Transfer, stable maintenance and expression of the mycolactone polyketide megasynthase mls genes in a recombination-impaired Mycobacterium marinum

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The human pathogen Mycobacterium ulcerans produces a polyketide metabolite called mycolactone with potent immunomodulatory activity. M. ulcerans strain Agy99 has a 174 kb plasmid called pMUM001 with three large genes (mlsA1, 51 kb; mlsA2, 7.2 kb; mlsB, 43 kb) that encode type I polyketide synthases (PKS) required for the biosynthesis of mycolactone, as demonstrated by transposon mutagenesis. However, there have been no reports of transfer of the mls locus to another mycobacterium to demonstrate that these genes are sufficient for mycolactone production because in addition to their large size, the mls genes contain a high level of internal sequence repetition, such that the entire 102 kb locus is composed of only 9.5 kb of unique DNA. The combination of their large size and lack of stability during laboratory passage makes them a challenging prospect for transfer to a more rapidly growing and genetically tractable host. Here we describe the construction of two bacterial artificial chromosome Escherichia coli Mycobacterium shuttle vectors, one based on the pMUM001 origin of replication bearing mlsB, and the other based on the mycobacteriophage L5 integrase, bearing mlsA1 and mlsA2. The combination of these two constructs permitted the two-step transfer of the entire 174 kb pMUM001 plasmid to Mycobacterium marinum, a rapidly growing non-mycolactone-producing mycobacterium that is a close genetic relative of M. ulcerans. To improve the stability of the mls locus in M. marinum, recA was inactivated by insertion of a hygromycin-resistance gene using double-crossover allelic exchange. As expected, the ΔrecA mutant displayed increased susceptibility to UV killing and a decreased frequency of homologous recombination. Southern hybridization and RT-PCR confirmed the stable transfer and expression of the mls genes in both wild-type M. marinum and the recA mutant. However, neither mycolactone nor its predicted precursor metabolites were detected in either strain. These experiments show that it is possible to successfully manipulate and stably transfer the large mls genes, but that other bacterial host factors appear to be required to facilitate mycolactone production.

INTRODUCTION

Mycolactone is an immunosuppressive and cytotoxic polyketide and primary virulence factor produced by Mycobacterium ulcerans, the aetiological agent of the emerging human disease Buruli ulcer (Johnson et al., 2005). M. ulcerans strain Agy99 possesses a 174 kb plasmid named pMUM001 that harbours a cluster of three large genes (mlsA1, 51 kb; mlsA2, 7.2 kb; and mlsB, 42 kb), encoding the type I polyketide synthases (PKS) required for mycolactone synthesis (Stinear et al., 2004). Transposon mutagenesis has confirmed predictions based...
on DNA sequence analysis that MlsA1 and MlsA2 synthesize the mycolactone core while MlsB synthesizes the acyl side-chain (Stinear et al., 2004). Type I PKS are large, multi-modular enzymic complexes that produce polyketides by the NADPH-dependent sequential condensation of acetate or propionate, presented to the synthon complex as activated malonyl or methylmalonyl-CoA thioesters, respectively (Staunton & Weissman, 2001). Each module within type I PKS is responsible for the addition and subsequent modification of either an acetate or a propionate unit before the growing chain is passed to the next module. The fully extended polyketide is unloaded from the PKS complex via the activity of a terminal thioesterase domain. Additional tailoring enzymes may then make further modifications to the polyketide, such as cyclization, oxidation, glycosylation and ligation with other compounds. PMU001 also has three accessory genes that are involved, or have a predicted role, in mycolactone synthesis. These genes include mup038, encoding a putative type II thioesterase, mup045, encoding a potential acyltransferase that might catalyse the C–O bond between the mycolactone core and side-chain, and mup053 (yp140A7), encoding a P450 monoxygenase that hydroxylates C12’ of the side-chain (Stinear et al., 2004, 2005a). The mlsA1, mlsA2 and mlsB genes span 105 kb and encode 11 different functional domains that are repeated across the two load modules (LMs) and 16 extension modules that comprise these PKS. An unusual feature of the PKS is their very high level of intra-domain sequence identity, such that domains of the same function share 97–100% amino acid and nucleotide identity. As a consequence, the entire 105 kb mls locus comprises only 9.5 kb of unique DNA sequence, suggesting that it has arisen by a series of in-frame duplication and recombination events (Hong et al., 2005; Pidot et al., 2008; Stinear et al., 2005a).

It has recently been discovered that mycolactones are produced not only by M. ulcerans but also by a variety of very closely related, pMUM-bearing mycobacteria, collectively referred to as mycolactone-producing mycobacteria (MPM) (Mve-Obiang et al., 2005; Ranger et al., 2006; Yip et al., 2007). Interestingly, different MPM produce mycolactone structural variants that differ in the composition of the acyl side-chain. Detailed DNA sequence analysis of different pMUM plasmids has uncovered the genetic basis for these variant mycolactones and shown how recombination-mediated swapping, duplication and deletion of the homologous PKS modules and domains within mlsB has led to the production of different mycolactone side-chain structural variants (Hong et al., 2005; Pidot et al., 2008). Intra- and inter-strain comparisons suggest that the extreme sequence homogeneity seen among the mls PKS genes is caused by frequent recombination-mediated intra-strain domain replacement. These observations have also raised the possibility that the mycolactone mls locus represents a genetic toolbox for production of novel bioactive polyketides through combinatorial biochemistry, an area of considerable worldwide research interest (Weissman & Leadlay, 2005).

However, it has been difficult to explore these ideas and to study the structure and biological effects of mycolactones because obtaining useful quantities of the molecule from M. ulcerans and other MPM is extremely problematic. These mycobacteria are slow-growing (doubling time >20 h), the yield of metabolite is variable and generally in the microgram range, and systems for their genetic manipulation are poorly developed. Heterologous expression, specifically the transfer of the entire mls locus to a faster growing and more genetically tractable host organism for the production of mycolactones, has been a major research objective to try and secure the supply of mycolactones for further study and to develop a platform for investigation of their potential for combinatorial biochemistry. In this study we set out to develop M. marinum as a heterologous host of the mls locus because of its very close relationship (>97% shared genome-wide nucleotide identity) to all MPM. Comparative genomics has shown that M. ulcerans and the other MPM evolved recently from a single M. marinum progenitor through acquisition of the pMUM plasmid and reductive evolution (Stinear et al., 2007; Yip et al., 2007). We reasoned that M. marinum was thus more likely than other mycobacteria, or indeed other actinobacteria such as Streptomyces that also naturally produce methyl-branched polyketides from type I PKS, to contain the requisite genetic background for mycolactone production.

Escherichia coli bacterial artificial chromosomes (BACs) have been used to facilitate the transfer of large secondary metabolite loci such including type I PKS from Streptomyces spp., through E. coli to an alternative host bacterium (Martinez et al., 2004). Furthermore, specific advances in recombineering have facilitated the modification of loci such that they can be manipulated to produce new metabolites (Fu et al., 2008). However, the unusually large size and extent of internal sequence duplication within the mls genes make their manipulation a particular challenge in molecular biology. We set out to develop two different E. coli/Mycobacterium shuttle vectors based on the BAC vector pBeloBAC11, combined with either a derivative of pMUM001 or a mycobacteriophage-based integration system. Using these BAC constructs in M. marinum strain M and also in a specifically constructed recombination-deficient M. marinum M, we sought to test the hypothesis that the mls PKS are not only necessary for mycolactone synthesis but also sufficient, and in so doing, to develop a platform for the stable production and study of mycolactones.

METHODS

Bacterial strains, plasmids and media. E. coli DH10B [F− mcrAΔ (mrv-5sdRMS-mcrBC) 80lacZAM15 ΔlacX74 deoR recA1 araD139 Δ(ara, leu)7697 galU galK rpsL endA1 mupG] was used for all experiments and cultured in Luria–Bertani broth or agar at 37 °C.
supplemented with antibiotics as required at the following concentrations: ampicillin, 100 μg ml⁻¹; apramycin, 50 μg ml⁻¹; hygromycin, 200 μg ml⁻¹; and kanamycin, 50 μg ml⁻¹. *M. marinum* M was cultivated at 30 °C in Middlebrook 7H9 broth or 7H10 agar (Becton Dickinson) supplemented with oleic acid albumin dextrose complex (OADC; Difco) and antibiotics as required at the following concentrations: apramycin, 50 μg ml⁻¹; hygromycin, 50 μg ml⁻¹; and kanamycin, 25 μg ml⁻¹. All plasmids and strains used in this study are listed in Tables 1 and 2, respectively. Electromobility mutant *M. marinum* wild-type (WT) was prepared as described elsewhere (Talaat & Trucksis, 2000), and 2–4 μg BAC DNA was used to transform mycobacteria. After electroporation, cells were incubated overnight at 30 °C and plated on 7H10 agar containing appropriate antibiotics, and incubation was continued at 30 °C for up to 3 weeks.

**Oligonucleotides and general DNA methods.** The oligonucleotides used for the construction of *M. marinum* ΔrecA are listed in Supplementary Table S1, and those used to PCR-screen for the presence of pMUM001 genes in recombinant *M. marinum* are listed in Supplementary Table S2. BAC DNA was extracted from *E. coli* using the method of Brosh et al. (1998) or using a plasmid DNA purification Nucleobond BAC 100 column, following the manufacturer’s instructions (Macherey-Nagel). The methods for preparation of genomic DNA from *M. marinum*, DNA probe preparation by PCR and Southern hybridization were performed as described previously (Yip et al., 2007). Conditions for PFGE, including DNA plug preparation, electrophoretic separation and restriction enzyme digestion (complete and partial) were essentially as described previously (Stinear et al., 2005a), with one modification. Prior to the final washing in 1× Tris-EDTA, DNA plugs for PFGE were exposed to an additional lysis treatment that included a wash for 3 h in a lysis solution of 1 M guanidine hydrochloride, 1% Tween 20 and 1% Nonidet P-40. In vitro transposon mutagenesis was performed using the Ex-Tn5 system following the manufacturer’s instructions (Epicentre Biotechnologies).

**Allelic replacement to inactivate recA using pDLS.** Allelic exchange was used to disrupt *recA* by insertion of a hygromycin-resistance gene. The entire *recA* gene (MMAR_1977) and flanking regions were amplified from *M. marinum* M by PCR as a 2 kb fragment incorporating XbaI termini and ligated into pGEM-T-Easy, resulting in plasmid pJKD2940. A hygromycin-resistance gene, modified by PCR to incorporate HindIII termini, was ligated into the unique HindIII site of recA within pJKD2940 to produce pJKD2941. A 3.2 kb fragment containing the disrupted *recA* gene and flanking sequence was then excised from pJKD2941 by *XbaI* digestion and ligated into the unique *XbaI* site of the mycobacterial allelic exchange vector pDLS (Lea-Smith et al., 2007) to create pJKD2960 (Table 1, Fig. 1). Following introduction of pJKD2960 into *M. marinum* M by electroporation, the bacteria were incubated for 2 weeks at 30 °C on 7H10 Middlebrook agar containing hygromycin and streptomycin to select for single-crossover recombination events. Colonies resistant to both antibiotics were then subcultured by dilution-streaking onto fresh 7H10 agar and incubated at 30 °C for 2 weeks without any selection. A 5 μl loopful of bacteria was harvested from each plate and resuspended in 7H9 broth, serially diluted, and plated out on 7H10 agar containing 2% sucrose and hygromycin. After 10 days incubation at 30 °C, potential double-crossover mutants were selected by patching sucrose- and hygromycin-resistant colonies onto 7H10 with and without streptomycin to identify colonies that were sucrose- and hygromycin-resistant but streptomycin-sensitive (Parish & Stoker, 2000). Colonies were screened by PCR using oligonucleotides DAPI039 and DAPI040, which span the site of the hygromycin insertion in *recA*. A complementation plasmid was prepared by cloning intact *M. marinum* recA/X genes and upstream sequence into the unique *XbaI* site of pMV261 to create pJKD3087.

**UV exposure assay.** *M. marinum* strains (Table 2) were grown to stationary phase and then diluted to OD 0.7 in sterile 1× PBS (Difco). Serial 10-fold dilutions of each strain were made between 10⁻¹ and 10⁻⁶. For each dilution from 10⁻⁴ to 10⁻⁶, six 50 μl aliquots were spread onto 7H10 containing appropriate antibiotics. Each strain was exposed to UV doses of 0, 5, 10, 15, 20 and 30 J m⁻² using a UVtech CL-580 cross-linker. Plates were then incubated at 30 °C for 10–14 days and colonies counted to assess viability.

**Recombination assay.** A 1576 bp partial fragment of an allele of *rpoB* that contained the F445S rifampicin-resistance-conferring mutation, obtained by PCR from a spontaneous rifampicin-resistant laboratory-derived *M. marinum* mutant, was cloned into the unique *NsiI* site of the integrating *E. coli/mycobacterial* vector pJKD8003 (Stinear et al., 2005b) and integrated into the chromosomes of different *M. marinum* strains (Table 2). Spontaneous mutation frequencies and recombination frequencies were calculated by dividing the number of rifampicin-resistant colonies by the number of colonies grown on non-selective media for each *M. marinum* construct.

**Plasmid stability assay.** Late-exponential-phase cultures of *M. marinum* harbouring pJKD2679 grown in the presence of kanamycin were diluted 1:100 into three 50 ml volumes of fresh 7H9 media.

**Table 1.** Plasmids used in this study

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<th>Name</th>
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<td>(1576 bp F445S mutation)</td>
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without kanamycin and incubation was continued at 30 °C for 12 days. Aliquots of each culture were then removed at successive 3-day time points, and appropriate dilutions were made and then plated on solid media with and without kanamycin. Colonies were counted after 10 days. The total cell number (expressed as c.f.u.) and the proportion of the total cell population that had maintained antibiotic resistance at each time point were calculated.

Quantitative RT-PCR (qRT-PCR). Reverse transcription was performed as described elsewhere (Gunesekere et al., 2006), except for the use of 1 μg RNA with 5.5 μg random hexamers. Reactions were stopped by heating to 70 °C for 15 min. cDNA from at least two biological repeats of each M. marinum strain, JKD8122 and JKD8090, at both exponential and stationary phase was tested. Each qPCR contained 10 μl SYBR green PCR master mix (Applied Biosystems), 500 nM of each forward and reverse primer, and 4 μl of a 1:100 dilution of cDNA in a total volume of 20 μl. Samples were cycled in a Realplex4 (Eppendorf) real-time PCR thermocycler at 95 °C for 2 min, 95 °C for 1 min. Following each run melt curves were performed. Analysis was performed utilizing the ΔΔCt method (Dussault & Poulriot, 2006).

Mycolactone extraction and analysis. Total lipids were extracted from bacterial cell preparations with chloroform : methanol (2:1, v/v) for 2 h at room temperature. After separation from the aqueous phase following the addition of 0.9 % NaCl (w/v), the organic phase was dried and the resulting material resuspended in ice-cold acetone. This acetone-soluble fraction was resuspended in methanol for analysis by electrospray ionization-liquid chromatography-MS (ESI-LC-MS) and ESI-LC-MS-MS (helium collision gas), using a Finnigan LCQ ion trap (Thermo Finnigan) coupled with an HP1100 liquid chromatograph fitted with a ThermoHypersil BDS C8 column (5 μm, 4.6 × 250 mm). Mycolactones were eluted with a 40 min gradient from 55 to 95 % acetonitrile in water. The presence of [M + Na]+ m/z 765.5 (mycolactone A/B) and [M + Na]+ m/z 749.5 (mycolactone C) was assessed by comparison of the MS-MS spectra with those of pure mycolactone preparations.

### RESULTS

**Inactivation of recA in M. marinum by allelic replacement**

We have previously shown that the mls locus is unstable in laboratory-passaged MPM, with loss-of-mycolactone mutants arising frequently and unpredictably through spontaneous deletion of fragments of the mls genes by intra-mls homologous recombination (Stinear et al., 2005a). To try and reduce the occurrence of these mutations we disrupted recA in M. marinum M by insertion of a hygromycin-resistance gene (Fig. 1) by allelic exchange, using a non-replicating plasmid containing a counter-selectable marker (sacB) to isolate a double-crossover recA mutant (Fig. 1). Eighteen hygromycin- and streptomycin-resistant single-crossover M. marinum isolates were obtained following transformation of M. marinum M (JKD8024) with 6 μg circular pJKD2960. After further passaging of these isolates, four potential double-crossover mutants that were hygromycin- and sucrose-resistant but streptomycin-sensitive produced a PCR product of the expected 1.7 kb, and one (JKD8074) was selected for further testing. The correct mutation in JKD8074 was confirmed by sequencing the PCR product and Southern hybridization (Fig. 2). The mutant was complemented by transformation of M. marinum ΔrecA JKD8074 with pJKD3087 (Fig. 1) to produce strain

<table>
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JKD8099. PCR, DNA sequencing and Southern hybridization confirmed that this strain was correct (Fig. 2).

*M. marinum* Δ*recA* JKD8074 displays increased sensitivity to UV killing

A RecA-deficient mutant is expected to display increased sensitivity to UV-induced DNA damage, as RecA is central to the SOS response and repair of DNA. *M. marinum* M WT (JKD8024), the Δ*recA* mutant (JKD8074) and the complemented mutant (JKD8099) were each exposed to increasing doses of UV, and colony counts were used to monitor the percentage of cells surviving UV exposure compared with the same strain that was unexposed. The *recA* mutant was significantly more sensitive to UV than the WT, with less than 10% of the population surviving the maximum dose tested of 30 J m\(^{-2}\) compared with 80% survival for the WT. Reintroduction of an intact copy of *recA* restored survival to nearly WT levels (Fig. 3).

*M. marinum* Δ*recA* JKD8074 is recombination-deficient

The primary motivation for inactivation of *recA* was to reduce homologous recombination in *M. marinum*, so we assessed the effect of inactivation of the *recA* mutation on
the frequency of homologous recombination by comparing the WT, mutant and complemented mutant in an rpoB recombination assay (Papavinasasundaram et al., 1998). These experiments showed that the recA mutant was completely defective for homologous recombination. The frequency of occurrence of recombination-mediated rifampicin resistance in M. marinum ΔrecA JKD8074 was not significantly different to the spontaneous rpoB mutation rate of $3.9 \times 10^{-9}$ (Fig. 4), but it was significantly lower than the WT homologous recombination frequency of $2.8 \times 10^{-7}$ ($P<0.05$). Complementation of the mutant restored homologous recombination to near WT levels (Fig. 4).

**Transfer of pMUM001 from M. ulcerans to M. marinum using BAC shuttle vectors**

Several faster-growing mycobacteria were considered as potential heterologous hosts for pMUM001, such as Mycobacterium smegmatis and Mycobacterium fortuitum; however, previous studies have shown that the pMUM ori is functional in M. marinum but not in these other species (Stinear et al., 2005b). We considered that 174 kb was probably too large and unstable to clone and transfer as a single DNA molecule, so we took advantage of two BAC clones that had been generated during the M. ulcerans Agy99 genome project (22D03 and 22B04) (Stinear et al., 2005a). Together, these two clones spanned the entire pMUM001 plasmid and are depicted in Supplementary Fig. S1. Clone 22D03 was a 109 kb deletion variant of pMUM001 that had arisen by recombination between the start of mlsA1 and mlsB that deleted the 65 kb region.

![Fig. 2. Confirmation of the recA insertional mutation in M. marinum JKD8074. (a) Nsil digestion and separation of genomic DNA from (lane 1) M. marinum M WT (JKD8024) and (lane 2) M. marinum ΔrecA (JKD8074); (b) Southern hybridization of (a) with a recA probe; (c) PCR analysis of genomic DNA from (lane 1) JKD80724 and (lane 2) ΔrecA JKD8074 with oligonucleotides DAP1039 and DAP1040. (d) Nsil digestion and separation of genomic DNA from (lane 1) M. marinum M WT (JKD8024), (lane 2) M. marinum ΔrecA (JKD8074), (lane 3) M. marinum ΔrecA-complemented (JKD8099), (lane 4) pJKD3087 and (lane 5) pMV261; (e) Southern hybridization of (d) with a probe from the kanamycin-resistance gene of Tn9.](image)

![Fig. 3. M. marinum M ΔrecA is sensitive to UV killing. Comparison of the sensitivity of M. marinum M WT (JKD8024, ■), M. marinum ΔrecA (JKD8074, ▲) and the complemented ΔrecA mutant (JKD8099, △) to increasing doses of UV irradiation. Each data point is the mean and se of at least three biological repeats.](image)

![Fig. 4. M. marinum M ΔrecA is recombination-deficient. Comparison of frequencies of homologous recombination and spontaneous mutation among M. marinum WT (JKD8024), M. marinum ΔrecA (JKD8074) and the complemented (compl) ΔrecA mutant (JKD8099). Depicted are the frequency of homologous recombination between a complete WT rpoB allele and an additional mutated partial copy of rpoB, calculated by the appearance of rifampicin-resistant mutants, and compared with the frequency of occurrence of spontaneous rifampicin-resistant mutants. The data are presented as the mean and se of at least three biological repeats. *P<0.05 by Student’s t test, comparing M. marinum strains carrying an additional partial copy of either the WT or the mutated rpoB allele.](image)
spanning mlsA1, mlsA2 and mup038 (Stinear et al., 2005a) (Supplementary Fig. S1). Thus, clone 22D03 was predicted to contain a functional pMUM replicon with an intact mlsB gene, and should be capable of expressing the MlsB synthase required to synthesize the fatty-acyl side chain of mycolactone. To facilitate transfer to M. marinum it was necessary to add a selectable antibiotic-resistance marker. In vitro transposon mutagenesis of 22D03 in E. coli using EZ-Tn5 resulted in a library of kanamycin-resistant E. coli 22D03::Tn5 KanR mutants, and one was selected that had the kanamycin-resistance gene inserted in a region of pMUM that would be unlikely to affect mycolactone synthesis or plasmid replication, between a copy of IS2606 and a hypothetical coding sequence. The plasmid was named pJKD3030 (Supplementary Fig. S1) and it was then used to transform both M. marinum M WT (JKD8024) and M. marinum M ΔrecA (JKD8074). For each strain, approximately 200 kanamycin-resistant colonies resulted, and 10 of these were patched and then screened by colony PCR for different markers of pMUM and the mlsB gene (parA, mup045, mlsB; LM and ketoreductase domains). More than 90 % of the colonies were PCR-positive for all markers, and further analysis by PFGE and Southern hybridization of one transformant from each group revealed that they contained a full-length plasmid (compared with the original construct pJKD3030 prepared in E. coli), resulting in strains JKD8070 and JKD8079 (Table 2, Supplementary Fig. S1).

The stability of the pMUM001-based replicon pJKD3030 was assessed by its ability to persist within M. marinum M in the absence of kanamycin selection. The results of this experiment are summarized in Fig. 5 and show that the pMUM001-based replicon was stably maintained and not lost from a population of cells in the absence of antibiotic selection. These data also indicate that maintenance of the plasmid had no discernible effect on growth rate in either of the recombinant M. marinum strains (Fig. 5).

The second E. coli BAC clone, 22B04, contained an 82 kb fragment of pMUM001 that included intact mlsA1, mlsA2 and mup038 genes (predicted to express the MlsA synthases required to produce the lactone core of mycolactone), and spanned the region of pMUM001 absent from 22D03 (Supplementary Fig. S1) (Stinear et al., 2005a). To transfer this region to M. marinum, a new BAC E. coli/Mycobacterium shuttle vector was developed by combining pBELOBAC11, the broad-host-range integrase attP of mycobacteriophage L5 (Hatfull & Sarkis, 1993) and the apramycin-resistance gene (Paget & Davies, 1996) to create pBEL5 (Supplementary Fig. S1).

The 82 kb region of pMUM001 within BAC clone 22B04 was then isolated by partial HindIII digestion and separation by PFGE and then ligation into the unique HindIII site of pBEL5, resulting in pJKD2679 (Supplementary Fig. S1). The construct was confirmed in E. coli by restriction fragment mapping, PCR, insert fragment size estimation by PFGE, BAC end-sequencing and Southern analysis (Fig. 6). M. marinum strains JKD8079 (M. marinum WT-pJKD3030) and JKD8080 (M. marinum ΔrecA-pJKD3030) that contained the 109 kb truncated pMUM001 replicon were transformed with pJKD2679 by electroporation, each strain yielding approximately 100 colonies resistant to apramycin and kanamycin. For both M. marinum strains, JKD8079 and JKD8080, 20 colonies were patched and screened by PCR for markers of the presence of pJKD2679 (mup038 and mlsA enoylreductase domain). Only one colony from each strain was PCR-positive for the two markers, but both of these strains were verified to contain full-length plasmid

![Fig. 5. A derivative of the pMUM001 plasmid is stable in M. marinum and has no effect on growth. Late-exponential-phase cultures of M. marinum JKD8074 and JKD8080, grown in the presence of kanamycin, were shifted to media without kanamycin and then monitored at successive time points by determining plate counts on media with and without the antibiotic. Shown is the stability of pJKD3030 in (a) M. marinum M WT (JKD8024) and M. marinum M-pJKD3030 (JKD8079), and (b) M. marinum M ΔrecA (JKD8074) and M. marinum M ΔrecA-pJKD3030 (JKD8080) in the absence of kanamycin selection. The percentage of c.f.u. containing the plasmid over successive time points is indicated by the persistence of cells resistant to kanamycin, expressed as a percentage of the total number of c.f.u. in the absence of kanamycin. For the total c.f.u. counts, each time point is the mean ± SEM of three biological repeats.](image-url)
(compared with the original construct pJKD3030 prepared in E. coli), resulting in strains M. marinum JKD8122 (M. marinum WT-pJKD3030-pJKD2679) and M. marinum JKD8090 (M. marinum ΔrecA-pJKD3030-pJKD2679). These constructs were confirmed correct by Dral digestion and RFLP analysis, which showed the predicted absence of the 253 397 bp Dral chromosomal fragment, which would arise following the specific integration of pJKD2679 at glyV (tRNA-glycine anticodon TCC, genome coordinates 4 732 971–4 733 044) (Fig. 6a). Southern hybridization analysis confirmed the presence of the full-length construct (Fig. 6b). These two strains, M. marinum JKD8122 and M. marinum JKD8090, were then tested for expression of the mls genes and the presence of mycolactones.

The mls genes are transcribed in M. marinum

To test whether the mls and other mycolactone-associated genes were transcribed in M. marinum, RNA was extracted from both late-exponential- and stationary-phase cultures of M. marinum JKD8122 and M. marinum JKD8090. qRT-PCR was used to measure the relative abundance of mRNA from mlsA1/mlsB, mup045 and mup053 compared with the expression of the housekeeping gene ppk. Levels of transcription, as measured by detecting a region of the ketosynthase domain within the LM at the 5’ end of the mls genes, were up to 10-fold higher than transcription of the terminal thioesterase (TEI) domain at the distal 3’ end (Fig. 7). Transcription was higher during exponential phase than during stationary phase. Both mup045 and mup053 appeared to be highly transcribed during the exponential phase, with levels 20 ± 3.7-fold higher than ppk for JKD8090 and 13 ± 2.9-fold higher for JKD8122 (Fig. 7). Stationary-phase transcripts appeared to be expressed at levels similar to that of the ppk gene for all genes.

Other host factors appear to be required for mycolactone synthesis

Acetone soluble lipids (ASLs) were extracted from whole-cell fractions of M. marinum JKD8090 and JKD8122 that had been cultured both in 7H9 Middlebrook broth and on Middlebrook 7H10 agar. ASLs were screened by high performance thin-layer chromatography (HPTLC) and LC-MS for the presence of the expected mycolactone A/B and the precursor compounds, mycolactone C and the macrolactone core; however, despite repeated testing, using different batches of culture, none of the expected metabolites was detected (Supplementary Fig. S2). In addition, there were no observed differences in colonial morphology or other gross phenotypic characteristics between the pMUM001-bearing M. marinum strains and M. marinum WT that might be expected if the strains were producing a hydrophobic compound such as mycolactone. These results suggest that the mls genes are not sufficient for mycolactone production in M. marinum.

DISCUSSION

An assured supply of mycolactone is essential to enable more comprehensive studies of its effects, its cellular target(s) and its role in the survival of bacteria, and also to conclusively demonstrate that the respective genes are both necessary and sufficient for toxin production. With these aims in mind, we developed a strategy to transfer the pMUM001 plasmid from M. ulcerans strain Agy99 to M. marinum M, a faster growing, genetically tractable and closely related species, and thus develop a heterologous host for the production of mycolactone. The amenability of M. marinum to genetic modification was demonstrated by the relative ease with which recA was inactivated in this study.
Our rationale for disruption of recA was based on the observation that the mls genes, when cloned in BAC vectors and hosted by E. coli DH10B, are surprisingly stable, with mls deletion mutants rarely observed during laboratory culture and passage. In contrast, the mls locus is notoriously unstable among M. ulcerans strains during laboratory passage (Stinear et al., 2005a). We proposed that the absence of homologous recombination in E. coli DH10B (a ΔrecA mutant) was increasing mls stability and that inactivating recA in M. marinum might confer similar constancy to the mls locus. We disrupted recA by using allelic exchange to insert a hygromycin-resistance gene within recA. As RecA is central to the SOS response, we confirmed that the mutant had the expected increased sensitivity to UV killing (Fig. 3). We also showed, using a previously described rpoB/rifampicin-resistance assay, that the mutant was recombination-deficient (Fig. 4). Both of these phenotypes were restored by complementation with an intact copy of recA, thus demonstrating for the first time the function of recA in M. marinum and showing that it is a non-essential gene under in vitro growth conditions.

We next developed a strategy to transfer pMUM001 from M. ulcerans Agy99 to M. marinum M WT and M. marinum M ΔrecA by using two modified pBELOBAC11 clones that could replicate in E. coli and M. marinum. These shuttle plasmids each contained large M. ulcerans Agy99 DNA fragments that together spanned all of pMUM001. BAC clone pJKD3030 was a 119 kb construct that contained all of pMUM001, including its origin of replication and putative partition locus, but lacked a 65 kb region that encompassed mlsA1, mlsA2 and mup038. Stability testing in the absence of antibiotic selection (Fig. 5), and Southern hybridization (Fig. 6b), indicated that pJKD3030 functions as a stable, autonomous replicon in M. marinum. Also of note was the high efficiency of transformation of M. marinum with this construct, suggesting that derivatives of pMUM001 could be more widely used as a tool for the transfer of large DNA fragments. In a variation on a method recently described using E. coli and multiple invertebrate hosts (Waterfield et al., 2008), the pMUM001–pBELOBAC11 shuttle system developed in this study could be exploited to study tuberculosis pathogenesis. For example, large genomic fragments could be transferred from Mycobacterium tuberculosis (or other mycobacterial pathogens) to M. marinum with testing for potential effects on the virulence of M. marinum.

The second heterologous expression construct, pJKD2079, was a 94 kb plasmid and also based on pBELOBAC11 but combined with the integrase (a tyrosine recombinase) from the broad-host-range L5 mycobacteriophage. This vector facilitated the successful transfer and integration of mlsA1, mlsA2 and mup038 from pMUM001 to M. marinum-pJKD3030 and M. marinum ΔrecA-pJKD3030. Transformation efficiencies in both strains were low with this construct, probably attributable to an inverse relationship between the efficiency of site-specific recombination and the length of the donor DNA molecule. Nevertheless, PCR, RFLP and Southern hybridization confirmed integration of the full-length constructs in both M. marinum WT and M. marinum ΔrecA (Fig. 6). This vector system too might find broader application as a tool for manipulation and transfer of mycobacterial genomic DNA. Similar phage integrate systems have been described for the successful heterologous expression of bacterial secondary metabolite clusters.
isolated from metagenomic libraries (Martinez et al., 2004).

Unfortunately, despite comprehensive screening of lipid extracts from these recombinant M. marinum strains, we failed to detect the presence of the predicted mycolactone A/B or its precursor metabolites such as mycolactone C and the lactone core (Supplementary Fig. S2). Expression of the mls genes in M. marinum was not the reason for this failure, as qRT-PCR revealed transcription from mlsA1, mlsA2, mlsB, mup045 and mup053 (Fig. 7). There was a negative correlation between transcript abundance and distance from the start of the long mls genes. Transcription of these genes was also greater during exponential growth than during stationary phase. These observations are consistent with a detailed study of mls gene expression that we have conducted in M. ulcers, which shows that the mls genes and mup045 are under the control of SigA-like promoters (N. J. Tobias and others, unpublished data). SigA is the principal mycobacterial sigma factor and promotes constitutive gene expression during exponential growth (Hu & Coates, 1999).

However, in this report we have not established that the mycolactone PKS enzyme complexes are synthesized. It remains a possibility that one or more point mutations leading to frame-shifts or premature stop codons within the mls genes occurred during the cloning process. Unfortunately, the extended homology within the mls genes means that DNA sequencing to ensure that reading frames are intact is not a trivial undertaking. We fully sequenced the original BAC clones used in this study, but subsequent subcloning and passaging may have introduced loss-of-function mutations. We are currently developing appropriate antibodies and employing proteomic approaches to check for production of the predicted Mls synthases, although again, detection of megadalton-sized proteins is not straightforward (Velkov et al., 2006).

It is possible that other chromosomally encoded factors are required to facilitate Mls enzymic activity. For example, a reductase that catalyzes mycolic motif oxidation was encoded by mrsAB, which provides evidence for additional toxin heterogeneity as a result of specific changes in the modular polyketide synthase.

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REFERENCES


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