinant M. marinum and screened for TKL by LC-MS, but disappointingly the expected metabolite was not detected in the heterologous host.

Fluorescence microscopy confirms a cell wall location for Mup045. Taking advantage of the specificity of the Mup045 antibody, we performed fluorescence microscopy to test our proposition that the mycolactone machinery is cell wall associated (Figure 10). A distinctive, focal, pericellular pattern of fluorescence was observed in M. ulcerans that was absent from M. marinum (Figure 10). These observations provide further support for a cell wall location of Mup045. We also attempted microscopy with the α-AT domain antibody, however, as seen with the Western immunoblots (Figure 7), this antibody was somewhat cross-reactive and stained M. ulcerans and M. marinum equally well (data not shown).

Discussion

In this study we have developed two modified mycolactone PKS genes and used them to gain a better understanding of the multienzymes they encode, as well as explore their potential for combinatorial polyketide biosynthesis. In the first instance we constructed a minimum version of the 51 kb mlsA1 gene (LM-M8). When introduced into M. ulcerans via a pYUB412-based integrating mycobacterial expression vector, the mlsA1 LM-M8 produced the expected triketide lactone by comparison with the authentic compound and high-resolution mass spectrometry analysis.
observed 115.0755, calculated for C₆H₁₁O₂⁺[M+H]⁺ 115.0754) (Figures 4 & 5). This experiment showed mlsA1 LM-M8 formed a functional PKS and demonstrated in principle that the mls genes can indeed be manipulated to produce novel small molecules, in this case a methyl triketide lactone (TKL). However, the amount of the methyl TKL obtained was low and when a second construct was tested using the same vector in the same M. ulcerans strain, but where the AT-acetate domain of the loading module was replaced with an AT-propionate version, the expected protein subunit was present (Figure 4), but the predicted ethyl TKL was not.

Figure 10. Imaging of Mup045 in association with the mycobacterial cell wall in M. ulcerans 06-3844 wild type compared with M. marinum M wild type, as revealed by DIC fluorescence microscopy. Cells were stained with DAPI and incubated with a primary anti-Mup045 antibody with visualization by a secondary antibody conjugated to Alexa fluor-568.
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There are several possible reasons for the low yield or absence of detectable TKL. It may be that the chain-terminating thioesterase (TE) of MlsA2 discriminates against the shorter chains or that transfer of the normal mycolactone F octaketide intermediate onto the endogenous MlsA2 outcompetes transfer of the diketide acyl chain from the truncated hybrid PKS (Figures 1 & 2). To try and test the latter hypothesis we transformed M. marinum M with mlsA1 LM-M8 and an epitope tagged version of mlsA2. M. marinum is potentially an ideal host to test expression of the mls PKS genes. It is very closely related to M. ulcerans, but does not contain the pMUM plasmid and does not make mycolactones. The M. marinum genome encodes a substantial secondary metabolome that includes many type 1 PKS loci and the necessary accessory enzymes required by these mega-enzymes such as 4’-phosphopantetheinyl transferase to transfer a 4’-phosphopantetheinyl (4’-PP) moiety from coenzyme A (CoA) to the ACP [30]. To facilitate independent expression of two, relatively large genes in M. marinum (mlsA1 LM-M8: 11 kb and mlsA2: 7.2 kb) we also developed a novel expression plasmid based on the pMUM001 ori. This plasmid has an active par locus and is stably maintained at low copy number even in the absence of antibiotic selection in M. marinum (Figure 6) [10,15]. The mlsA1 LM-M8 gene was cloned into this pMUM-based plasmid and this construct, together with mlsA2 cloned into pYUB412 was used to transform M. marinum M. Each plasmid expressed their respective mls PKS genes from the mlsA1/mlsB promoter; a regulatory sequence we have previously shown to function in M. marinum M [26]. Immunoblotting confirmed the presence of both MlsA1 LM-M8 and MlsA2 in M. marinum (Figure 7B). The identity of MlsA2 was additionally confirmed by immunodetection of its C-terminal 5x His epitope (Figure 7C). However, while the Mls PKS appeared to be expressed at reasonable levels (proteins visible by Coomassie staining), this recombinant M. marinum did not produce detectable methyl TKL, even in the absence of competing mycolactone intermediates. Supplying M. marinum with the mycolactone accessory enzymes encoded by mup045 and mup038 on pMUM001 did not activate TKL synthesis. Mup045 resembles an atypical acyltransferase and Mup038, a type II thioesterase with a proposed role in ensuring the processivity of the Mls system [10].

The absence of the expected TKL prompted us to examine the cellular distribution of the Mls proteins. Immunohistochemistry showed co-localization of the Mls PKS and mup045 with the mycobacterial cell wall components in both M. ulcerans 06-3044 and the recombinant M. marinum TPS0334 (Figures 4, 8 &9). Very low levels of Mulp038 were detected in wild type M. ulcerans strains (data not shown), consistent with our previous study that showed low mup038 promoter activity compared to mlsA1, mlsB and mup045 [26]. Fluorescence microscopy also supported a cell wall location for the mycolactone synthesis machinery, with Mup045 present in distinct foci around the M. ulcerans cell wall (Figure 9). Attempts to similarly visualize the Mls PKS were not successful due to a lack of specificity with our anti-AT serum. This serum preparation was produced using the entire AT domain, a domain widely present in mycobacterial PKS and fatty-acid synthases (e.g. in M. marinum there are 16 proteins with this domain, sharing >50% aa sequence similarity). Future research to explore our initial observations will require Mls PKS antibodies with improved specificity. Nevertheless, the data we present here suggest that mycolactone synthesis is occurring at or within the cell wall, consistent with previous reports describing mycolactone blebbing from the bacterial cell in lipid and protein-rich vesicles [31] and an M. ulcerans proteomic investigation that also detected the Mls PKS in the cell wall [32]. It is interesting to speculate how these megadalton-sized molecular machines with their predicted homodimeric and interconnected structure are arranged within the mycobacterial cell wall. Only a handful of reports have uncovered PKS linked to the cell wall [33,34]. In mycobacteria there is a proposed pathway linking an RND superfamily transporter protein (MmpL7) with the type I PKS, PpsE [34]. It is possible that the Mls system is similarly dependent on MmpL-like proteins for mycolactone export.

A recent comparative genomic study of the evolution of M. ulcerans has revealed that cell wall-associated genes are undergoing significant diversifying selection compared to M. marinum [35]. Maybe this evolutionary signature is a response to selective pressures that are shaping M. ulcerans to accommodate the mycolactone synthesis machinery. Furthermore, it is possible that some of these changes are essential for the correct positioning, location, and thus functioning of the Mls PKS within the mycobacterial cell wall. M. marinum, despite its high shared genetic identity with M. ulcerans, may therefore not have the requisite genetics and cell wall composition for the Mls PKS to function correctly.

Our findings illustrate the promise of the Mls PKS for combinatorial polyketide biochemistry but also underline the experimental challenge. While a minimal Mls PKS produced the expected TKL, yields of metabolite were low or undetectable. To better understand the limits of modularity in this remarkable system, the next step should be direct assessment of the activity and specificity of individual mls domain and module activity, using recombinant proteins and diverse synthetic substrates, as recently described for a fully reducing module of the nanchangmycin type I PKS [36].

Meanwhile, the Mls and accessory enzyme expression constructs we have developed here have provided intriguing additional evidence that, in mycolactone formation at least, polyketide assembly on the multienzyme modular PKS and subsequent tailoring and export are carried out in intimate association with the cell wall.

Supporting Information

Table S1 Oligonucleotides used in this study. (DOCX)

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Author Contributions

Conceived and designed the experiments: JLP NJT SJP SF KLT AV HH PFL TPS. Performed the experiments: JLP NJT SJP SF KLT HH TPS. Contributed reagents/materials/analysis tools: JLP NJT SJF SF KLT HH AFL TPS. Wrote the paper: JLP NJT PFL TPS.

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