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# Introduction of *Mycobacterium ulcerans* Mycolactone Genes into a Heterologous Host

Samantha Elizabeth Wirth
*University of Tennessee - Knoxville*

To the Graduate Council:

I am submitting herewith a thesis written by Samantha Elizabeth Wirth entitled "Introduction of *Mycobacterium ulcerans* Mycolactone Genes into a Heterologous Host." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.

Pamela Small, Major Professor

We have read this thesis and recommend its acceptance:

Todd Reynolds, Chunlei Su

Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Carolyn R. Hodges,
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# Introduction of *Mycobacterium ulcerans* Mycolactone Genes into a Heterologous Host

A Thesis

Presented for the

Master of Science Degree

The University of Tennessee, Knoxville

Samantha Elizabeth Wirth

August 2008

## DEDICATION

Graduate school has been both a rewarding and challenging endeavor. The research described in this thesis is the culmination of three years of dedication and hard work in the laboratory. I could not have persevered through the good times and the bad without the steady support and love of my parents. I dedicate this thesis to them.

## ACKNOWLEDGEMENTS

When I first began my graduate career in microbiology, it was Brian Ranger who helped me to develop the skills that would be crucial to this project. I will always be thankful for his patience, guidance, and his quirky sense of humor. My lab mates have been like a second family to me throughout my graduate career, through the bad times as well as the many good times. I will always remember the BBQ's, potlucks, lunches at Nama, heart-to-heart talks, brainstorming sessions about Buruli ulcer disease, road trips, music, movies, and most importantly, the friendships. The advice, support, and approachability of my committee members, Dr. Su and Dr. Reynolds, is not something I take for granted. They have helped to make my graduate experience one which I will remember fondly in years to come. Elizabeth McPherson has been an amazing mentor throughout my six years as a graduate teaching assistant and has both exemplified and shared with me the art teaching. More than that, I especially value her friendship. Finally, I would like to thank my mentor, Dr. Pamela Small, whose guidance and support have been central to my graduate work. Dr. Small's passion for research is contagious and it has been an honor to work with her.

## ABSTRACT

*Mycobacterium ulcerans* is the causative agent of Buruli ulcer (BU), a necrotizing skin disease endemic to West Africa and Australia. The cytopathicity, cell cycle arrest and immunosuppression characteristic of BU are attributed to the production of a plasmid-encoded, macrolide toxin, mycolactone. The core of mycolactone is a product of two large polyketide synthases (PKS) and is conserved among all mycolactone congeners. Heterogeneity of the toxin is a result of differences in the polyketide side chain, the product of a third PKS. The mycolactone plasmid (MP) was initially thought to be restricted to *M. ulcerans*. However, other mycolactone producing mycobacteria (MPMs) have now been identified in association with diseased frogs and fish. Although plasmids are common in environmental mycobacteria, nothing is known about the ability of these plasmids to participate in horizontal transfer. This work investigates the expression of mycolactone in heterologous hosts, *M. fortuitum* and *M. marinum,* as well as the ability of MPMs to participate in conjugation.

In this work a 152 kb fragment of the 154 kb MP plasmid from *M. ulcerans* 1615 was cloned into pBeloBAC11 and introduced into *E. coli*. This plasmid, pMYCO7017, contains the mycobacterial plasmid origin of replication as well as the mycolactone gene cluster. A Kanamycin resistance gene was introduced into pMYCO7017 by transposon mutagenesis. A construct, pMYCO7017::TnKm, containing an insertion outside the mycolactone gene cluster, was isolated for further work. Electroduction was used to transfer pMYCO7017:TnKm from its *E. coli* host into both *M. marinum* 1218 and a plasmid minus strain of *M. fortuitum*.

One *M. marinum* and four *M. fortuitum* transformants were verified by PCR analysis. Lipids were extracted from the transformants, and then analyzed by TLC and cytotoxicity assay, but results were inconclusive. However, when lipid extracts were analyzed by mass spectrometry and HPLC, a novel molecule was discovered indicating that one transformant, *M. fortuitum* 10394.6 (pMYCO7017::TnKm) was able to produce the mycolactone core. This work is the first example of heterologous expression of an *M. ulcerans* mycolactone PKS genes. Importantly, the gene cluster contained on pMYCO7017::TnKm is expressed. These results further suggest that the requirements for synthesis of the mycolactone core differ from those for the side-chain.

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# CHAPTER 1 : LITERATURE REVIEW

## 1.1 Buruli ulcer disease

### 1.1.1 Ecology and environment

Buruli ulcer is an emerging disease associated with lethargic rivers, stagnant swamps and other inert water bodies in tropical (Van der werf, 1999) and temperate climates (Johnson, 1999) in over thirty countries around the world (WHO). In highly endemic areas of West Africa, the prevalence of Buruli ulcer may be as high as 151 cases per 100,000 people and may be equal to or greater than rates of tuberculosis infection (Amofah, 2002). Although the exact mode of transmission remains elusive, it is thought that *Mycobacterium ulcerans*, the causative agent of Buruli ulcer, is introduced into the human host through trauma to the skin. This hypothesis has been supported by case-control studies in Benin (Debacker, 2006), Cameroon (Pouillot, 2007), southeastern Australia (Quek, 2007), and Ghana (Raghunathan, 2005). These studies revealed that the risk of Buruli ulcer disease is decreased when people wear protective clothing, namely long trousers and long sleeve shirts, which help to shield the skin.

The specific ecological niche of *M. ulcerans* has not yet been determined and after dozens of years, there is only one example of an *M. ulcerans* culture obtained from the environment (Portaels, 2008). Additionally, the exact mode of transmission of Buruli ulcer disease is still unknown, but several different routes of infection are currently being investigated. *M. ulcerans* has been shown to form biofilms on aquatic plants (Marsollier, 2004) and the bacteria may be introduced into the skin by a prick from sharp grasses or weeds (Meyers, 1974). While direct contact with endemic water bodies in certain African countries is a documented risk factor for Buruli ulcer disease (Raghunathan, 2005), only a small percentage of cases in Australia are associated with direct contact with contaminated water. However, many Australian Buruli ulcer patients reported spending time within close proximity to swampy or marshy water bodies. This observation

suggests that direct contact with contaminated waters is not always necessary for a person to acquire Buruli ulcer disease.

Scientists are now searching for potential vectors, namely insects that may harbor the bacterium and transmit it from contaminated water sources to the human host. Marsollier and colleagues have shown that a predacious water insect, Naucoridae, can be experimentally infected with *M. ulcerans* and that the bacterium is able to colonize the salivary glands of these predacious aquatic insects. The insects can then transmit *M. ulcerans* to mice through a bite (Marsollier, 2002), raising the possibility that these predacious, biting insects may be potential vectors in Buruli ulcer transmission. In the Daloa region of the Ivory Coast, an area heavily endemic for Buruli ulcer, up to 5% of Naucoridae collected from the environment test positive for *M. ulcerans* DNA (Marsollier, 2002).

Mosquitoes are known to be a vector for several diseases of human importance including Malaria, Dengue Fever, and Yellow Fever, to name a few. Consequently, mosquitoes are also being investigated as potential vectors for transmission of Buruli ulcer disease. Mosquitoes were collected and pooled into groups of 1 – 23 individuals from six different locations during a Buruli ulcer outbreak in Australia (Fyfe, 2007). Two out of 42 mosquito pools tested PCR positive for IS2404, IS2606, and KR, *M. ulcerans* - specific DNA sequences (Fyfe, 2007). Further research is needed to elucidate the extent of the role of mosquitoes in transmission of Buruli ulcer.

During the on-going search for the environmental niche of *M. ulcerans*, PCR has been used to examine a variety of environmental samples, resulting in *M. ulcerans* DNA being detected in water filtrate, biofilms, vertebrates and invertebrates (Williamson, 2008). This illustrates that *M. ulcerans* is widely distributed in aquatic environments and suggests that there may be some kind of trophic role in transmission of the bacterium to humans. It is possible that there is not simply one mode of transmission and, in fact, there may be multiple pathways of infection occurring simultaneously in endemic sites around the world.

### 1.1.2 Pathology and clinical presentation of Buruli Ulcer Disease

Buruli ulcer disease often begins as an innocuous nodule or papule, similar in appearance to a spider bite, and surgical excision at this stage is usually curative (Van der werf, 1999). However, the harmless initial appearance of the disease, often in combination with various socio-economic factors and cultural beliefs, may partially explain why individuals in this stage of infection may not seek medical treatment. Next, the infection may progress to large areas of indurated skin, called plaques, or sizeable areas of edema. If left untreated, these preulcerative lesions can develop into large, necrotic ulcers within weeks to months (Raghunathan, 2005). The exact incubation time for the disease is unclear due to the slow growth of the causative organism and the uncertainty of exactly how the disease is transmitted. **Figure 1** shows both the non-ulcerative and ulcerative stages of Buruli ulcer disease.

Buruli ulcer disease can be clinically diagnosed by (1) culture of *M. ulcerans* from ulcerative lesions, (2) detection of acid fast bacilli (AFB) in lesions, (3) histopathological features, and/or (4) PCR detection of *M. ulcerans* DNA in patient sample (Guarner, 2003). Differential diagnoses that must be ruled out include squamous cell carcinoma, tropical phagedemic ulcer, actinomycosis, noma, leishmaniasis, yaws, and scrofuloderma (Guarner, 2003).

Buruli ulcers can cover up to 15% of the skin surface (George, 1999) and will have undermined edges. There is generally necrosis of the subcutaneous fat, which leads to subsequent sloughing of the overlying skin (Van der werf, 1999). Another defining characteristic of Buruli ulcers is the relative lack of immune cells present at the site of infection and the absence of an inflammatory response. Antibiotic therapy has shown little success in treatment of Buruli ulcer. Surgical excision of diseased tissue, with margins extending into healthy tissue, followed by skin grafting is currently the best treatment available for patients.

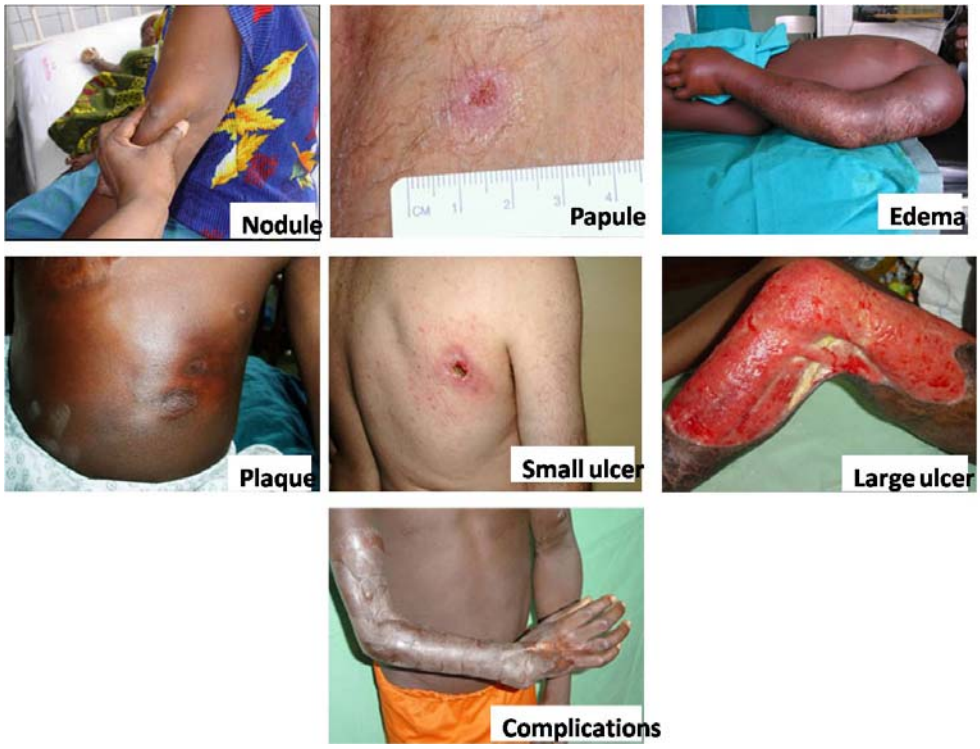


**Figure 1. The non-ulcerative and ulcerative stages of Buruli ulcer disease.** Surgical excision and skin grafting are often the only treatment option for patients with the ulcerative stage disease. This surgical treatment is usually curative, but often leaves patients with complications, such as joint contractures. (Photographs from the World Health Organization website, http://www.who.int/buruli/photos/en/index.html)

Acid-fast bacilli can be readily detected in the sloughed skin (Van der werf, 1999) and extracellular *M. ulcerans* can be isolated from discrete infectious foci within the center of ulcerative regions. However, tissue necrosis usually extends a great distance from the actual site of bacterial infection, which led researchers to hypothesize that *M. ulcerans* may produce a diffusible toxic substance (Connor, 1965).

## 1.2 Mycolactone, a polyketide-derived macrolide toxin

### 1.2.1 Background information and mycolactone discovery

As clinicians continued to study the pathology of Buruli ulcer disease, it became clear that the tissue damage characteristic of Buruli ulcers often extends beyond the scope of discrete *M. ulcerans* infectious foci. In 1965, Connor and Lunn hypothesized that a bacterial exotoxin may be responsible for the expansion of these necrotic ulcers (Connor, 1965). There were multiple attempts to isolate, identify and characterize the *M. ulcerans* toxin with preliminary reports describing it as a heat-stable substance present in sterile culture filtrate (Hockmeyer, 1978), (Krieg, 1974), (Olson, 1995). In 1974, Krieg and colleagues postulated that the *M. ulcerans* toxin was a phospholipoprotein – polysaccharide complex.

The toxin was known to be present in sterile filtrate (SF) and preliminary reports suggested that the toxin was proteinacious. George et al exposed SF from *M. ulcerans* broth cultures to protease enzymes with the intent to inactivate the toxin (George, 1998). However, protease – treated SF showed the same level of cytotoxic activity on L929 fibroblast cell cultures as untreated SF. Additionally, proteins are inactivated by excessive heat, as this type of treatment causes irreversible denaturing. The cytotoxic activity in *M. ulcerans* sterile filtrate was not heat sensitive. These two experiments, taken together, illustrated that the *M. ulcerans* toxin was not a protein (George, 1998).

In the same study, log phase cultures of *M. ulcerans* were subjected to an extraction process, which utilized a 2:1 ratio of chloroform and methanol, in order to further purify the toxic substance from the SF. Proteins, salts, and highly polar molecules separate into the aqueous phase, while the organic phase retains most of the cell-derived lipids

(George, 1998). The organic phase was dried down and acetone was added to precipitate the non-soluble phospholipids, leaving less polar lipid molecules in solution. Thin layer chromatography (TLC) was used to further separate and purify the various lipid species present in the sample. Two separate lipid bands isolated from the TLC plates were cytotoxic to L929 murine fibroblasts in cell culture (George, 1998). Early suspicions were confirmed; *M. ulcerans* does produce an exotoxin, which was ultimately characterized as a lipid (George, 1998). In 1999, the *M. ulcerans* lipid toxin was further described as a lipid-like polyketide and given the name, "Mycolactone" (George, 1999).

### 1.2.2 Biological activity of mycolactone

Mycolactone is a polyketide – derived macrolide consisting of a 12 membered macrolide ring connected to a polyketide side chain (Stinear, 2004). Macrolides are lipid like molecules that are smaller than proteins, but have extremely potent biological activities (George, 1999). *M. ulcerans* belongs to the order *Actinomycetales* and members of this order are well known to produce macrolides as secondary metabolites, which, in turn, have diverse biological effects on eukaryotic cells. Erythromycin (antibiotic), rapamycin (immunosuppressant), FK506 (immunosuppressant), amphotericin B (antifungal), and avermectin (antihelmetic) are all examples of well known macrolides (George, 1999).

Buruli ulcers are characterized by extensive necrosis of the dermis and subcutaneous adipose tissue, with the edges of the ulcerative lesions undermined (Read, 1974). There is a surprising lack of inflammatory response and ulcers are non-purulent and painless. The same observations can be made when *M. ulcerans* bacterial cells are injected into the dermis of guinea pigs (Read, 1974), (George, 1999). More importantly, since the isolation and characterization of the mycolactone toxin, it has been shown that injection of the toxin alone is sufficient to produce the redness, edema, and painless ulceration characteristic of Buruli ulcer disease (*M. ulcerans* infection) (George, 1999). Several studies, using both the guinea pig model of infection and cell culture, have investigated the biological effects of *M. ulcerans*, sterile culture filtrate, and purified mycolactone (Adusumilli, 2005), (Coutanceau, 2007), (Dobos, 2001), (En, 2008), (George, 1999),

(George, 2000), (Guarner, 2003), (Gooding, 2001), (Synder, 2002), (Torrado, 2007), (Read, 1974).

In 1974, Read and colleagues first utilized cell cultures of L929 murine fibroblasts to more closely study the cytopathic effect (CPE) of *M. ulcerans* culture filtrate (CF). The biological effects of mycolactone on L929 murine fibroblasts in cell culture are now well documented and this method of cytopathicity assay is commonly used to ascertain the CPE of *M. ulcerans* cells, CF, lipid extracts, and purified mycolactones. After 24 hours of exposure to CF (which contains mycolactone as well as multiple bacterial-derived proteins) (Read, 1974) or pure mycolactone (George, 1999), L929 murine fibroblasts will round up and arrest in the $G_0/G_1$ stage of the cell cycle. This is largely due to radical alterations in cytoskeletal rearrangement (Synder, 2002). Forty eight hours post-exposure, L929 cells (1) decrease in cell number and size, (2) have pyknotic nuclei that are displaced toward the cell wall, (3) display karyorrhexis (fragmentation of the nucleus), and (4) lift off the cell culture plates as they become necrotic and undergo apoptosis (Read, 1974), (George, 1999), (Synder, 2002).

At high doses above 15 μg/ml, mycolactone will cause L929 cell necrosis, as detected by lactate dehydrogenase release, after 4 – 24 hours of exposure (Adusumilli, 2005). Exposure to much lower amounts of mycolactone (15 ng/ml to 150 ng/ml of mycolactone) causes L929 murine fibroblasts to undergo apoptosis (Adusumilli, 2005). Additionally, work with mycolactone negative mutants of *M. ulcerans* has shown that the pathology of *M. ulcerans* can be replicated by mycolactone alone (Adusumilli, 2005) Mycolactone, a small hydrophobic molecule, is thought to enter cells by passive diffusion since no evidence of receptor mediated active transport has been observed (Snynder, 2002). Once inside the cells, mycolactone localizes to the cytoplasm. A dose-dependent increase in intracellular calcium levels is observed, although this is most likely secondary to mycolactone-induced cytoskeletal remodeling and damage to calcium containing compartments within the cell (Synder, 2002).

Mycobacterial infections with *M. marinum*, *M. tuberculosis*, and *M. bovis* BCG, are typically intracellular and can be found residing within resident macrophages. There has been one report suggesting an intra macrophage growth stage of *M. ulcerans* in the human host, but *M. ulcerans* cells remain mostly extracellular during the disease process. This may be partially due to the immunosuppressive properties of the mycolactone toxin, which has been shown to (1) decrease TNF-α production by monocytes, (2) inhibit dendritic cell migration to the site of infection, and (3) inhibit dendritic cell maturation, which in turn prevents T cell priming (Gooding, 2001), (Coutanceau, 2007).

Mycolactone also has analgesic properties, but the exact mechanism has not been well studied. The painlessness of Buruli ulcers may lead patients to underestimate the seriousness of *M. ulcerans* infection, which in turn may explain how ulcerative lesions are allowed to expand to over 15% of the skin surface (En, 2008). Recently, researchers have used a murine model to investigate the analgesic properties of mycolactone. Intraneural hemorrhage, vascular changes, thinning of the myelin lining of neural cells, histological damage, and general hyposensitivity are observed when mycolactone is injected into the footpads of mice (En, 2008). These observations indicate that the lack of pain present at the site of *M. ulcerans* infection is due, at least in part, to actual nerve damage caused by exposure to mycolactone (En, 2008).

### 1.2.3 Differences and similarities between *M. marinum* and *M. ulcerans*

Mycobacteria can be broadly classified into the fast – growers, including *M. marinum* (doubling time, 6 to 8 hours (Yao, 2006)) and the slow – growers like *M. ulcerans* (doubling time approximately 36 to 48 hours (Mve-Obiang, 2003)). *M. marinum* is commonly found in pools, aquaria, and marine environments where it is a natural pathogen of fish. *M. marinum* is zoonotic, as it can also cause limited, intracellular, granulomatous infections in humans as a result of handling infected fish or exposure to infected environments (Ferreira, 2006). Mycobacteriosis due to *M. marinum* infection occurs after abraded skin is exposed to contaminated water, and is characterized by skin lesions which are usually limited to the extremities (Stinear, 2008). Human cutaneous infections caused by *M. marinum* can be effectively treated with drugs such as rifampin,

ethambutol, quinolones, doxycycline, and/or clarithromycin (Stinear, 2008). Untreated infections or infections in immunocompromised individuals may develop into more serious conditions including tenocynovitis, arthritis, bursitis, and osteomylitis, but these conditions are rare (Ferreira, 2006).

*M. marinum* is classically photochromogenic, meaning that exposure to light induces the bacterium to produce pigments, such as carotenoids. The production of these pigments is protective against exposure to ultra violet light through the reduction of singlet oxygen species (Stinear, 2008) and supports the bacterium's widespread distribution in the environment. *M. marinum* cannot grow at temperatures above 37°C, with optimal temperatures generally ranging between 30°C and 32°C (Ferreira, 2006). Thus ectotherms, such as fish and frogs, are suitable hosts. In frogs, *M. marinum* infection causes a chronic, granulomatous, non-lethal disease and as a result, it has been hypothesized that frogs may be the natural environmental host for *M. marinum* (Ferreira, 2006).

*M. ulcerans*, on the other hand, has strikingly different phenotypic and growth characteristics when compared to *M. marinum*. *M. ulcerans* is extremely difficult to isolate and culture directly from the environment, but it is known to be widely distributed in aquatic environments around the world, with clusters of endemic foci (Stinear, 2000). The scope of the environmental distribution of *M. ulcerans* has been ascertained from the collection and screening of a wide variety of environmental samples, including both flora and fauna, which have tested PCR positive for *M. ulcerans* DNA (Williamson, 2008).

*M. ulcerans* is not photochromogenic, which can be explained by the interruption of the gene, *crtI*, involved in the production of light-inducible carotenoids (Stinear, 2007). In contrast to other mycobacterial infections, such as mycobacteriosis caused by *M. marinum* where the bacterium is largely intracellular, *M. ulcerans* remains primarily extracellular throughout the course of Buruli ulcer disease. *M. ulcerans*, like *M. marinum*, has optimal growth between 30°C and 32°C, which explains the cutaneous nature of *M. ulcerans* infections. The extracellular location of *M. ulcerans* during human

infection may be due to the production of the mycolactone toxin, which is cytotoxic, has anti-phagocytic activity and has been shown to induce apoptosis of antigen presenting cells (Stinear, 2007). Additionally, the intracellular mycobacteria, *M. tuberculosis* and *M .marinum*, possess an ESX-1 protein secretion system responsible for the export of proteins involved in the formation of granulomas (Stinear, 2007). Inactivation of this protein secretion system in *M. marinum* and *M. tuberculosis* reduces phagocyte uptake and intracellular spread. The natural loss of this system in *M. ulcerans* may contribute to its predominantly extracellular lifestyle in the human host (Stinear, 2007).

Despite their phenotypic differences, the recent publication of the genome sequences of both *M. ulcerans* and *M. marinum* has shed light on the close genetic relationship between these two organisms. The 16S rRNA gene from *M. ulcerans* is greater than 99.8% identical to the same gene in *M. marinum* (George, 1999), suggesting that *M. ulcerans* is an ecotype of *M. marinum* (Jenkin, 2003). This close genomic relationship has also been confirmed by multilocus sequence typing (MLST) (Stinear, 2000). These two species are phenotypically very different and cause distinct disease etiologies, and this is, in part, due to the ability of *M. ulcerans* to produce the polyketide toxin, mycolactone. Mycolactone is critical for the destructive and necrotic pathogenesis of *M. ulcerans*, whereas *M. marinum* does not produce mycolactone and does not produce such a devastating ulcerative disease.

It was anticipated that *M. ulcerans* would possess a unique mycolactone biosynthetic gene cluster, which would be absent in *M. marinum*. Suppressive subtractive hybridization (SSH) has been utilized previously to identify genomic differences between closely related mycobacteria including *M. tuberculosis*, *M. bovis*, and *M. bovis* BCG (Jenkin, 2003). Jenkin and colleagues took advantage of the high degree of genomic similarity between *M. ulcerans* and *M. marinum* and used suppressive subtractive hybridization (SSH) in order to identify the mycolactone synthesis gene cluster in *M. ulcerans*. A unique type I polyketide synthase locus was identified in *M. ulcerans* as the genetic basis for mycolactone production (Jenkin, 2003).

#### 1.2.3.1 Polyketide synthase systems

Polyketides are large, structurally diverse lipid-like compounds and are often made as secondary metabolites by bacteria in the order *Actinomycetales* (Smith, 2007), (George, 1999). Over 10,000 polyketides have been identified so far and are synthesized by assorted organisms, both prokaryotic and eukaryotic (Smith, 2007). Polyketide synthase systems are common in mycobacteria, where they are often involved in the production of cell wall mycolates (Stinear, 2008). Within the genome of *M. marinum*, there are 27 coding sequences predicted to encode polyketide synthases (Stinear, 2008) and in the genome of *M. ulcerans* there are 12 predicted polyketide synthases (Stinear, 2007). The biosynthetic pathways and enzymes necessary for polyketide production can be broadly grouped into categories based on their architectural organization (Smith, 2007). Type III polyketide synthases (PKS) are freestanding enzymes that act alone in a repetitive manner (Smith, 2007). Type III PKS synthesize polyketide molecules through a series of multiple condensation reactions in which malonyl-CoA serves as the primary starting molecule and extender unit (Song, 2006).

Type II PKS are freestanding, monofunctional enzymes that act in a repetitive manner to synthesize polyketides using malonyl-CoA as the main building block. The main difference between type II PKS systems and type III PKS systems is that type II PKS systems utilize an acyl-carrier protein (ACP) domain. The ACP is responsible for the translocation of the growing polyketide molecule from one free standing enzyme to the next for iterative condensation reactions (Smith, 2007).

The system utilized by *M. ulcerans* to produce mycolactone is a type I PKS (Stinear, 2003). Unlike type II and type III PKS whose enzymes are freestanding, the enzymes of type I PKS are covalently linked into large, multi-functional megasynthases (Smith, 2007). The main polyketide enzymes necessary for mycolactone biosynthesis are mlsA1 and mlsA2, responsible for production of the 12 membered toxin core, and mlsB, which is responsible for assembly of the side chain (Stinear, 2004). Each enzyme contains a specific number of discrete catalytic domains. The catalytic domains are grouped

together to form an assembly line arrangement of modules, with each module containing its own unique set of catalytic domains.

The arrangement and order of the catalytic domains within each module directly influences the structure, stereochemistry, and β carbon processing of the polyketide molecule (Bali, 2006). Each module has a core set of catalytic domains including: an acyl transferase (AT) domain that recruits chain extension units from malonyl or methyl-malonyl; a β-ketosynthase (KS) domain which catalyzes the elongation of the polyketide chain by forming carbon – carbon bonds; and an acyl carrier protein (ACP) domain that serves as an attachment site for the extender units as well as the growing polyketide chain (Bali, 2006). Each module within the enzyme may also contain optional domains that mediate the various types of reduction of the growing polyketide, such as: a ketoreductase (KR) domain that adds hydrogen to –C=O groups to give -C-OH; a dehydratase (DH) domain responsible for removing oxygen and hydrogen, as a water molecule, from the polyketide; and/or an enoyl reductase (ER) domain that reduces the C=C double bond to give $CH_2$-$CH_2$ (Bali, 2006). **Figure 2** shows the arrangement of the type I PKS utilized by *M. ulcerans* to produce the core of mycolactone.

#### 1.2.3.2 The mycolactone plasmid

In 2004, Stinear and colleagues determined that the unique type I polyketide synthase locus, including all polyketide synthases and associated polyketide modifying enzymes responsible for the synthesis of mycolactone, are encoded on a large plasmid, pMUM001. This plasmid was first discovered as a defined band of roughly 170 Kb that was observed during pulsed field gel electrophoresis analysis of undigested whole cell DNA from *M. ulcerans* Agy 99 (Stinear, 2004). Southern hybridization experiments showed that this DNA band hybridized to *M. ulcerans* – specific polyketide synthase probes (Stinear, 2004). After BAC end sequencing, insert sizing and restriction fragment profiling, the polyketide synthase locus was confirmed to be located on a circular plasmid of 174,155 bp (Stinear, 2004).

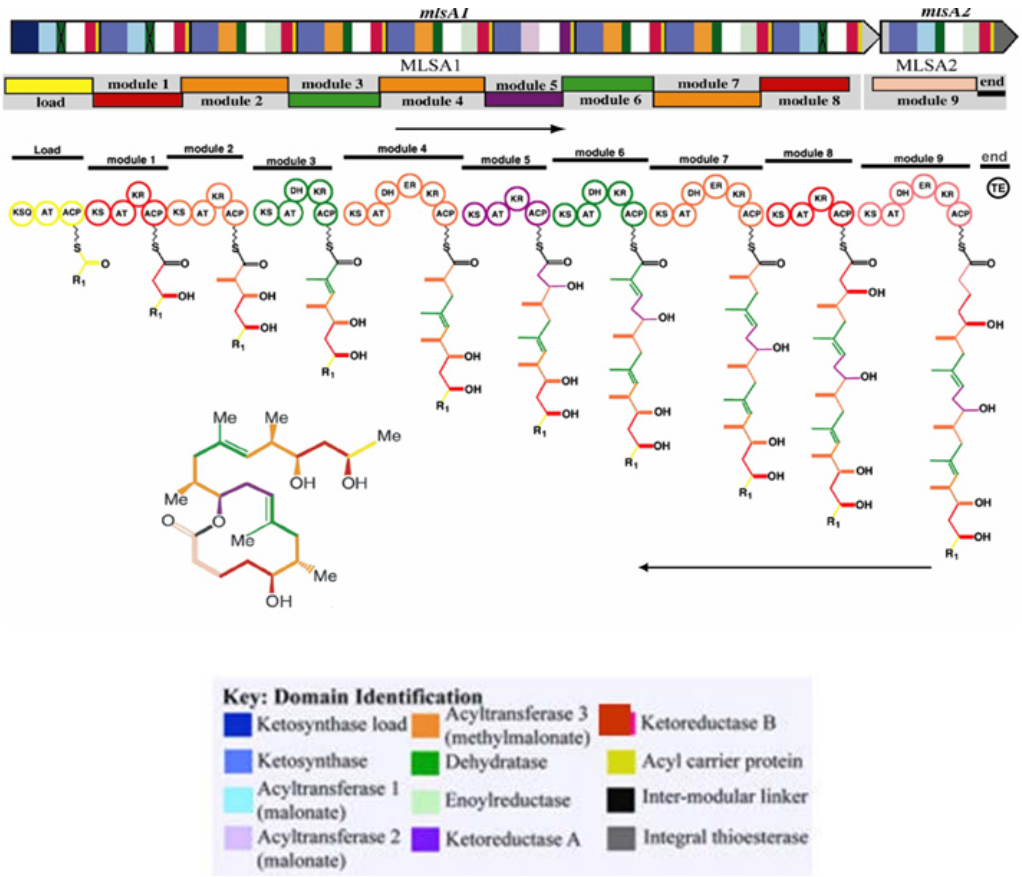


**Figure 2. Arrangement of the catalytic domains within *mlsA1* and *mlsA2*, the genes responsible for synthesis of the mycolactone core.**
(Adapted from www.med.monash.edu.au/microbiology/research/stinear.html)

The *M. ulcerans* plasmid, pMUM001, is the first example of plasmid – mediated virulence in mycobacteria (Stinear, 2004), however, there is a precedent for plasmid borne genes involved in the biosynthesis of secondary metabolites (Stinear, 2005). For example, *Streptomyces rochei* possesses a plasmid, pSLA2-1 that encodes both type I and type II polyketide synthase clusters (Stinear, 2005). However, the *M. ulcerans* polyketide synthase genes are particularly interesting for several reasons. These genes encode some of the largest proteins ever reported (Stinear, 2005). The *mlsA1* PKS gene is 50,973 bp long and encodes a 1.8 mega Dalton (MDa) protein. The *mlsA2* PKS gene is 7,233 bp long and encodes a 0.26 MDa protein. These two genes together are responsible for synthesis of the mycolactone core. The *mlsB* gene is 42,393 bp long and encodes a 1.2 MDa protein responsible for production of the mycolactone side chain (Stinear, 2005).

Another striking feature of the *M. ulcerans* mycolactone polyketide synthase (PKS) locus is that these three polyketide synthase genes are highly repetitive, with large segments of nucleotides with 99.7% sequence identity. Only 9.5 Kb out of the total 105 Kb of coding sequence dedicated to these three PKS genes consists of unique, non-repetitive DNA sequence (Stinear, 2004). Additionally, there is also greater than 97% sequence identity among the functional domains of the 18 PKS modules of the mycolactone type I polyketide synthase system (Stinear, 2004).

There are 81 predicted protein coding sequences located on pMUM001, with roughly 105,000 bp out of 175,155 bp dedicated to the main PKS genes (Stinear, 2005). The plasmid also has multiple copies of IS2404 and IS2606, insertion sequence elements (IS) that are generally positioned on either side of the PKS genes (Stinear, 2004). Other accessory genes involved in mycolactone production are: *mup053*, which encodes a p450 monooxygenase thought to hydroxylate the twelfth carbon of the mycolactone side chain and the *mup045* gene that encodes a Fab-H like type III ketosynthase thought to catalyze the ester bond formation necessary to join the mycolactone core and side chain (Stinear, 2004). Additionally, pMUM001 has genes that are crucial the

replication, *repA*, and partioning, *parA*, of the plasmid so it may be passed on to future daughter cells (Stinear, 2004). **Figure 3** illustrates a circular representation of pMUM001 as constructed by Stinear et al.

Currently, scientists believe that *M. ulcerans* has evolved from a *M. marinum* progenitor and diverged into a separate clonal lineage through acquisition of foreign DNA, namely the mycolactone plasmid, pMUM001 (Stinear, 2005). There is a precedent for this type of evolutionary bottleneck, as it has been observed for *M. leprae*, *Y. pestis*, and *B. pertussis*, to name a few (Stinear, 2007). *M. ulcerans* has all the genomic characteristics of a bacterium that has experienced such an event, including: (1) the proliferation of insertion sequence elements (there are 209 partial or complete copies of IS2404 and 83 copies of IS2606 in the *M. ulcerans* genome); (2) accumulation of pseudogenes (*M. ulcerans* has 743 predicted genomic pseudogenes); (3) chromosomal rearrangements; (4) genome downsizing (*M. ulcerans* genome is 5.6 Mb compared to the *M. marinum* genome of 6.6 Mb (Stinear, 2008); and (5) the acquisition of foreign genes, often via plasmids or bacteriophages (Stinear, 2007).

Although, the consensus is that *M. ulcerans* acquired the mycolactone plasmid through some type of horizontal gene transfer, the exact origin of the mycolactone plasmid has not been determined. The *repA* gene of pMUM001 shares 68.3% amino acid identity with the repA of pJAZ38, a plasmid harbored by *M. fortuitum* (Stinear, 2004). The polyketide synthase system possessed by *M. ulcerans* has strong homology to two different PKS systems within the genome of *M. marinum*, but seems to be more closely related to PKS systems harbored by *Streptomycetes* (Stinear, 2008). It is possible that *M. ulcerans* acquired a core set of PKS genes from a bacterium in the *Streptomycetes* genra (Stinear, 2005) and that the mycolactone PKS system, as it exists today, has evolved as a result of multiple recombination and duplication events (Stinear, 2004). Insertion sequence elements, like those found on the mycolactone plasmid, are known to mediate such duplications and rearrangements (Stinear, 2005).

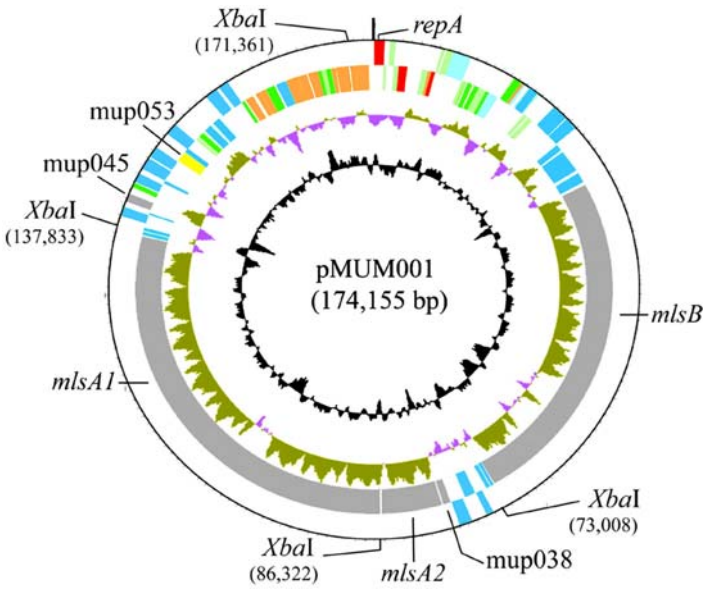


**Figure 3. Circular map of the mycolactone plasmid, pMUM001.**
Colors represent the functional classification of each gene (red, replication; light blue, regulation; light green, hypothetical protein; cyan, insertion sequence elements; yellow, intermediate metabolism; gray, lipid metabolism) (Stinear, 2004).

Even though the exact manner of plasmid acquisition and evolution of the mycolactone gene cluster are not fully understood, it is clear that the ability of *M. ulcerans* to produce mycolactone has provided a survival advantage for the bacterium. Mycolactone could play a role in the colonization and/or survival of *M. ulcerans* in an as-of-yet undiscovered environmental niche, as it is unlikely that mycolactone's cytotoxicity to human cells is part of the bacterium's primary survival strategy (Stinear, 2005).

*M. ulcerans* isolates from diverse geographic locations around the world produce a heterogeneous mixture of mycolactone congeners that have been designated A through D (Mve-Obiang, 2005). The structure of the toxin core remains constant, but *M. ulcerans* strains have been shown to produce variations in the toxin's side chain, which may be linked to the severity of disease process (Mve-Obiang, 2005). Multiple different *M. ulcerans* isolates have been screened in order to characterize their mycolactone profiles. Mycolactone A/B is produced as the major mycolactone species by isolates from Malaysia and Western Africa and as a minor toxin species

by *M. ulcerans* strains from Australia, Japan, and China. Mycolactone A/B is not produced by strains from Mexico (Mve-Obiang, 2005). *M. ulcerans* isolates from Australia produce mainly mycolactone C, while strains from Japan and China produce mainly mycolactone D (Mve-Obiang, 2005). **Figure 4** shows the structure of some of the mycolactone congeners.

### 1.2.4 Other mycolactone producing mycobacteria

The pathology of Buruli ulcer disease is now better appreciated since the discovery that *M. ulcerans* produces a cytotoxic polyketide, mycolactone, which is responsible for a majority of the tissue damage characteristic of Buruli ulcers (George, 1999). Additionally, it is exciting that the mycolactone PKS gene cluster has been discovered and that this locus is located on an independently replicating plasmid, which may have been acquired by *M. ulcerans* through horizontal gene transfer (Stinear, 2004). Plasmids are generally considered to be movable genetic elements, but this particular plasmid was thought to be unique and limited to the *ulcerans* species of mycobacteria. Additionally, horizontal transfer systems are not well documented in mycobacteria, with only a few examples of conjugation demonstrated in *M. smegmatis* (Parsons, 1998), (Bhatt, 2002), (Bhatt, 2003), (Wang, 2003), (Coros, 2008).

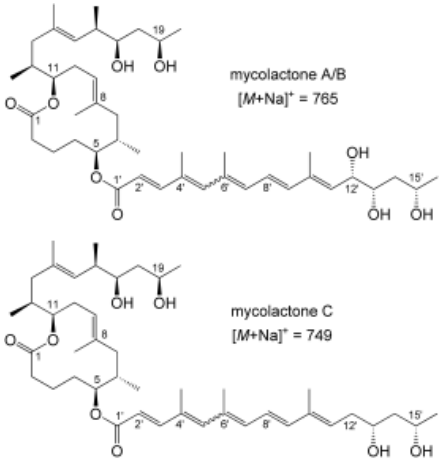


**Figure 4. The structure of mycolactone.**
The structure of two mycolactone congeners produced by *M. ulcerans* isolates. Notice that the structural differences are limited to the toxin's fatty acid sidechain.

#### 1.2.4.1 *M. liflandii*

In 2001, an *M. ulcerans*-like infection spread through a colony of African *Xenopus tropicalis* and *Xenopus laevis* frogs at the University of California in Berkley (Trott, 2004). The disease presented as cutaneous ulcers, with some incidence of generalized edema. Ziehl-Neelson staining and microbiological culture of bacteria collected from diseased frogs clearly indicated that the frogs were suffering from a mycobacteriosis (Trott, 2004). Sequence analysis was performed on the following genes: *hsp65* (heat shock protein), *16sA*, *16sB*, *16sC* (highly conserved regions of the 16s rRNA gene), *ITS* (an internal spacer between the 16s and 23s rRNA), *rpoB* (RNA polymerase B subunit), *IS2404*, and *IS2606*. Genetic examination of mycobacterial isolates did not provide a clear-cut identification of the etiological agent, with sequence homology to both *M. ulcerans* and *M. marinum*, two very closely related species (Trott, 2004).

Mve-Obiang and colleagues further characterized this non-*ulcerans*, mycolactone producing mycobacterium (MPM) as a new species, *M. liflandii* (Mve-Obiang, 2005). *M. liflandii* was PCR positive for five pMUM001-specific genes including, *mlsA1*, *mlsB*, *mup045*, *mup037* (a type II thioesterase), and *repA* (Mve-Obiang, 2005). These genes had 99% sequence identity to the same genes in *M. ulcerans*. Additionally, pulsed field gel electrophoresis of *M. liflandii* DNA plugs showed a unique band of 180 Kb that hybridized to pMUM001-specific genes (Mve-Obiang, 2005). Lipids were extracted and analyzed by mass spectrometry, which detected a molecule of *m/z* 737.7. Further analysis by tandem MS-MS revealed the mycolactone core at *m/z* 429.5 and a peak representing the mycolactone side chain at *m/z* 331.4, 28 daltons less than the side chain of mycolactone A/B. This new mycolactone molecule was designated mycolactone E (Mve-Obiang, 2005). The mycolactone plasmid possessed by this newly discovered MPM is roughly 180 Kb, slightly larger than the *M. ulcerans* mycolactone plasmid (Yip, 2007).

#### 1.2.4.2 *M. marinum* MPM species

Mycobacteriosis in fish caused by infection with *M. marinum* has been well documented for decades, but in the early 1990s, *M. marinum* infections became especially problematic

in Israeli aquaculture systems (Ucko, 2002). Mycobacteria have been isolated from fish in Israel and compared to *M. marinum* on the basis of 16s rRNA and *hsp65* gene sequence alignments. All Israeli fish isolates have been categorized as *M. marinum*, but were clearly divided into two strains; mycobacterial strains infecting marine fish and those strains infecting freshwater fish (Ucko, 2002). Furthermore, mass spectrometry analysis of lipids extracted from the Israel fish isolates proved that these *M. marinum* strains were unique based on their ability to produce mycolactone F, *m/z* 723 (Ranger, 2006). MS/MS analysis of these lipids detected the mycolactone core *m/z* 429, the structure of which remains constant throughout all mycolactone congeners described to date (Ranger, 2006).

These newly discovered MPM strains produce a novel mycolactone molecule that differs from other toxin species due to differences in the fatty acid sidechain. **Figure 5** shows the structure of mycolactone F compared to mycolactone A/B. Like other mycolactone producing mycobacteria, these *M. marinum* Israeli fish isolates possess a form of the mycolactone plasmid that ranges in size from 180Kb to 200 Kb, slightly larger than the plasmid harbored by *M. ulcerans* strains (Yip, 2007).

#### 1.2.4.3 *M. pseudoshottsii*

Since the discovery and characterization of *M. liflandii*, several MPMs have been discovered and characterized. 1997 saw the beginning of a mycobacteriosis outbreak of striped bass, *Morone saxatilis*, collected from the Chesapeake Bay and some of its tributaries. Researchers were able to obtain multiple different mycobacterial isolates from diseased fish, as polyinfections within individual fish were observed in 25% of samples (Rhodes, 2004). Approximately 12% of fish were infected with a previously uncharacterized mycobacterial species (Rhodes, 2005).

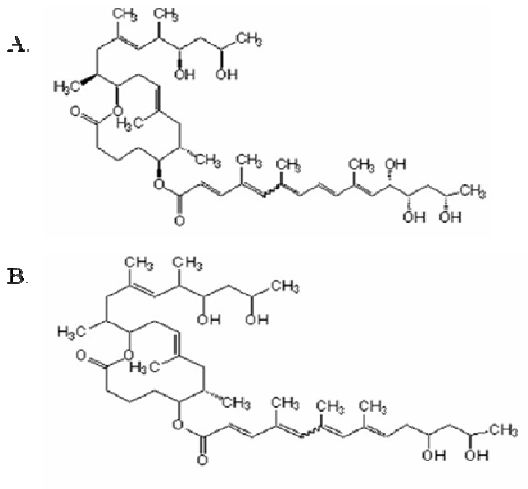

**Figure 5. The structures of mycolactone A/B and mycolactone F.**
Comparison of the structure of (A) mycolactone A/B, produced by *M. ulcerans* strains, and (B) mycolactone F, produced by *M. marinum* strains isolated from Israeli fish. Notice that structural differences are limited to the fatty acid sidechain. (Ranger, 2006)

After high performance liquid chromatography analysis of mycolic acid profiles, biochemical testing, and observation of growth characteristics, one new isolate seemed to most closely resemble *M. shottsii*, also a new species discovered during the outbreak in the Chesapeake Bay (Rhodes, 2003). However, the new mycobacterial isolate was PCR positive for IS2404 and IS2606, a genotypic trait of *M. ulcerans*. The new isolate was named *M. pseudoshottsii* to reflect its similarity to *M. shottsii*, but clearly designate it as a new species (Rhodes, 2005).

The 16s rRNA sequence of *M. pseudoshottsii* showed that this isolate was closely related to *M. ulcerans*, *M. marinum* MPM strain DL240490 isolated from Israeli fish, and *M. liflandii* (Ranger, 2006). *M. pseudoshottsii* was PCR positive for several mycolactone associated genes including *repA*, *mlsA*, *mlsB*, *mup045*, and pMUM001 insertion sequence elements IS2404 and IS2606 (Ranger, 2006). *M. pseudoshottsii* was differentiated from *M. ulcerans* due to the absence of an *M. ulcerans*-specific hypothetical membrane protein gene, *uhp-mem* (Ranger, 2006). Mass spectrometry analysis of lipids extracted from *M. pseudoshottsii* isolate L15 detected mycolactone, *m/z* 723, and the mycolactone core, *m/z*

429, was detected by MS/MS (Ranger, 2006). Further analysis of the mycolactone molecule produced by *M. pseudoshottsii* led to the discovery that this MPM was producing yet another congener of mycolactone, designated mycolactone F (Ranger, 2006). The mycolactone plasmid harbored by *M. pseudoshottsii* was determined to be 200 Kb, larger than pMUM001 found in *M. ulcerans* strains (Yip, 2007).

DNA-DNA hybridization (DDH) studies have been utilized to compare these newly discovered MPMs with *M. ulcerans* in the hopes of shedding some light on the evolutionary story of mycolactone production. This method of genetic comparison has been used for over 30 years in bacterial taxonomy to determine the degree of relatedness between different species and/or strains (Yip, 2007). Based on this method of comparison, MPMs are very closely related to *M. ulcerans* (Yip, 2007). Moreover, it is significant that MPMs are 99% identical to each other at the nucleotide level, as shown by multilocus sequence analysis (MLSA) and variable number tandem repeat loci (VNTR) (Yip, 2007). MLSA has been used to compare and contrast the frequency of mutation found in chromosomal genes versus the frequency of mutation of genes found on the mycolactone plasmid.

In MPMs, the frequency of mutation of chromosomally encoded genes was synonymous with the mutation frequency of plasmid-borne genes, suggesting that the mycolactone plasmid co-evolved with all mycolactone producing mycobacteria (Yip, 2007). These data imply that MPMs have evolved from a common progenitor after acquisition of the mycolactone virulence plasmid and not as a result of multiple exchanges of the plasmid (Yip, 2007). Present day differences between *M. ulcerans* and other MPMs have evolved as these mycobacteria have evolved to occupy different ecological niches (Yip, 2007).

## 1.3 Plasmids in mycobacteria

### 1.3.1 Plasmids in non-*ulcerans* mycobacterial species

Plasmids have been described in multiple mycobacterial species isolated from both environmental and clinical settings. *M. avium* is predominantly a pathogen of birds and is commonly found in the environment, but in recent years, this mycobacterium has been causing infections in immunocompromised patients (Beggs, 2005). Crawford and colleagues were the first to identify plasmids in *M. avium* (Crawford, 1979) and five years later they characterized a 15.3 Kb plasmid, pLR7 (Beggs, 1995). *M. avium*'s plasmid, pLR7, is very simple and is known to harbor genes encoding a Rep protein and a surface-associated protein (Beggs, 1995). Although preliminary data suggests that *M. avium* strains that possess this plasmid may experience enhanced intracellular survival in the human host (Beggs, 1997), more research is needed to fully understand if pLR7 enhances virulence.

Plasmids have also been found in *M. scrofulaceum*, *M. chelonae*, and *M. abscessus* species and subspecies (Bachrach, 2000), (Gavigan, 1997), (Labidi, 1984), (Labidi, 1992), (Meissner, 1984). The plasmids range in size from 7 Kb up to 115 Kb and are typically found in low copy numbers within bacterial cells. The 115 Kb plasmid, pVT1, harbored by *M. scrofulaceum*, is known to contain a gene encoding mercuric reductase, which presumptively enhances the bacterium's survival in contaminated aquatic environments (Meissner, 1984). *M. scrofulaceum* also has another plasmid, pMSC262 (Bachrach, 2000). These plasmids have not been fully annotated and the exact function of most of the genes remains largely unknown.

*M. fortuitum* isolates may completely lack plasmids or can possess up to six or more individual plasmids ranging in size from 7 Kb to 112 Kb (Labidi, 1984). Three *M. fortuitum* plasmids, pAL5000, pJAZ38, and pMF1, have been extensively studied for their potential utility as tools for mycobacterial genetics studies (Bachrach, 2000), (Gavigan, 1997), (Labidi, 1992). Linear plasmids have been identified in other, lesser known mycobacterial species, including *M. xenopi*, *M. celatum*, and *M. branderi*,, but

there is no indication that these plasmids are essential to the metabolism of these bacteria (Picardeau, 1997).

### 1.3.2 Plasmid biology and host range

Mycobacteria, namely *M. tuberculosis* and *M. leprae*, were some of the first bacteria to be recognized as the causative agents of human disease, and yet knowledge of the molecular biology of mycobacteria continues to lag behind that of most other groups of pathogenic bacteria (Bachrach, 2000). Many of the genetic tools used in microbial genetics are ill suited for genetic manipulation of mycobacteria. Mycobacteria have a thick, waxy, hydrophobic cell wall that makes traditional prokaryote cell lysis methods and genetic transformations extremely difficult. Additionally, medically important pathogens, such as *M. tuberculosis* and *M. leprae*, grow extremely slow, making work with these microorganisms very time-consuming.

The Rep regions of several of the plasmids identified in mycobacteria have been sequenced and show high degrees of sequence homology with each other (Gavigan, 1997), (Bachrach, 2000). Scientists are diligently investigating the host range of these plasmids to assess their potential use in mycobacterial transformations and heterologous expression of genes (Bachrach, 2000). For example, the plasmid pCLP, harbored by *M. celatum*, cannot be replicated by *M. smegmatis*, but the plasmid pMF1 isolated from *M. fortuitum*, can be carried and replicated by both *M. tuberculosis* and *M. smegmatis* (Bachrach, 2000). *M. fortuitum*'s plasmid, pJAZ38, has a rep region similar to pLR7 (*M. avium*) and pMSC262 (*M. scrofulaceum*) and can be successfully replicated by *M. smegmatis* (Gavigan, 1997). *M. fortuitum*'s plasmid pAL5000 is one of the most well-studied mycobacterial plasmids and has served as the basis for several mycobacterial vectors (Gavigan, 1997).

Nucleotide sequencing has enabled a broader understanding of mycobacterial plasmids. The ability to compare the nucleotide similarity of the rep regions of various mycobacterial plasmids may provide useful insight into their host range. For example, the *repA* gene from the *M. ulcerans* plasmid, pMUM001, shares greater than 68% amino

acid identity with *rep* from the *M. fortuitum* plasmid pJAZ38 and 55.6% amino acid identity with the same *rep* gene in pVT2, a plasmid harbored by *M. avium* (Stinear, 2005).

Although plasmids are common in mycobacteria outside of the *M. tuberculosis* complex, none of these plasmids have ever been directly linked to virulence until the discovery of the *M. ulcerans* plasmid, pMUM001 (Stinear, 2005). In fact, there are very few reports that have even assigned functions to the genes on these plasmids and consequently, it is thought that lateral gene transfer of plasmid DNA between mycobacteria is not essential to pathogenesis or virulence (Stinear, 2005). pMUM001, first isolated from *M. ulcerans*, is the first example of a mycobacterial plasmid that confers the ability to produce a toxin that is responsible for pathogenesis of the mycobacterial disease, Buruli ulcer. Importantly, other mycobacterial species have been discovered that possess forms of this plasmid and that can also produce the virulence-enhancing toxin, mycolactone.

### 1.3.3 pMYCO7017 contains the majority of the *M. ulcerans* plasmid

Insight into the biology and host range of pMUM001 has been hindered by (1) the slow growth of the plasmid's native host, *M. ulcerans*, (2) difficulty performing genetic manipulations with mycobacteria due to the high G + C nucleotide content, and (3) the extremely large size of the plasmid, 174 Kb. Additionally, like other mycobacterial plasmids, pMUM001 is also present in very low copy numbers within mycobacterial cells. For these reasons, very little work has been done to investigate the ability of other non-*ulcerans* mycobacterial species to replicate the mycolactone plasmid and heterologously produce mycolactone.

However, Stinear et al have done some work to investigate the stability of the *ori* region of pMUM001 (Stinear, 2005). A 6 Kb region of pMUM001, encompassing the putative *parA*, *repA*, and *ori*, was cloned into the vector pCDNA2.1 and the construct was marked with an apramycin gene. Electroporation was used in an attempt to introduce this construct into *M. marinum*, *M. smegmatis*, and *M. fortuitum*; however, transformation was only successful with *M. marinum*. The *M. marinum* transformants were cultured

with apramycin as selective pressure for the transformants to maintain the vector construct along with the pMUM001-derived DNA. Late log phase cultures of *M. marinum* transformants were switched to antibiotic-free media for 12 days in order to test the stability of the pMUM001-derived genes, *ori, repA,* and *parA* in the absence of selective pressure. In the absence of antibiotic selection, *M. ulcerans*' *ori, repA,* and *parA* genes were not sufficient to maintain the construct in *M. marinum*.

The aim of this current work was to further investigate the stability of *M. ulcerans* mycolactone genes in other, faster growing mycobacterial species. In order to circumvent the difficulty of direct manipulation of pMUM001, a whole-genome *M. ulcerans* bacterial artificial chromosome (BAC) library was previously constructed at the Clemson University Genomics Institute (Tomkins, 2001). The hope was to create a BAC, with a selectable marker and an *E. coli* origin of replication, as well as the mycolactone synthesis genes. The BAC used for this study, pMYCO7017, contains a 152 kb fragment of *M. ulcerans* plasmid DNA and includes the majority of the *M. ulcerans* virulence plasmid, pMUM001. **Figure 6** shows a linear representation of pMYCO7017 depicting the size and location of the genes necessary for mycolactone production. Notice that the pMYCO7017 construct also contains the mycobacterial replication (*rep*) and partioning (*par*) genes. The creation of this construct, pMYCO7017, has, for the first time, enabled investigation of the host range and stability of the *M. ulcerans* mycolactone plasmid in heterologous hosts.


**Figure 6. Linear arrangement of pMYCO7017, total length 152 Kb.**
* *mup027c*, similar to the IS116 / IS110 / IS902 family of transposases

## 1.4 Conjugation

DNA can be transferred between prokaryotic cells by transformation, transduction or conjugation. During transformation, a bacterial cell may take up free DNA that is present in the environment. Bacteriophages are responsible for transferring fragments of nucleic acid between bacteria through the process of transduction. Conjugation, on the other hand, is the direct transfer of DNA from one cell to another. Each of these processes have been vital to inter and intra-species spread of virulence determinants, such as antibiotic resistance, throughout microbial communities.

A crucial step of successful conjugative DNA transfer is intimate cell surface contact between the donor and recipient cells (Grohmann, 2003). In gram negative bacteria such as *E. coli*, this close cell-to-cell contact is often established with specialized conjugative structures called sex pili (Grohmann, 2003). Sex pili are similar to the structures involved in the type IV secretion system (Grohmann, 2003). The donor cell uses a sex pilus to penetrate the gram negative outer membrane, periplasm, and inner membrane. To date, no sex pili have been elucidated in mycobacteria, although mycobacteria may possess homologous structures capable of facilitating intimate cell contact that have yet to be characterized.

Plasmid DNA is almost always the centerpiece of conjugative transfer; however, it cannot be transferred through the narrow sex pilus to the recipient cell in its circularized form. Most detailed work on plasmid conjugation apparatus has been conducted with gram negative organisms such as *E. coli*. In gram negative bacteria, the plasmid DNA must be nicked and a single strand of DNA may be lead through the sex pilus to the recipient cell, where it is copied and re-circularized. The genes necessary for conjugation are often cis-acting elements, encoded on the same DNA element involved in the transfer. There may also be trans-acting, chromosomally encoded elements necessary for conjugative plasmid DNA transfer.

Various gram negative conjugation systems have been described, but the IncP transfer system present on the broad host range plasmid, RP4, serves as an excellent example to help better understand this process. Two protein complexes are essential for the conjugative transfer of RP4; the relaxosome and the mating-pair formation (mpf) complex (Grohmann, 2003). The relaxosome is a "multiprotein-DNA complex" that localizes to the plasmid's origin of transfer site, *oriT* (Grohmann, 2003). The relaxosome cleaves a specific phosphodiester bond of the double stranded plasmid DNA at a unique *nic* site within the *oriT* (Grohmann, 2003). The relaxosome remains covalently bound to a single strand of the plasmid DNA and is then linked to the mpf complex by a TraG coupling protein. It is the mpf complex that is responsible for trafficking the relaxosome-TraG-donor DNA complex from the donor, through the sex pilus, to the recipient cell.

*Enterococcus faecalis*, a gram positive bacterium, possesses a pheromone-inducible conjugation system. Horizontal transfer of the pCF10 plasmid involves chromosomally encoded pheromones which act on plasmid-encoded response machinery. Horizontal transfer of the pCF10 plasmid occurs when donor cells encounter high concentrations of cCF10 pheromone produced by recipient *Enterococcus* cells. Self-induction of conjugation by endogenous production of cCF10 pheromone by donor *Enterococcus* cells is reduced by the plasmid encoded membrane protein, PrgY. Additionally, donor cells also produce endogenous iCF10, an inhibitor peptide that neutralizes residual endogenous pheromone that escapes the control of PrgY (Kozlowicz, 2006).

Both cCF10 (pheromone) and iCF10 (inhibitor) are taken into donor *Enterococcus* cells where they bind to PrgX. PrgX represses initiation of transcription of the plasmid-encoded conjugation operon under normal conditions when iCF10 concentrations are far greater than cCF10 pheromone concentrations. However, when recipient cells are nearby, the concentration of cCF10 increases and overwhelmingly binds PrgX within the donor cells. cCF10 prevents the repression activity of PrgX and leads to expression of the conjugation operon, followed by conjugative transfer of the pCF10 plasmid (Kozlowicz, 2006).

Although conjugation has been well-studied in gram negative bacteria, especially in *E. col*i conjugation between gram positive bacteria is rare and not well understood. The thick cell wall of gram positive bacteria is one of the major hurdles limiting their conjugative capabilities. The cell wall of acid fast mycobacteria is even more complex and extremely thick. Mycobacteria possess an inner cell membrane, which is then covered by a thin layer of peptidoglycan. Next, there is a complex matrix of galactan, arabinan, mycolic acid, and other hydrophobic molecules (Brennan, 2007). Despite this formidable barrier to conjugation, relaxases have been described in some gram positive bacteria, such as the mobilization (Mob) protein family. This type of relaxase has been described in *Streptococcus*, *Bacillus*, *Clostridium*, *Staphylococcus*, and proteobacteria (Grohmann, 2003). Additionally, a putative conjugative relaxase has been discovered on the *M. avium* plasmid, pVT2 (Grohmann, 2003). The presence of relaxase genes on plasmids harbored by these gram positive bacteria suggests that conjugative plasmid transfer may proceed in a manner similar to the IncP system (Grohmann, 2003).

Conjugative transfer of plasmids is known to occur between bacteria of the *Streptomyces* genra and the Tra protein is essential to this process. *Streptomyces* Tra proteins are similar to known septal DNA translocator proteins. Plasmids harbored by *Streptomyces* do not typically encode any resistance traits or virulence determinants, but simply confer the ability to replicate and transfer the plasmid (Grohmann, 2003), although there are examples of large linear plasmids which encode antibiotic biosynthetic pathways. Movable plasmids are often small and exist in multiple copies within a cell. Interestingly, one large, low-copy number plasmid has been isolated from *Streptomyces coelicolor*. SCP2 is 31,317 bp and is able to accept large fragments of DNA, such as entire antibiotic biosynthetic gene clusters (Grohmann, 2003).

### 1.4.1 Mycobacterial Conjugation

The evolutionary history of *M. ulcerans*, namely the origin and acquisition of the mycolactone plasmid, continues to be investigated. Current research postulates that *M. ulcerans* evolved from a *M. marinum* progenitor through acquisition of the mycolactone plasmid, although the original source of this plasmid remains a mystery. However, it has

been shown that the *repA* gene of pMUM001 shares 68.3% amino acid identity with the repA of pJAZ38, a plasmid harbored by *M. fortuitum* (Stinear, 2004). The polyketide synthase system possessed by *M. ulcerans* has strong homology to two different PKS systems within the genome of *M. marinum*, but seems to be more closely related to PKS systems harbored by *Streptomycetes* (Stinear, 2008). It is possible that *M. ulcerans* acquired a core set of PKS genes from a bacterium in the *Streptomycetes* genra (Stinear, 2005) and that the mycolactone PKS system, as it exists today, has evolved as a result of multiple recombination and duplication events (Stinear, 2004).

Conjugative systems in gram negative bacteria have been extensively studied and most often involve plasmid transfer from donor to recipient through a conjugative sex pilus. Double stranded plasmid DNA is nicked at a specific *nic* site within the *oriT*. A relaxase binds to one strand of the nicked DNA and facilitates transfer of the single stranded DNA into the recipient cell. Once in the recipient, re-circularization of the DNA is mediated by the same donor relaxase and does not require recipient cell recombination functions (Wang, 2003).

An exception to this plasmid only rule has been discovered in Hfr (high frequency of recombination) strains of *E. coli*. In Hfr *E. coli* strains, *oriT* (origin of transfer) sites have been introduced into the chromosome via integration of conjugative elements, and the *oriT*, in turn, allows *E. coli* to mobilize their chromosomes at a high frequency (Parsons, 1998). A similar phenomenon has been discovered in the fast-growing mycobacterium, *M. smegmatis*. A DNA transfer system has been described in which multiple cis-acting sequences on the chromosome, designated *bom* (basis of mobility), mobilize sections of donor chromosomal DNA to recipients (Wang, 2003).

Studies on the plasmid biology of mycobacteria and conjugation are in their infancy (Derbyshire). However it is clear that the mechanism of DNA transfer in *M. smegmatis* is quite different from the classical plasmid conjugative transfer systems described in gram negative bacteria for two main reasons. First of all, DNA transfer in *M. smegmatis* only occurs between distinct donor and recipient strains, as transfer of *bom* sequences from donor to recipient does not confer ability to become a donor. This is different from

conjugative transfer in *E. coli*, where F+ donor strains transfer plasmid DNA to F- recipient strains. When the F plasmid re-circularizes in the recipient strain, the *oriT* is restored, thus converting the F- recipient to an F+ donor.

The second major difference in *M. smegmatis* DNA transfer is the requirement for recombination events in the recipient cells. DNA transfer between *M. smegmatis* donor and recipient strains occurs as follows (1) multiple *bom* sequences in the donor chromosome mobilize segments of DNA in preparation for transfer to the recipient; (2) once donor chromosomal DNA is in the recipient, the recA protein facilitates a gap-repair like process of recombination with homologous regions of the recipient chromosome (Wang, 2003), (Bhatt, 2003). This mechanism has been supported by experiments in which *M. smegmatis bom* sequences have been cloned into non-mobilizable plasmids and consequently these plasmids were mobilized to recipient strains (Bhatt, 2003). Following the conjugal transfer of the *bom*-containing test plasmids into the recipient *M. smegmatis* strains, the plasmid is re-circularized not by relaxase-mediated ligation (as seen in gram negative conjugation systems), but rather by a mechanism similar to gap repair (Bhatt, 2003). The donor test plasmid is rescued by homologous recombination using the recipient's chromosome as a template for gap repair of the double stranded DNA break (Bhatt, 2003), (Wang, 2004). Chromosome mobilizing systems similar to the *M. smegmatis bom* system have also been observed in *Streptomyces* species (Parsons, 1998). This realization is extremely intriguing considering the fact that the *M. ulcerans* mycolactone polyketide synthase genes have homology to polyketide synthases located within the genome of *Streptomyces*.

A recent publication reported the existence of a *M. tuberculosis*-specific insertion sequence, IS6110, in a recipient strain of *M. smegmatis* (Coros, 2008). The IS6110-like insertion sequence has 67% amino acid identity to the native element found in *M. tuberculosis* (Coros, 2008). The authors stipulate that the presence of this *M. tuberculosis*-specific IS element within the genome of a recipient strain of *M. smegmatis* suggests a horizontal DNA transfer event. This is supported by the fact that many of the genes required for DNA transfer in *M. smegmatis* have been identified in *M. tuberculosis*

(Coros, 2008). This discovery has exciting implications toward elucidating the existence of horizontal DNA transfer between mycobacterial species and the phenomenon will certainly be investigated further.

# CHAPTER 2 : MATERIALS AND METHODS

## 2.1 Bacterial strains and culture conditions

The strains used in this study are listed in **Table 1**. *Escherichia coli* EC100 (Epicentre, Madison, Wisconsin) was cultured on Luria-Bertani (LB) with 1.5% agar (wt/vol). LB media was made using reagents obtained from Fisher Scientific (Fairlawn, New Jersey). *Escherichia coli* strains carrying pMYCO7017 were grown overnight at 37°C on LB agar or in broth containing 12.5 μg per ml Chloramphenicol (Sigma Chemical Company, St. Louis, Missouri). *E. coli* pMYCO7017::TnKm was cultured overnight at 37°C on LB agar or in broth containing 50 μg per ml Kanamycin (Sigma Chemical Company). Mycobacterial strains were cultured at 32°C in Middlebrook 7H9 broth medium (Difco Labs, Sparks, Maryland) or on solid media containing 1.5% wt/vol agar (Fisher Scientific). Middlebrook 7H9 media was always supplemented with 10% (vol/vol) oleic acid, albumin, dextrose supplement (OADC). Mycobacterial transformants carrying pMYCO7017::TnKm were grown in Middlebrook 7H9 broth medium or on agar (Difco) supplemented with OADC (10% vol/vol) and Kanamycin at a concentration of 50 μg per ml and were incubated at 32°C.

## 2.2 BAC library construction and isolation of pMYCO7017 DNA

A whole-genome *M. ulcerans* bacterial artificial chromosome (BAC) library was constructed at the Clemson University Genomics Institute as previously described (Tomkins, 2001). Briefly, DNA from *M. ulcerans* 1615 was prepared in agarose plugs, then partially digested with HindIII. DNA fragments were separated by PFGE, cloned into pBeloBAC11, and then transformed into *E. coli* DH10B by electroporation. The BAC used for this study, pMYCO7017, contains a 152 kb fragment of *M. ulcerans* plasmid DNA and includes the majority of the *M. ulcerans* virulence plasmid, pMUM001.

**Table 1. Bacterial strain information.**

| Species | Strain | Source [a] | Reference |
|---|---|---|---|
| *E. coli* | DH10B (pMYCO7017) | CUGI | This work |
| | EC100 | Epicentre | Epicentre |
| | EC100 (pMYCO7017::TnKm) | UTK | This work |
| *M. fortuitum* | 10394 | UAM | (Bachrach, 2000) |
| | 10394.1 (pMYCO7017::TnKm) | UTK | This work |
| | 10394.4 (pMYCO7017::TnKm) | UTK | This work |
| | 10394.6 (pMYCO7017::TnKm) | UTK | This work |
| | 10394.10 (pMYCO7017::TnKm) | UTK | This work |
| *M. marinum* | 1218 [c] | ATCC 927 [b] | (Collins, 1975) |
| | 1218 white mutant [d] | UTK | This work |
| | DL240490 | NCM | (Ucko, 2005) |
| | 1218Y(pMYCO7017::TnKm) [e] | UTK | This work |
| *M. smegmatis* | mc2 155 | AECM | (Lee, 1991) |
| *M. ulcerans* | 1615 | ATCC 35840 [b] | (Pettit, 1966) |
| | 1615::Tn118 | UTK | (Stinear, 2004) |

[a] AECM, Howard Hughes Medical Institute, Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, NY; CUGI, Clemson University Genomics Institute, Clemson, SC, USA (Jeff Tomkins); NCM, Israel Oceanogrphic and Limnological Research, Ltd., National Center for Mariculture, Eilat, Israel; UTK, Department of Microbiology, University of Tennessee, Knoxville, TN; UAM, Universidad Autonoma de Madrid, Spain (M. J. Garcia);

[b] ATCC, American Type Culture Collection

[c] Light-inducible pigment production

[d] No pigment production

[e] Constitutive pigment production

## 2.3 Pulsed field gel electrophoresis

A freezer stock of *E. coli* DH10B(pMYCO7017) (Clemson University) was revived by thawing on ice for 10 minutes, then 100ul of cells were transferred onto LB agar containing Chloramphenicol at a concentration of 12.5 μg per ml. The cultures were incubated overnight at 37°C. One colony was picked and inoculated into a 1 liter flask containing 500 ml LB broth and 12.5 μg per ml Chloramphenicol. Cultures were incubated at 37°C with shaking at 250 rpm for 10 hours. The QIAGEN Large Construct Kit was used to extract BAC DNA according to the manufacturer protocol in the 2002 handbook (QIAGEN, Valencia, California) (**Figure 7**).

The integrity and size of the DNA was verified by pulsed field gel electrophoresis (PFGE) using a CHEF-DR III system with a cooling module (BioRad, Hercules, California). An agarose gel was prepared using 1 gram of Sea Prep GTG Agarose (FMC BioProducts, Rockland, Maine) and 100 ml 0.5X TBE buffer (for one liter: 4.5 g tris base, 2.75 g boric acid, 2 ml 0.5M EDTA (pH 8.0)). 2.4 liters of 0.5X TBE buffer was added to the electrophoresis cell (BioRad). The buffer was pre-chilled in the chamber before the electrophoresis with a coolant pump, which was turned on and the temperature was set to 14°C. A 1 microliter slice (25 ng of DNA) of a 194 Kb low range PFG marker (New England BioLabs,Ipswich, Massechusetts) was loaded into the first well in the gel and the well was sealed with 1% agarose.

The pMYCO7017 sample DNA was prepared as follows: 8 μl DNA, 1 μl loading dye, 1 μl distilled, deionized water (dd$H_2O$). Electrophoresis was run according to the following parameters: current 6 volts per cm, pulse angle 120°, and temperature 14°C. The ramp times were 3 seconds to 15.5 seconds for 12 hours followed by 15.5 seconds to 50 seconds for 8 hours. The DNA was visualized by ethidium bromide staining and ultraviolet transillumination. A graph was made of the molecular weight of the band sizes of the 194 Kb low range PFG marker versus the distance they migrated in the gel. This graph was used to extrapolate the size of the pMYCO7017 DNA band, 152 Kb.

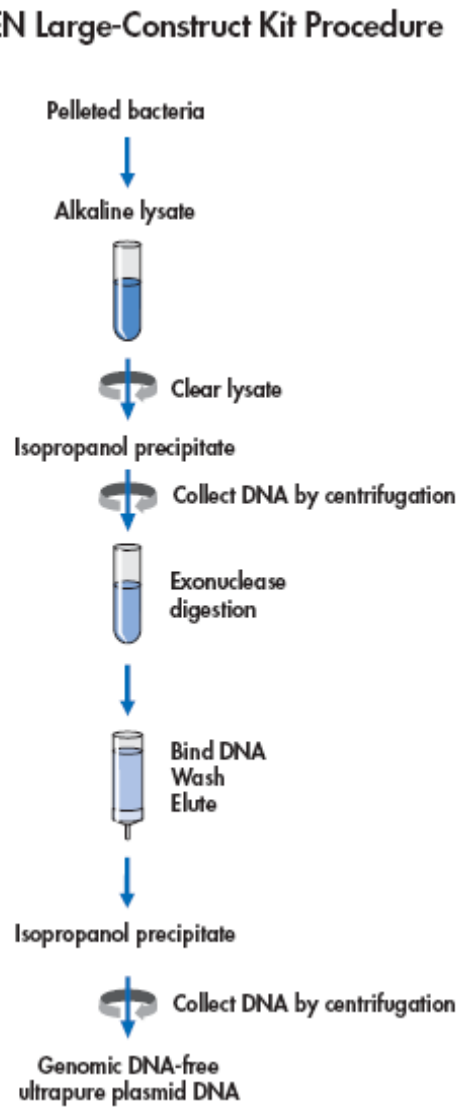


**Figure 7. Qiagen Large Construct Protocol.**
The Large Construct Kit (QIAGEN) was used to isolate pMYCO7017 BAC DNA from *E. coli* DHB10(pMYCO7017). The protocol involves a modified alkaline lysis procedure followed by an ATP-dependent exonuclease digestion. DNA is bound to a resin column, washed, and eluted. The DNA is further concentrated by isopropanol precipitation, and then resuspended in an appropriate volume of TE buffer or water. (Adapted from 2002 QIAGEN handbook.)

### 2.4 Creation of pMYCO7017::TnKm by transposon mutagenesis

The EZ::TN$^{TM}$ <KAN-2> Insertion Kit (Epicentre, Madison, Wisconsin) was used to introduce a Kanamycin resistance transposon (Km) into pMYCO7017 by random mutagenesis according to the manufacturer protocol (**Figure 8**). Briefly 77 ng BAC DNA, 1 µl 10X reaction buffer, 1 µl KAN-2 transposon, and 1 µl transposase enzyme were combined in a 0.2 ml tube (Eppendorf, Westbury, New York) and incubated at 37°C for 2 hours. The transposition reaction was stopped by adding 1 µl EZ-TN5 10X Stop Solution and heating at 70°C for 10 minutes.

The new construct, pMYCO7017::TnKm, was transformed into *E. coli* ElectroMAX EC100 (Epicentre) by electroporation according to the manufacturer protocol. Briefly, 1µl pMYCO7017::TnKm DNA and 50 µl bacterial cells were mixed and transferred to an electroporation cuvette. The cells were pulsed with 2.5 kV and 200 Ω resistance, then recovered in S.O.C. broth medium (Invitrogen) for 1 hour at 37°C with shaking at 150 rpm. Electroporation was performed using a Gene Pulser II with pulse controller (BioRad) and 2mm electroporation cuvettes (Fisher Scientific, Fairlawn, New Jersey). The recovered bacterial cells were plated onto LB agar containing 50 µg per ml Kanamycin and incubated at 37°C overnight.


**Figure 8. Creation of pMYCO7017::TnKm.**
The EZ-Tn5 <KAN-2> transposon (Km) was randomly inserted into the DNA by incubating 77 ng pMYCO7017 DNA with 1 µl 10X reaction buffer, 1 µl KAN-2 transposon, and 1 µl transposase enzyme. (Adapted from Epicentre catalogue number EZI982K.)

### 2.5 PCR screening of bacterial transformants

PCR was used to select true *E. coli* EC100 (pMYCO7017::TnKm) transformants by probing for the enoyl reductase (ER) domain found within *mlsA*, a polyketide synthase gene found on pMYCO7017::TnKm. Additionally, PCR was used to find a clone containing a Km insertion outside the mycolactone gene cluster, specifically within the insertion sequence IS2606. For this particular screening, each clone subjected to two separate PCR reactions using primers in the following combinations; Kan-F with IS2606-R, and Kan-R with IS2606-F (**Figure 9**). Primer sequences are listed in **Table 2**.

Reaction conditions for PCR amplification were as follows: each 50 µl reaction contained 1 µl each of forward and reverse primer (1.0 µM), 25 µl FailSafe$^{TM}$ PCR 2X PreMix G buffer (Epicentre), 22.6 µl dd$H_2O$, 1 unit of FailSafe$^{TM}$ PCR Enzyme Mix (Epicentre). A sterile toothpick was used to obtain bacterial cells which were directly added to each PCR reaction tube as a source of template DNA. Cycling conditions for ER were carried out as described previously (Williamson, 2008) in a Mastercycler (Eppendorf). Cycling conditions for both Kan-F/IS2606-R and Kan-R/IS2606-F reactions was as follows: initial denaturation at 98°C for 5 minutes, followed by 34 cycles of denaturation at 98°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute and 30 seconds, and a final extension of 72°C for 10 minutes. The 7 µl of each PCR reaction was combined with 1 µl 10X loading dye and samples were analyzed by electrophoresis (100 volts for 60 minutes) using a 1% agarose (Invitrogen) and ethidium bromide. Band sizes were compared to a 1 kb DNA ladder (Invitrogen).


**Figure 9. Nucleotide sequence for the <KAN-2> transposon and insertion sequence IS2606.**

The DNA sequence for the EZ-Tn5 <KAN-2> transposon was obtained from Epicentre (catalogue number EZ1982K). The boxes indicate the location of the Kan2-F and Kan2-R primers. The nucleotide sequence for IS2606 was obtained from the BuruList Web Server (http://genolist.pasteur.fr/BuruList). The highlighted areas indicate the location and sequence of the IS2606Long-F and IS2606Long-R primers.

**Table 2. Oligonucleotides used in this study.**

| Oligonucleotide | Sequence (5′→ 3′) | Description |
|---|---|---|
| Kan2-F | ACCTACAACAAAGCTCTCATCAACC | Kanamycin transposon |
| IS2606Long-R | TAAGCGGCGTTTTCGGTTAG | Insertion sequence |
| IS2606Long-F | CAAGACTGTCGTGGCTGTGC | Insertion sequence |
| Kan2-R | GCAATGTAACATCAGAGATTTTGAG | Kanamycin transposon |
| parA-F | CGTAGCCGTTTGGACGAC | Plasmid partitioning |
| parA-R | GTCCTGGCGGATCTTTGAAC | |
| repA-F | GCCTGGAACTCGACACCAAC | Plasmid replication |
| repA-R | GCATCGACGCTCGCTACTTC | |
| ER-F | GAGATCGGTCCCCGACGTCTAC | *pks* enoyl reductase domain |
| ER-R | GGCTTGACTCATGTCACGTAAG | |
| mlsB-F | CAGCCAACTGCGCTACTACA | *pks* loading module |
| mlsB-R | AGGAGACACGGTTGGCTATG | |
| fabH-F | GGAGATCGCCACCACCAGTGG | Type III ketosynthase |
| fabH-R | GCACCACTTGCGCCGCATAGC | |
| mup027c-F | AGCTGACCGAAGCCCTCTAC | Plasmid insertion sequence |
| mup027c-R | CAGTGCTCCTTGGAGGTAGG | |
| Kan118-F | ACAGGATGAGGATCGTTTCG | Mariner Kanamycin transposon |
| Kan118-R | CAATAGCAGCCAGTCCCTTC | |

## 2.6 Making bacterial cells electrocompetent

Procedures were adapted from protocols described by Tanya Parish (Parish, 1998). *E. coli* EC100 (pMYCO7017::TnKm) cells were grown to mid log phase in 100 ml LB broth containing 50 μg per ml Kanamycin under the conditions described above. Cells were harvested by centrifugation in an MX-200 high speed microcentrifuge (TOMY, Tokyo, Japan) for 10 minutes at 3,000 x g, 4°C, washed once with a wash solution (10 mM Tris-HCl (pH 7.5) plus 1 mM $MgCl_2$). Washed cells were pelleted by centrifugation, then resuspended in 3 ml wash solution and chilled on ice for 10 minutes before use.

Recipient mycobacteria were grown to mid log phase in 100 ml Middlebrook 7H9 broth under the conditions described above. Cells were harvested by centrifugation for 15 minutes at 5,000 x g, 4°C. Cells were then made electrocompetent through a series of washes with ice-cold 10% sterile glycerol (ICSG) as follows; cell pellet was resuspended in 100 ml ICSG by vortexing, then collected by centrifugation for 15 minutes at 5,000 x g, 4°C. This procedure was repeated 3 times with decreasing volumes of ICSG; 50 ml, 25 ml, and 10 ml, respectively. Mycobacterial cells were resuspended in a final volume of 3 ml ICSG and chilled on ice for 10 minutes before use.

## 2.7 Electroduction procedure

Supplies for electroduction reactions were heat sterilized when possible and all external surfaces of supplies were decontaminated with 70% isopropanol. Electroduction reactions were performed inside a laminar flow biosafety cabinet. 350 μl of electrocompetent mycobacterial recipient cells (*M. marinum* 1218 or *M. fortuitum* 10394) and 50 μl electrocompetent *E. coli* EC100 (pMYCO7017::TnKm) donor cells were mixed in a sterile 2 ml Eppendorf tube, then transferred to an electroporation cuvette with a 0.2 mm gap (FisherBiotech). A Gene Pulser II (BioRad) was used to carry out the electroduction under the following conditions; 2.5 kV, 25 μF, and 1000 Ω pulse-controller resistance. For transfer of the pMYCO7017::TnKm construct from mycobacterial transformants back into electrocompetent *E. coli*, electroduction was carried out at 2.5kV, 45 μF, without a pulse controller.

After the electric pulse, the cuvette was placed on ice for 10 minutes, then the electroduction suspension was transferred to a 50 ml Falcon tube (Becton Dickinson Labware, Franklin Lakes, New Jersey) containing 5 ml Middlebrook 7H9 broth supplemented with 10% OADC. Bacterial cells were allowed to recover for 2 hours at 32°C. Cells were then plated onto Middlebrook 7H9 agar with 10% OADC and 50 μg per ml Kanamycin and incubated at 32°C for 12 to 14 days. Potential transformant colonies were subcultured onto fresh selective media for further analysis.

### 2.8 Conjugation procedure

*M. ulcerans* 1615::Tn118 was used as the donor strain for the mycobacterial conjugation experiment. It is a mycolactone negative mutant that was created by random mutagenesis using a mariner Kanamycin transposon. *M. ulcerans* 1615::Tn118 and *M. fortuitum* 10394 were grown to mid stationary phase in Middlebrook 7H9 broth supplemented with 10% OADC under the appropriate conditions as described above. Filter mating between the donor and recipient strains was carried out as previously described (Lessard, 2004) with the following exceptions: media used was Middlebrook 7H9 broth or agar supplemented with 10% OADC; conjugation mixtures were incubated overnight at 32°C; following the final resuspension, 10 fold dilutions were made ($10^{-1}$, $10^{-2}$, and $10^{-3}$) and 100 μl of each dilution were plated onto selective media; plates were incubated at 37°C for 7-14 days or until potential transconjugant colonies appeared. Plates were incubated at 37°C on selective media to kill the *M. ulcerans* 1615::Tn118 donor cells and encourage growth of and *M. fortuitum* 10394 transconjugants. Each potential transconjugant colony was picked and vortexed in 1 ml of 0.01% SDS, then passed through a 25 gauge needle three times to break up large clumps of bacterial cells. Next, ten-fold dilutions of each potential transconjugant suspension were made ($10^{-1}$, $10^{-2}$, and $10^{-3}$) using M7H9 broth. Ten microliters of each $10^{-3}$ suspension was spread onto Kanamycin selective media and incubated at 37°C for 7 – 14 days.

DNA was obtained from each potential transconjugant by the boil preparation method. One loopfull of each potential transconjugant was suspended in 400 microliters of sterile distilled, deionized water in a 2 ml free-standing, screw cap microtube (Denville

Scientfic, Inc., Metuchen, NJ). The screw cap tubes were placed in a plastic floatation device and exposed to boiling water for 20 minutes. The boil preparation tubes were removed from the boiling water and allowed to cool to room temperature, then centrifuged for 5 minutes at 4600 x g. Three hundred microliters of supernatant was transferred to a new, sterile 0.2 ml Eppendorf tube and stored at 4.0°C until use. The supernatant from each boil preparation contained whole cell DNA from each potential transconjugant.

Whole cell DNA from each potential transconjugant colony was screen by PCR for both the ER domain of mycolactone gene cluster and the mariner Kanamycin transposon. The oligonucleotide sequences for both ER and the mariner Kanamycin transposon can be found in **Table 2**. Each PCR reaction was as follows: each 50 µl reaction contained 1µl each of forward and reverse primer (1.0 µM), 10 µl GoTaq$^{TM}$ 5X Green reaction buffer (Promega, Madison, Wisconsin), 17.6 µl dd$H_2O$, 1 unit of GoTaq$^{TM}$ DNA Polymerase (Promega, Madison, Wisconsin), 1 µl dNTPs (Promega), and 5 µl DNA template. The thermocyling program used for the mariner Kanamycin transposon is as follows: initial denaturation at 94°C for 5 minutes, followed by 34 cycles of denaturation at 47°C for 1 minute, 55°C for 45 seconds, 72°C for 30 seconds, and a final extension at 72°C for 10 minutes. The PCR amplicons were analyzed by 1% agarose gel electrophoresis with ethidium bromide staining and band sizes were compared to a 1 kb DNA ladder (Invitrogen).

### 2.9 DNA extraction and PCR

BAC DNA was extracted from mycobacterial cells by a protocol adapted from Lamour and Finley as described previously by Williamson et al (Williamson, 2008). Procedure was followed as described except that three loopfulls of each potential mycobacterial clone were vortexed in 400 µl lysis solution and one gram 1.0 mm glass beads (Sigma-Aldrich). PCR was used to probe for the following genes located on pMYCO7017; *parA*, *repA*, enoyl reductase domain (ER), *mlsB*, *fabH*, and *mup027c*. Primers used to screen transformants are listed in **Table 2**. The thermocyling program used for *mup027c* is as follows: initial denaturation at 97°C for 3 minutes, followed by 35 cycles of denaturation

at 97°C for 1 minute, 59°C for 45 seconds, 72°C for 1 minute, and a final extension at 72°C for 10 minutes. The thermocyling program used for ER was as followed described previously. All other thermocyling was as follows: initial denaturation at 98°C for 5 minutes, followed by 32 cycles of denaturation at 98°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute and a final extension at 72°C for 10 minutes.

Reaction conditions for PCR amplification were as follows: each 50 µl reaction contained 1µl each of forward and reverse primer (1.0 µM), 25 µl FailSafe™ PCR 2X PreMix G buffer (Epicentre), 17.6 µl dd$H_2O$, 1 unit of FailSafe™ PCR Enzyme Mix (Epicentre), and 5 µl DNA template. The PCR amplicons were analyzed by 1% agarose gel electrophoresis with ethidium bromide staining and band sizes were compared to a 1 kb DNA ladder (Invitrogen).

### 2.10 Isolation and Analysis of lipids

Lipid extraction from mycobacterial cells was performed as described previously (Mve-Obiang, 2005). Mycobacterial cultures were grown to confluence on Middlebrook 7H9 agar, with or without antibiotics, as described above. Lipids were extracted from mycobacterial pellets using chloroform : methanol (2:1) and the organic phase was dried down using nitrogen gas and a 50°C heat block. The lipid extract was solubilized in acetone (ASL) and ASLs were analyzed by thin layer chromatography as described previously using a solvent of chloroform : methanol : water (90:10:1) (Mve-Obiang, 2005).

Cell rounding and cell cycle arrest in the $G_0/G_1$ phase are typical affects observed when L929 murine fibroblasts are exposed to mycolactone in cell culture. In order to ascertain the biological activity of the ASLs, and thus the presence or absence of mycolactone within the ASL extract from mycobacterial transformants, cypathicity assays were carried out as previously described (Snyder, 2003). Briefly, murine L929 were grown overnight in Dulbecco Modified Eagle Medium (DMEM) supplemented with 5% heat-inactivated calf serum (Gibco BRL, Grand Island, New York) at 37°C with 5% carbon dioxide.

Serial 1:5 dilutions of ASLs, resuspended in ethanol, were added to L929 cultures, which were then observed by microscope for any cytopathic changes.

### 2.11 Mass spectrometry of lipid extracts

ASLs extracted from the different bacteria strains listed in Table 1 were analyzed using Shimadzu LC-20AD analytical HPLC system. An autosampler was used to inject 50 µl into a reverse phase Phenomenex –luna 250X4.6 mm $C_{18}$ column. Separation was obtained using acetonitrile /water mobile phase in a linear gradient of 55% -100% acetonitrile over 45 minutes at a flow rate of 2 ml per minutes. Column elution was monitored using UV detection at both 254 and 360 nm. Data acquisition was controlled and analyzed using EZ-start 7.3 software.

# CHAPTER 3 : RESULTS

## 3.1 Analysis of pMYCO7017

The *M. ulcerans* mycolactone plasmid has been difficult to study due to its low copy number and large size, which ranges from 155 Kb to 200 Kb across different mycolactone producing mycobacteria. To circumvent this difficulty, we constructed a bacterial artificial chromosome (BAC) library using *M. ulcerans* whole-genome DNA in collaboration with the Jeff Tompkins and the genome center at Clemson University. During this process we were fortunate to clone nearly the entire mycolactone plasmid into the BAC vector as a single fragment. The resulting BAC, pMYCO7017, included the entire 110 kb mycolactone gene cluster.

pMYCO7017 DNA was isolated from the *E. coli* transformant harboring it through the use of the Qiagen Large Construct kit. The size and integrity of the DNA was checked by PFGE. pMYCO7017 was determined to be 152 Kb in size when compared to the 194 Kb low range PFG marker (**Figure 10)**. Additionally, the fragility of this large BAC construct is illustrated in **Figure 10** by the presence of the upper DNA band, which represents nicked and non-supercoiled DNA.

## 3.2 Transposon mutagenesis of pMYCO7017

The goal of this work was to introduce the *M. ulcerans* mycolactone gene cluster into a faster growing mycobacterial species as a first step towards achieving heterologous expression of the mycolactone gene cluster. Construction of a strain with a mycolactone plasmid containing a selectable marker for use in mycobacteria was an essential first step for both studies on heterologous expression, and conjugation. In addition, it was important that introduction of a selectable marker did not interrupt the genes for mycolactone biosynthesis. The first problem encountered was that the BAC pMYCO7017 did not contain a good selectable marker for use in mycobacterial species. The BAC, pMYCO7017, contained a selectable marker chloramphenicol, but this is only useful in enteric bacteria and can not be used as a selectable marker in *Mycobacteria* species. However, kanamycin provides strong selection in most species of mycobacteria. In order to provide selection for pMYCO7017 in mycobacterial species, the EZ::TN$^{TM}$ <KAN-2> insertion kit from Epicentre was used to introduce a Kanamycin transposon into pMYCO7017 by random mutagenesis as described in the materials and

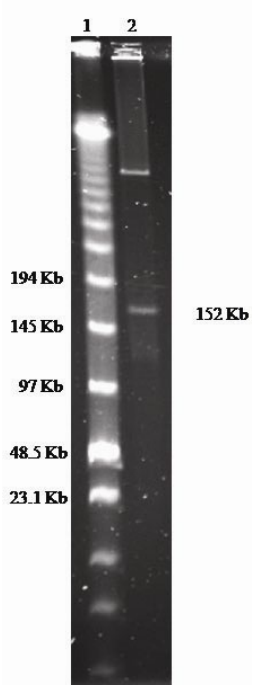


**Figure 10. The size and integrity of pMYCO7017 was verified by pulsed field gel electrophoresis.**

Pulsed field gel electrophoresis of pMYCO7017. BAC DNA was isolated from *E. coli* using the Qiagen Large Construct Kit and analyzed by PFGE under the conditions described. Lane 1: 194 Kb Low range PFG Marker. Lane 2: pMYCO7017 DNA, upper band contains nicked and non-supercoiled DNA; lower band contains intact, supercoiled pMYCO7017 DNA.

methods section. Two separate mutagenesis reactions were carried out. After each mutagenesis reaction, the resulting pMYCO7017::TnKm constructs were transformed into electrocompetent *E. coli* EC100 by electroporation. Each set of *E. coli* EC100 (pMYCO7017::TnKm) transformants was cultured on LB agar plates containing 50 μg per ml of Kanamycin.

### 3.3 PCR screening of *E. coli* transformants carrying pMYCO7017::TnKm

#### 3.3.1 *E. coli* EC100 (pMYCO7017::TnKm) transformants from first reaction

The first Kanamycin transposon mutagenesis reaction and electroporation into *E. coli* resulted in fourteen potential *E. coli* EC100 (pMYCO7017::TnKm) transformants. The first step in analyzing the *E. coli* EC100 (pMYCO7017::TnKm) transformants was to probe them for the presence of ER, the enoyl reductase domain of the polyketide synthase genes *mlsA1* and *mlsA2*. ER can be found on pMYCO7017::TnKm and would therefore only be present in *E. coli* after a successful transformation reaction. Through colony PCR, it was determined that thirteen out of the fourteen colonies from the first mutagenesis and electroduction reaction were positive for ER (**Figure 11**). These thirteen colonies were subcultured onto fresh LB + Kanamycin plates for further analysis.

DNA was isolated from *E. coli* EC100 (pMYCO7017::TnKm) transformant number 2 and quantified by spectrophotometry to be 85 ng/μl. The nucleotides were sequenced outwards from the Kanamycin transposon located on pMYCO7017::TnKm using the Kan2-F primer. NCBI BLAST was used to determine that the closest match to the resulting DNA sequence was module 9 within a polyketide synthase gene of pMYCO7017::TnKm. Transformant number 2 contained a pMYCO7017::TnKm construct in which the Kanamycin transposon was interrupting a gene necessary for the production of the mycolactone toxin, and thus was not of interest for this study.

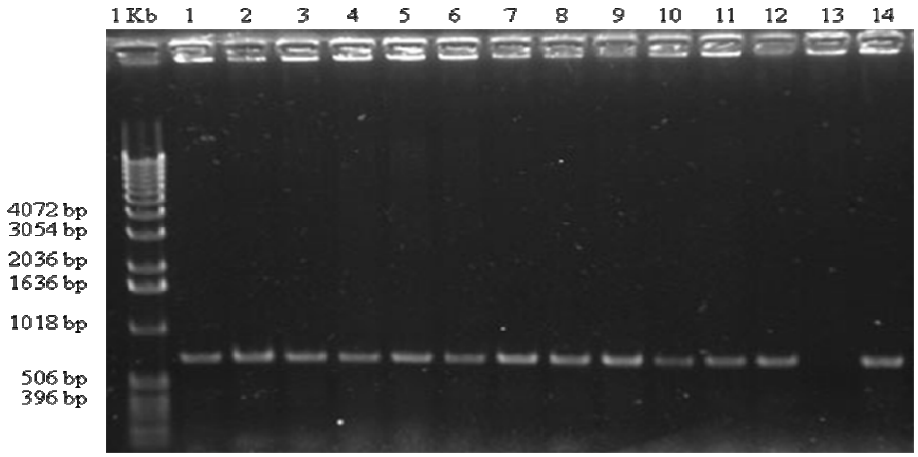


**Figure 11. ER PCR results for potential *E. coli* EC100 (pMYCO7017::TnKm) transformants from the first mutagenesis reaction.**
Lanes 1 – 14 represent individual *E. coli* EC100 colonies that grew on LB + Kanamycin plates after the first electroporation reaction with pMYCO7017::TnKm.

### 3.3.2 *E. coli* EC100 (pMYCO7017::TnKm) transformants from second reaction

A second reaction, completed exactly as described previously, was carried out in order to introduce a Kanamycin transposon into pMYCO7017 by random mutagenesis. The resulting construct, pMYCO7017::TnKm, was transformed into *E. coli* EC100 by electroporation. Thirty eight potential transformant colonies grew on LB + Kanamycin and were subcultured onto fresh selective media for analysis.

A different strategy was used for screening the second round of *E. coli* EC100 pMYCO7017::TnKm) transformants than was used previously. Colonies were specifically screened for the presence of the Kanamycin transposon (TnKm) within the insertion sequence IS2606. This was accomplished by carrying out two colony PCR reactions for each transformant. Reaction one involved the use of a forward primer located within IS2606 and a reverse primer located near the 5' end of TnKm. Reaction two utilized a forward primer located near

the 3' end of TnKm and a reverse primer located within IS2606. If present, the molecular weight of the PCR amplicon from each reaction was calculated by gel electrophoresis when compared to a 1 Kb molecular weight standard. Additionally, if a PCR product was obtained from both reactions, this indicated that TnKm was located completely within the boundaries of the IS2606 element.

A total of 38 *E. coli* EC100 pMYCO7017::TnKm) transformants were screened in this manner. Two transformants were PCR positive for both Kan/IS2606 reactions (**Figure 12**). By adding together the combined total size of both amplicons, it was determined that the entire Kanamycin transposon was contained within an IS2606 element in the pMYCO7017::TnKm constructs harbored by transformants 2 and 10 (**Figure 13**).

PCR products from transformant number 10 for both reactions (IS2606Long-F/Kan2-R and Kan2-F/IS2606Long-R) were purified using the QIAQuick PCR Purification Kit (QIAGEN) and sequenced using primers specific for the Kanamycin transposon. The resulting nucleotide sequence was compared to sequences in the database of NCBI BLAST, resulting in a match to *Mycobacterium ulcerans* insertion sequence IS2606. Thus, the pMYCO7017::TnKm construct carried by *E. coli* transformant number 10, contained a selectable Kanamycin marker in an IS2606 element and not interrupting the mycolactone gene cluster. This pMYCO7017::TnKm construct was utilized for all subsequent electroductions, with the goal of introducing the mycolactone gene cluster, with a selectable marker, into faster growing mycobacterial species.

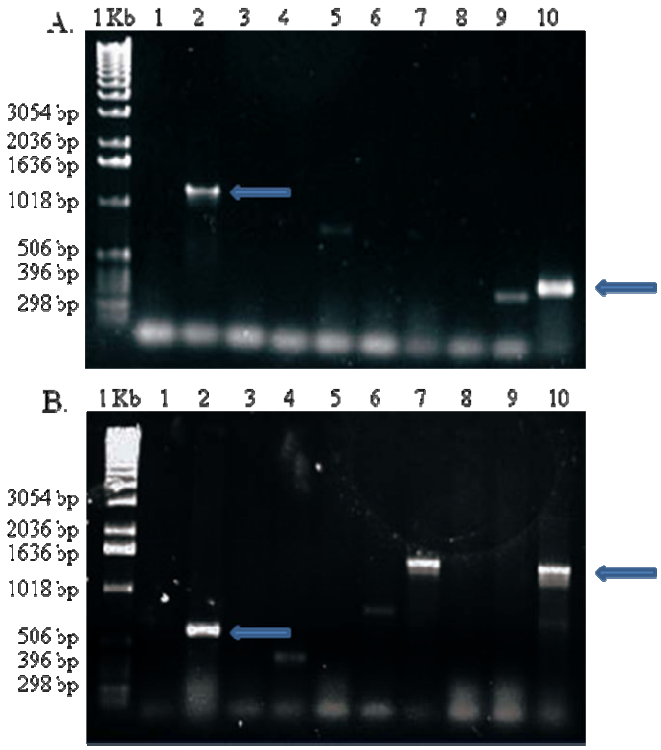


**Figure 12. Results of PCR screening of *E. coli* EC100 (pMYCO7017::TnKm) transformants from the second Kanamycin transposon mutagenesis reaction.** PCR screening of *E. coli* EC100 (pMYCO7017::TnKm) transformants 1 through 10. Each lane represents the colony PCR results of an individual colony. (A) Results of reaction one, using primers IS2606Long-F and Kan2-R. Colony 2 amplicon 1 size: 1200 bp. Colony 10 amplicon 1 size: 350 bp. (B) Results of reaction two, using primers Kan2-F and IS2606Long-R. Colony 2 amplicon 2 size: 600 bp. Colony 10 amplicon 2 size: 1450 bp.

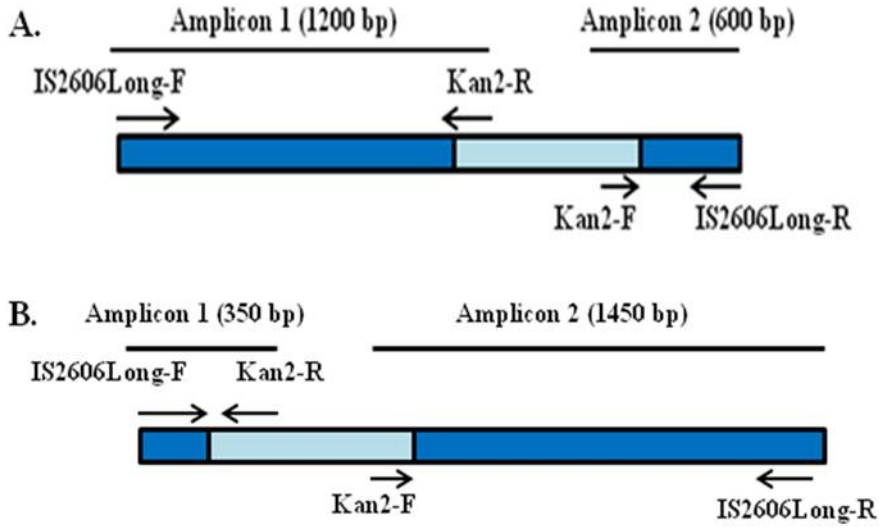


**Figure 13. Schematic representation of the location of the Kanamycin transposon within IS2606.**

These pMYCO7017::TnKm constructs were harbored by two different *E. coli* transformants. Blue bars represent IS2606 DNA, aqua bars represent the Kanamycin transposon. (A) Transformant 2: Schematic map of TnKm location in pMYCO7017::TnKm harbored by *E. coli* transformants 2, as determined from PCR amplicon sizing. (B) Transformant 10: Schematic map of TnKm location in pMYCO7017::TnKm harbored by *E. coli* transformant 10, as determined from PCR amplicon sizing.

### 3.4 Transfer of pMYCO7017::TnKm into mycobacteria by electroduction

Following construction of pMYCO7017::TnKm, which contains the *M. ulcerans* polyketide synthases and polyketide-modifiying enzymes necessary for mycolactone production, in addition to a Kanamycin resistance gene, the next step was to try to introduce pMYCO7017::TnKm into a faster growing species of mycobacteria. The most common way to introduce naked DNA into bacterial cells is transformation. The first step of transformation is to make the bacterial cell competent to take up foreign DNA by chemically weakening the cell wall, usually with calcium chloride or rubidium chloride. Competent cells are then mixed with the DNA of interest and transformed, either by heat or electrical shock. In this study, the DNA of interest was pMYCO7017::TnKm, an extremely large, low copy number plasmid harbored by *E. coli*. Obtaining the microgram amounts of intact pMYCO7017::TnKm plasmid DNA needed to carry out a successful transformation reaction was exceptionally difficult. Additionally, the thick, waxy cell wall of mycobacteria makes transformation difficult and consequently, mycobacterial transformation frequency is often very low.

In order to circumvent these difficulties, DNA can be introduced into mycobacteria by a process called electroduction. "Electroduction is the direct transfer of plasmid DNA from one organism to another by electroporation" (Parish, 1998). Electroduction was used to introduce pMYCO7017::TnKm plasmid DNA into mycobacteria. The cell walls of the donor cells, *E. coli* EC100 (pMYCO7017::TnKm), and the recipient cells, either *M. marinum* 1218 or *M. fortuitum* 10394, were weakened through a series of washings, then they were mixed together and exposed to an electrical shock. **Table 3** contains results from the multiple electroduction attempts to introduce pMYCO7017::TnKm into *M. marinum*, *M. fortuitum*, *M. smegmatis*, and *M. ulcerans*. Out of 39 separate electroduction reactions, a total of 114 potential electroductants grew on selective media and were screened for the presence of pMYCO7017::TnKm. Eleven out of the 114 potential electroductants were PCR-confirmed to be true, having acquired pMYCO7017::TnKm by electroduction. There was 1 *M. marinum* electroductant and 10 *M. fortuitum* electroductants. (**Table 4**). A more detailed description of the analysis of these electroductants is detailed in the next sections.

**Table 3. Electroduction of pMYCO7017::TnKm into Mycobacterial species.**

| Recipient strain | Electroduction reactions | Genotype ER[a] |
|---|---|---|
| *M. marinum* 1218 | 16 | 1/1 |
| *M. marinum* DL240490 | 8 | 0/0 |
| *M. smegmatis* $mc^2155$ | 7 | 0/0 |
| *M. fortuitum* 10394 | 6 | 10/113 |
| *M. ulcerans* 1615A | 2 | 0/0 |

[a] ER, enoyl reductase domain of the polyketide synthase gene, *mlsA*

### 3.5 Analysis of *M. marinum* electroductant

After sixteen separate electroduction reactions involving billions and billions of *M. marinum* cells, there was one successful electroductant, which was named *M. marinum* 1218 (pMYCO::TnKm). The genotype of *M. marinum* 1218 (pMYCO::TnKm) was confirmed by PCR, specifically probing for 6 pMYCO7017::TnKm genes (**Figure 14**). The electroductant remained PCR positive for the pMYCO7017::TnKm genes after multiple subcultures, proving that the construct could be replicated in *M. marinum* 1218 and effectively passed to successive generations (**Figure 14**). This suggests that pMYCO::TnKm is stably maintained in *M. marinum.*

Wild type *M. marinum* 1218 is characterized by light-inducible carotenoid production whereas *M. ulcerans* has a mutation in a key gene required for carotenoid biosynthesis. However, the successful transductant was unique due to its constitutive carotenoid production, even in the absence of light. The severity of electrical shock used during the electroduction procedure may result in cellular damage or mutations of this sort (personal communication). Additionally, most slow-growing mycobacteria have only one copy of the rDNA operon (Parish, 1998). A direct mutation to this operon can often lead to

spontaneous resistance to chemotherapeutic agents, such as Kanamycin (Parish, 1998).

The ability of *M. marinum* 1218 (pMYCO7017::TnKm) to heterologously produce the mycolactone toxin was investigated in several ways. Lipids were extracted from *M. marinum* 1218 (pMYCO7017::TnKm) cells using chloroform:methanol (2:1). The acetone soluble lipids (ASLs) were analyzed by thin layer chromatography, cytotoxocity assay, and mass spectrometry and compared with ASLs from *M. ulcerans* 1615 or pure mycolactone. **Figure 15** shows that mycolactone was not detected by TLC in ASLs from *M. marinum* 1218 (pMYCO7017::TnKm).

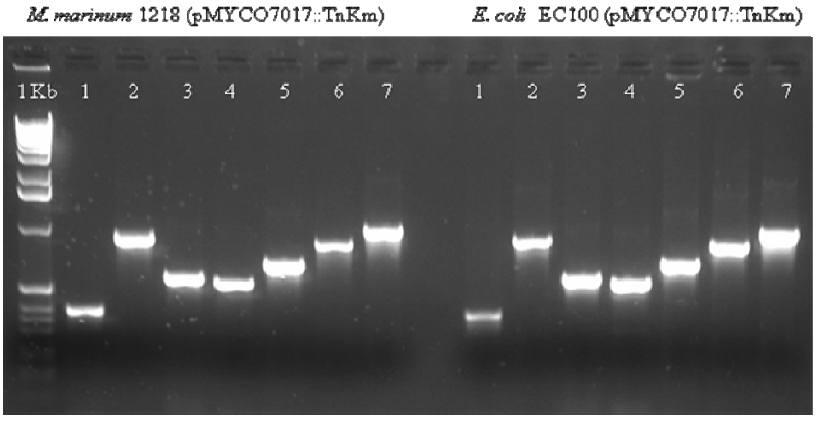


**Figure 14. Gel electrophoresis results of PCR screening of the *M. marinum* transformants.**
PCR amplicons were run on a 1.5% agarose gel at 100 volts for 45 minutes. (1) Kanamycin transposon, (2) *parA*, (3) *repA*, (4) *mlsB*, (5) ER, (6) *fabH*, (7) *p450*.

**Table 4. Mycobacterial transformants positive for pMYCO7017::TnKm genes.**

| | PCR target | | | | | | |
|---|---|---|---|---|---|---|---|
| | *kan* | *parA* | *repA* | ER | *mlsB* | *fabH* | *mup027c* [a] |
| *M. fortuitum* 10394.1 (pMYCO7017::TnKm) | - | + | + | + | - | + | - |
| *M. fortuitum* 10394.4 (pMYCO7017::TnKm) | - | + | + | + | - | + | - |
| *M. fortuitum* 10394.6 (pMYCO7017::TnKm) | + | + | + | + | + | + | + |
| *M. fortuitum* 10394.10 (pMYCO7017::TnKm) | + | + | + | + | + | + | + |
| *M. marinum* 1218 (pMYCO7017::TnKm) | + | + | + | + | + | + | ND[b] |

[a] putative transposase with homology transposases in the IS116/IS110/IS902 family identified in other *Actinomycetales*
[b] ND, not done

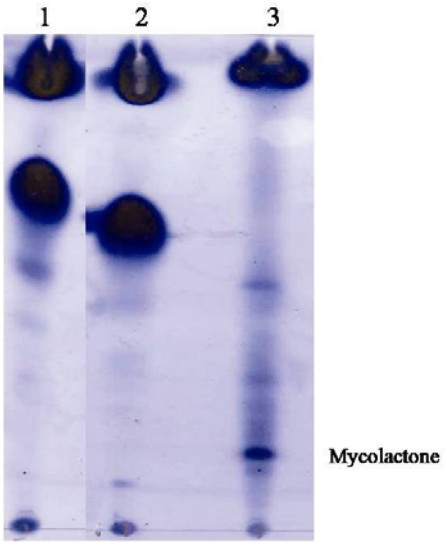


**Figure 15. Thin layer chromatography analysis of acetone soluble lipids from *M. marinum* 1218 (pMYCO7017::TnKm).**
Mycolactone was not present in the lipid extract from the *M. marinum* electroductant. (1) ASLs from *M. marinum* 1218 (pMYCO7017::TnKm), (2) ASLs from *M. marinum* 1218, and (3) ASLs from *M. ulcerans* 1615.

Next, L929 murine fibroblasts were exposed to 1:5 serial dilutions of ethanol soluble lipids from *M. marinum* 1218 (pMYCO7017::TnKm). L929 cells were observed for any cytopathic phenotypes and compared to cells exposed to pure mycolactone. **Figure 16** shows the results of the cytopathicity assay after 15 hours. Cells exposed to pure mycolactone rounded up and contained intra-cellular "blebs." L929 cells exposed to ASLs from the *M. marinum* electroductant are obviously unhealthy or even dead, but did not show the distinct effects seen after mycolactone exposure. Mycobacterial ASL extracts are a complex mixture of lipids, and even L929 cells exposed to ASLs from wild type *M. marinum* 1218 were adversely affected. In summary, ASLs from *M. marinum* 1218 (pMYCO7017::TnKm) are damaging to L929 cells, but the affects are different from those seen as a result of exposure to mycolactone.

Finally, lipids from *M. marinum* 1218 (pMYCO7017::TnKm) were sent to Richard Lee and Engy Mahrous at the University of Tennessee Health Science Center in Memphis, TN for mass spectrometry analysis. Depending on the conditions of the mass spectrometrometry analysis, mycolactone may be detected as an intact molecule with a mass of 765 ($M^+$ + Na), 742.5, or 725 ($M^+$ $^-$OH) (George, 1999) (**Figure 17**). MS-MS produces fragments characteristic of the core lactone with an *m/z* peak at 423.3, 424, 429.5 or 447 ($M^+$ + Na) and the side chain may be detected in small amounts as an *m/z* peak of 359.4. No mycolactone derived molecules were detected when the mass spectrum for ASLs from *M. marinum* 1218 (pMYCO7017::TnKm) was compared to that of *M. ulcerans* 1615 and *M. marinum* 1218 (**Figure 18**).

Taken together, all data collected through analysis of *M. marinum* 1218 (pMYCO7017::TnKm) confirms the following: (1) the *M. marinum* 1218 electroductant possesses the mycolactone gene cluster contained on pMYCO7017::TnKm; (2) *M. marinum* 1218 (pMYCO7017::TnKm) is able to replicate *M. ulcerans* mycolactone genes and pass them on to future generations; and (3) *M. marinum* 1218 (pMYCO7017::TnKm) does not produce mycolactone.

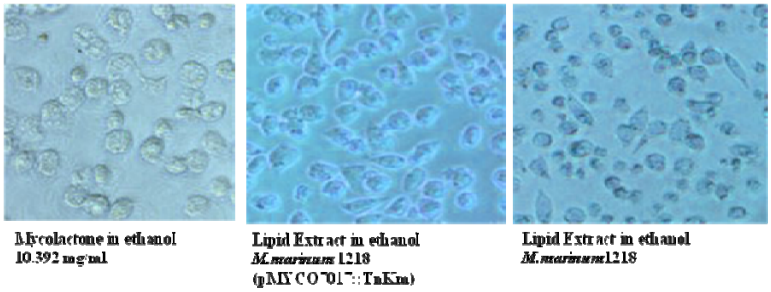


**Figure 16. Cytotoxicity assay of lipids extracted from *M. marinum* 1218 (pMYCO7017::TnKm).**
L929 murine fibroblast cells after 15 hours of exposure to pure mycolactone, ASLs from *M. marinum* 1218 (pMYCO7017::TnKm), or ASLs from *M. marinum* 1218.

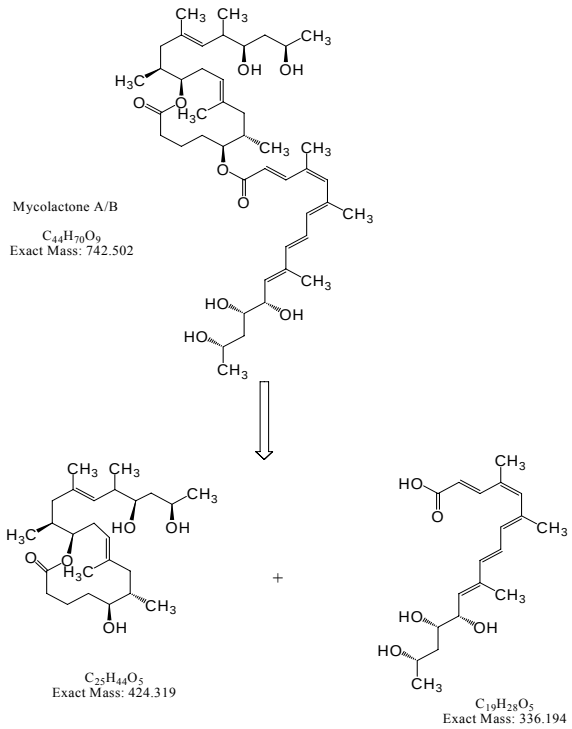


**Figure 17. Possible fragmentation pattern of mycolactone.**
During mass spectrometry analysis, mycolactone may be detected as an intact molecule or it can break up to give two fragments. (Adapted from Engy Mahrous, University of Tennessee, Memphis, TN)

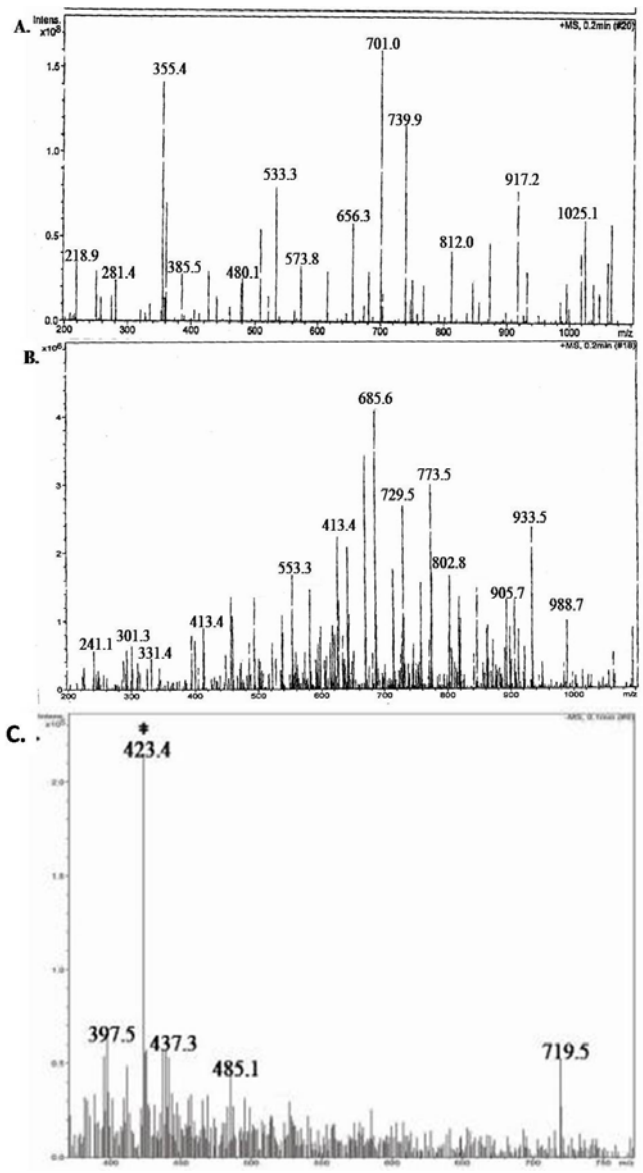


**Figure 18. Mass spectra of lipids extracted from *M. marinum* 1218 (pMYCO7017::TnKm) vs. wild type *M. marinum* 1218.**

(A) wild type *M. marinum* 1218, (B) *M. marinum* 1218 (pMYCO7017::TnKm), and (C) *M. ulcerans* 1615.

### 3.6 Transfer of pMYCO7017::TnKm from mycobacteria back into *E. coli*

In order to test the stability of the pMYCO7017::TnKm construct and to provide definitive proof of successful transduction, electroduction was used to transfer this BAC DNA from the electroductant *M. marinum* 1218 (pMYCO7017::TnKm) back into electrocompetent *E. coli*. THIS IS OUT OF SEQUENCE. IT NEEDS TO GO WITH THE MARINUM EXPERIMENT., Two electroduction reactions were carried out and 100 µl aliquots of the reaction mixtures were spread onto 18 separate LB + Kanamycin agar plates and incubated at 37°C overnight. After incubation, all plates had colonies that were too numerous to count. Seventeen were screened by colony PCR and found to be positive for TnKm, *parA*, *repA*, ER, and *mlsB*. This experiment exemplifies the stability of pMYCO7017::TnKm, in that it was successfully transferred from *E. coli* to mycobacteria and back again.

### 3.7 Spontaneous Kanamycin resistance

Ideally, the outcome of attempts to transfer pMYCO7017::TnKm into *M. fortuitum* should have been easily evaluated by the ability of the organism to grow in the presence of the selectable marker, Kanamycin. However, this work elucidated the fact that 80% of *M. fortuitum* colonies growing on solid media with 50 µg/ml of Kanamycin were spontaneously resistant to Kanamycin and did not possess the Kanamycin resistance gene or pMYCO7017::TnKm. This background level of spontaneous Kanamycin resistance was observed in *M. fortuitum* colonies after both the electroduction experiments and conjugation experiments. Spontaneous kanamycin resistance is commonly found in *M. tuberculosis* but is rare in *M. ulcerans.*

### 3.8 Analysis of *M. fortuitum* electroductants

Wild type *M. fortuitum* is known to possess multiple plasmids (Labidi, 1984), which may be problematic when attempting to introduce yet another, non-native plasmid into this species. To avoid any potential molecular competition, a plasmid minus strain, *M. fortuitum* 10394 (**Table 1**) was utilized in electroduction experiments. Six separate electroduction reactions were carried out between *E. coli* EC100 (pMYCO7017::TnKm)

and *M. fortuitum* 10394, resulting in hundreds of potential electroductants that grew in the presence of Kanamycin, the selectable marker for pMYCO7017::TnKm. One hundred and thirteen colonies were screened by PCR for the presence of ER, the enoyl reductase domain of the polyketide synthase genes. Out of all colonies screened, 10 *M. fortuitum* 10394 colonies were PCR positive for ER, as illustrated in **Table 4**.

Two electroductants, *M. fortuitum* 10394.1 (pMYCO7017::TnKm) and *M. fortuitum* 10394.4 (pMYCO7017::TnKm), were PCR positive for 4 out of the 6 pMYCO7017::TnKm-specific genes, including *parA*, *repA*, ER, and *fabH* (**Table 4**). Two additional electroductants, *M. fortuitum* 10394.6 (pMYCO7017::TnKm) and *M. fortuitum* 10394.10 (pMYCO7017::TnKm), were PCR positive for all 6 pMYCO7017::TnKm-specific genes (**Table 4**). The electroductants remained PCR positive for the pMYCO7017::TnKm genes after multiple subcultures, proving that the construct could be replicated in *M. fortuitum* 10394 and effectively passed to successive generations.

The ability of the *M. fortuitum* 10394 (pMYCO7017::TnKm) electroductants to heterologously produce the mycolactone toxin was investigated in the same manner as described for the *M. marinum* electroductants. Lipids were extracted from the *M. fortuitum* cells using chloroform:methanol (2:1). The acetone soluble lipids (ASLs) were analyzed by thin layer chromatography, cytotoxocity assay, and mass spectrometry and compared with ASLs from *M. ulcerans* 1615 or pure mycolactone. **Figure 19** shows that mycolactone was not detected by TLC in ASLs from *M. fortuitum* 10394.1 (pMYCO7017::TnKm) or *M. fortuitum* 10394.4 (pMYCO7017::TnKm). A faint band of significance may be visible in ASLs from *M. fortuitum* 10394.6 (pMYCO7017::TnKm), but this is speculative. **Figure 20** shows the TLC results of ASLs obtained from *M. fortuitum* 10394.10 (pMYCO7017::TnKm) compared to ASLs from *M. ulcerans* 1615. Again, there is not a distinct mycolactone band for the electroductant, however, there is the shadow of a band present in area of interest.

Acetone soluble lipids from all four *M. fortuitum* 10394 electroductants were resuspended in ethanol in preparation for cytotoxicity assays (**Figure 21**). L929 murine

fibroblasts were grown overnight at 37°C with 5%, in 96 wells plates containing DMEM. L929 cells were exposed to serial 1:5 dilutions of ASLs from each electroductant and observed for cytopathic changes. L929 cells exposed to ASLs from *M. fortuitum* 10394.4 (pMYCO::TnKm) showed different affects than cells exposed to ASLs from wild type *M. fortuitum* 10394. However it was unclear if these affects were the same affects seen after exposure to pure mycolactone. L929 fibroblast cells did not show any cytotoxic affects or cell rounding after exposure to ASLs from *M. fortuitum* 10394.1 (pMYCO7017::TnKm), *M. fortuitum* 10394.6 (pMYCO7017::TnKm), or *M. fortuitum* 10394.10 (pMYCO7017::TnKm).

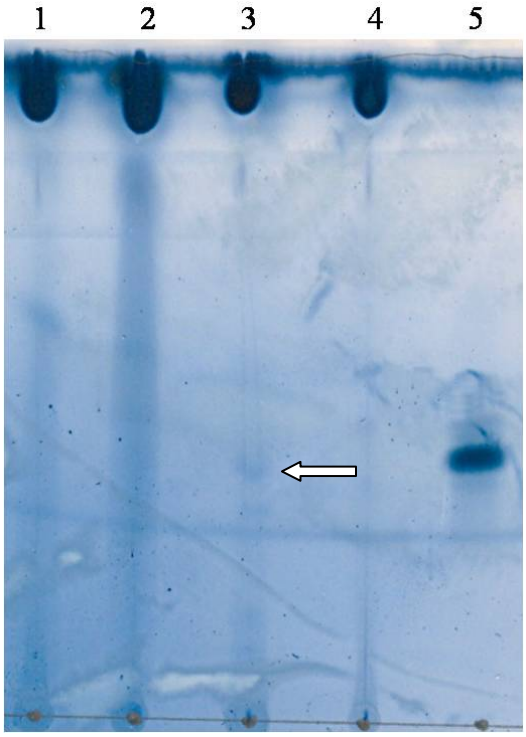


**Figure 19. TLC analysis of acetone soluble lipids from *M. fortuitum* electroductants 1, 4, and 6.**

TLC analysis of acetone soluble lipids from *M. fortuitum* electroductants 10394.1 (lane 1), 10394.4 (lane 2), and 10394.6 (lane 3) compared to wild type *M. fortuitum* 10394 (lane 4) and pure mycolactone (lane 5). The arrow in lane 3 points to a faint band of significance indicating a molecule with a similar rf to mycolactone.

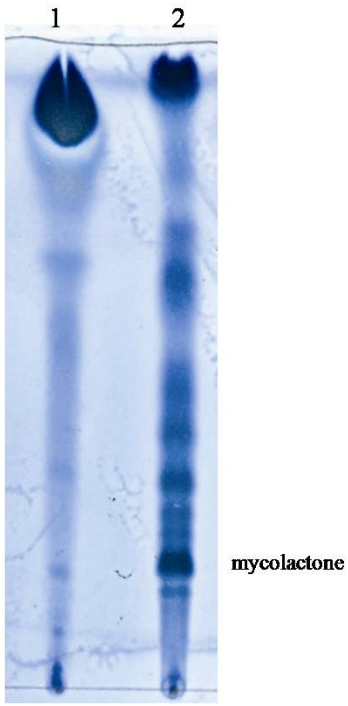


**Figure 20. TLC analysis of *M. fortuitum* electroductant 10.**
TLC analysis of acetone soluble lipids from *M. fortuitum* 10394.10 (pMYCO7017::TnKm) (lane 1), compared to *M. ulcerans* 1615 (lane 2).

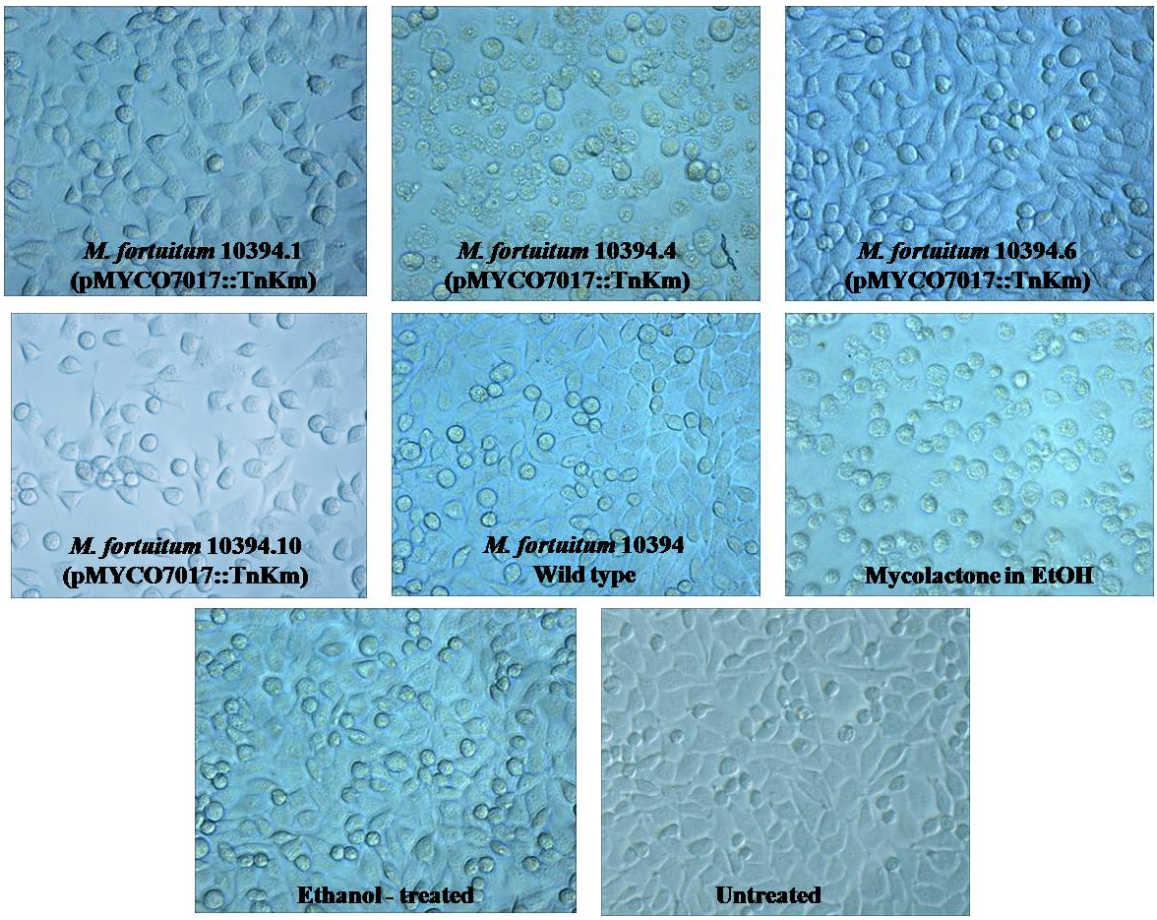


**Figure 21. Cytotoxicity assay of lipids extracted from *M. fortuitum* electroductants.** Representative pictures of cytotoxicity results after L929 murine fibroblasts were exposed to various compounds for 26 hours.

Lipid extracts from the four *M. fortuitum* 10394 (pMYCO7017::TnKm) electroductants were sent to Richard Lee and Engy Mahrous at the University of Tennessee Health Science Center in Memphis, TN for high performance liquid chromatography (HPLC), mass spectrometry (MS) analysis, and/or tandem MS/MS analysis. **Figure 22** shows the mass spectra from wild type *M. fortuitum* 10394, *M.fortuitum*10394.1 (pMYCO7017::TnKm), *M.fortuitum*10394.4 (pMYCO7017::TnKm), and *M. fortuitum* 10394.10 (pMYCO7017::TnKm). There were no molecules with appropriate mass or fragmentation patterns characteristic of mycolactone.

High performance liquid chromatography was performed on lipids extracted from the *M. fortuitum* 10394 electroductants and compared to the HPLC profile of lipids from *M. ulcerans* 1615. The HPLC analysis of lipids from *M. ulcerans* 1615, *M. fortuitum* 10394.6 (pMYCO7017::TnKm), and wild type *M. fortuitum* 10394 are illustrated in **Figure 23**. Panel A shows the typical HPLC results of lipids extracted from *M. ulcerans* 1615, where the asterix indicates the mycolactone toxin eluting off the column at 21.6 minutes.

There was a unique molecule contained within the lipids from *M. fortuitum* 10394.6 (pMYCO7017::TnKm) that also eluted off the column at 21.6 minutes that was not found in wild type *M. fortuitum* 10394. The lipid species eluted at 21.6 minutes was isolated for further analysis by ESI-mass spectrometry.

ESI-mass spectrometry analysis of the lipid species eluted off the HPLC column at 21.6 minutes detected a molecule with a *m/z* of 423.3, signifying the core of the mycolactone toxin (**Figure 24**). *M. fortuitum* 10394.6 (pMYCO7017::TnKm) possesses the mycolactone gene cluster and is able to successfully utilize the *M. ulcerans*-derived genes to heterologously produce the mycolactone core.

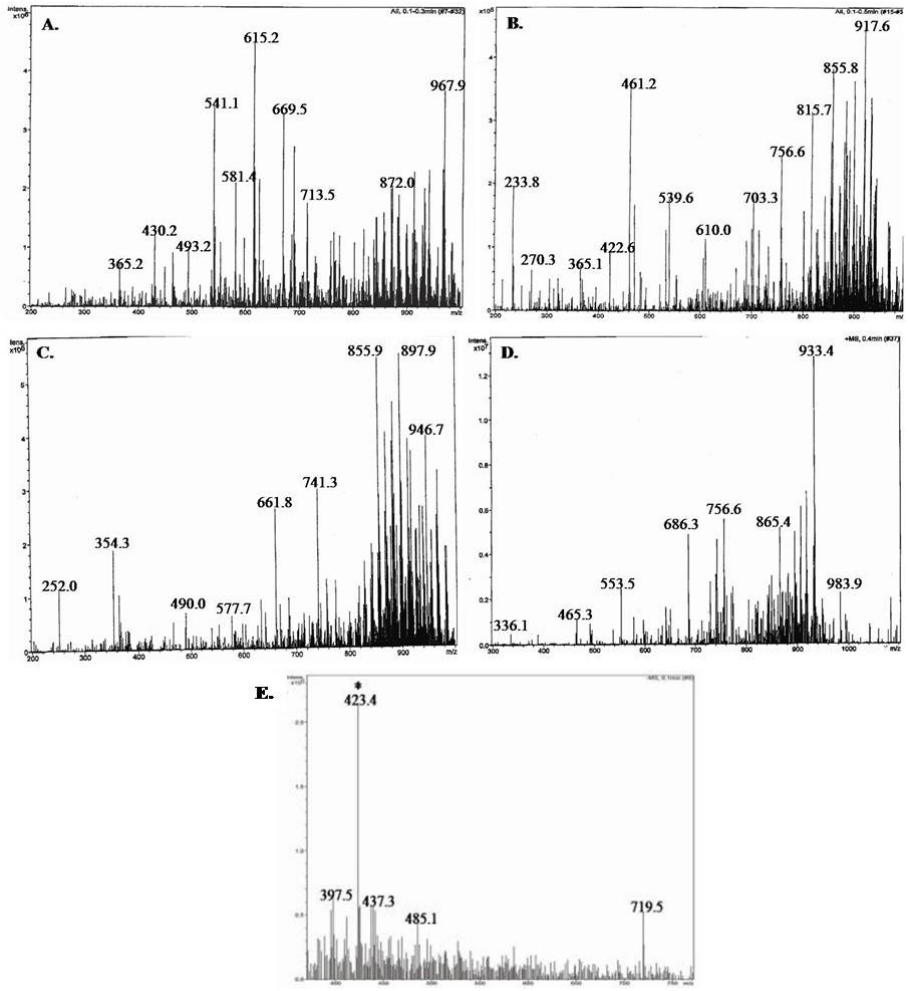


**Figure 22. Mass spectra of lipids extracted from *M. fortuitum* electroductants 1, 4, and 10 compared to wild type *M. fortuitum* 10394.**

Mass spectra of lipid extracts from (A) wild type *M. fortuitum* 10394, (B) *M. fortuitum*10394.1(pMYCO7017::TnKm), (C) *M.fortuitum*10394.4 (pMYCO7017::TnKm), (D) *M. fortuitum* 10394.10 (pMYCO7017::TnKm, and (E) and *M. ulcerans* 1615. The starred peak in panel E represents the mycolactone core.

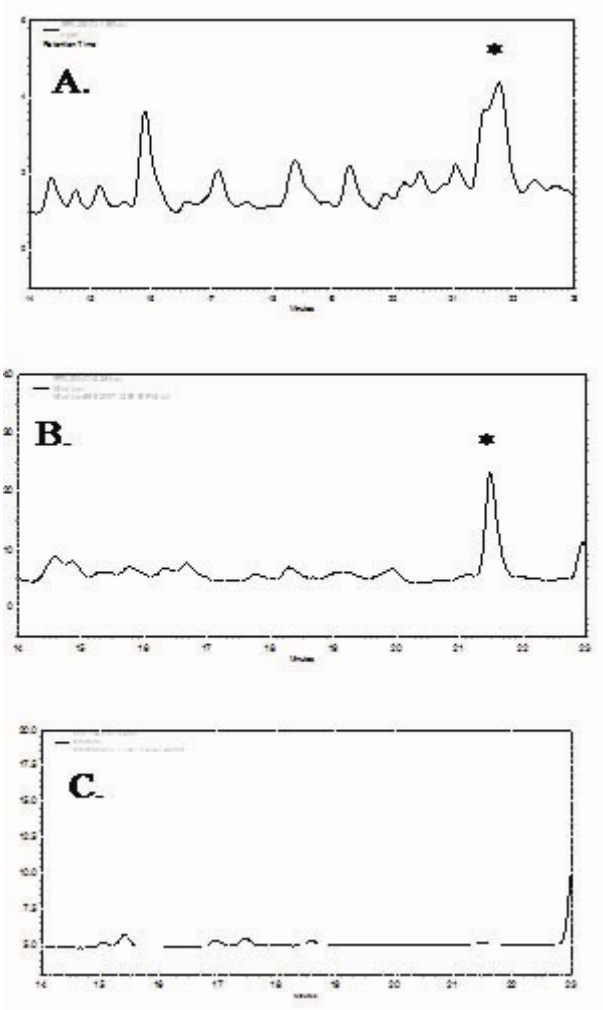


**Figure 23. High performance liquid chromatography of lipids extracted from *M. fortuitum* 10394.6 (pMYCO7017::TnKm) compared to controls.**

High performance liquid chromatography of lipids extracted from (A)*M. ulcerans* 1615, (B) *M. fortuitum* 10394.6 (pMYCO7017::TnKm), and (C) wild type *M. fortuitum* 10394. The asterisk in panel A points out the time, 21.6 minutes, when mycolactone elutes from the column. The asterisk in panel B indicates a unique molecule, not found in wild type *M. fortuitum* 103941034, which also eluted at 21.6 minutes. This molecule was isolated for further analysis by ESI-mass spectrometry.

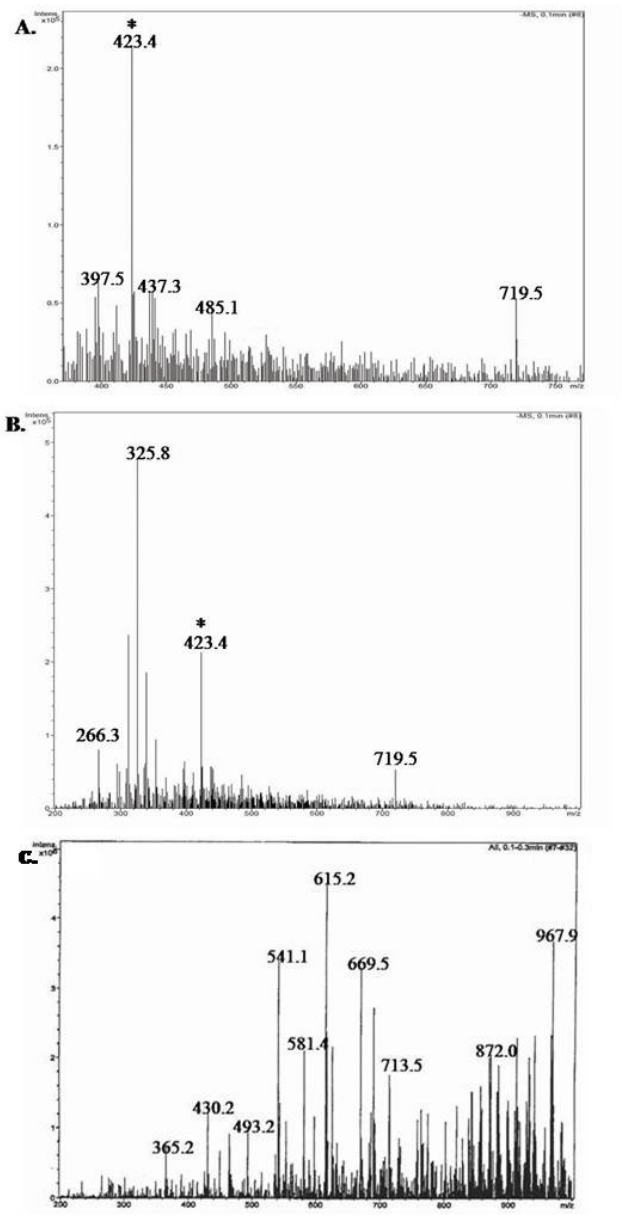


**Figure 24. ESI-MS analysis of the lipid species eluted at 21.6 minutes during HPLC of *M. fortuitum* 10394.6 (pMYCO7017::TnKm).**

ESI-MS analysis of the lipid species eluted at 21.6 minutes during HPLC for (A) *M. ulcerans* 1615, (B) *M. fortuitum* 10394.6 (pMYCO7017::TnKm), and (C) wild type *M. fortuitum* 10394. The asterisk in each panel indicates the core of the mycolactone toxin.

### 3.9 Conjugation using *M. ulcerans* 1615::Tn118 as a donor

Electroduction reactions attempting to introduce pMYCO7017::TnKm into the mycolactone negative mutant, *M. ulcerans* 1615A, were unsuccessful. A *M. ulcerans* 1615A (pMYCO7017::TnKm) electroductant would have been the ideal donor for conjugation experiments since pMYCO7017::TnKm contains the mycolactone gene cluster and it harbors a Kanamycin selectable marker in a non-coding region of the DNA. Instead, *M. ulcerans* 1615::Tn118 was used as the donor for the conjugation experiments. This strain was created previously by Armand Mve-Obiang in Dr. Pamela Small's lab at the University of Tennessee in Knoxville. *M. ulcerans* 1615::Tn118 is the result of random mutagenesis using a mariner transposon which interrupted the *FabH* gene of the mycolactone gene cluster. Thus transfer of this plasmid could be evaluated on the basis of growth temperature and kanamycin resistance although expression of mycolactone could not be evaluated.

After filter mating between *M. ulcerans* 1615::Tn118 and *M. fortuitum* 10394, reaction mixture dilutions were plated on selective media and incubated at 37°C. *M. fortuitum* 10394 can grow at this temperature, while *M. ulcerans* species cannot grow at 37°C. Four hundred and eighteen colonies grew on the Kanamycin selective media and 100 of those colonies were subcultured for further study. DNA was extracted by boil preparation as described and PCR was performed to probe for ER and the mariner Kanamycin transposon. None of the 100 colonies screened were PCR positive for ER or the mariner Kanamycin transposon.

# CHAPTER 4 : DISCUSSION

Buruli ulcer is a devastating disease that affects thousands of people every year and yet the mode of transmission remains elusive. Endemic foci are consistently found associated with slow moving or stagnant waters in both tropical and subtropical regions of the world. Buruli ulcer disease is caused by a cutaneous infection with *Mycobacterium ulcerans*, a slow-growing, acid fast positive bacterium. Attempts to culture the bacterium directly from the environment have been largely unsuccessful despite PCR detection of *M. ulcerans* DNA in various environmental samples. Consequently, most laboratory research has centered on *M. ulcerans* isolates obtained directly from Buruli ulcer patient lesions.

The polyketide toxin, mycolactone, is responsible for the extensive necrosis, immunosuppression, and painlessness that are characteristic of Buruli ulcers. *M. ulcerans* harbors a large, low copy number plasmid that contains the genes necessary for the production of mycolactone. Plasmids are common in mycobacteria, but this is the first example of a mycobacterial plasmid that is directly associated with virulence. The mycolactone plasmid was thought to be restricted to *M. ulcerans* until the recent discovery of several non-*ulcerans* mycolactone producing mycobacteria (MPM), which also possess versions of the mycolactone plasmid. These MPMs have been isolated from frogs and fish, both in the U.S. and abroad, illustrating that mycobacteria harboring the mycolactone plasmid are more widely distributed in the environment than previously thought.

The repertoire of tools available for the genetic manipulation of mycobacteria has been limited by the thick, waxy cell wall, slow growth rate, and the genome's high G + C content. Very little is known about the host range or biology of the *M. ulcerans* mycolactone plasmid for the reasons stated above, as well as its large size and low copy number. Previously, experiments were carried out by Stinear and colleagues to test the stability of *M. ulcerans*' plasmid-derived genes in the heterologous hosts *M. marinum* (M

strain), *M. smegmatis* ($mc^2155$), and *M. fortuitum* 10394. *M. marinum* was an appealing candidate for this experiment because of its extremely high level of genetic sequence similarity with *M. ulcerans*, including only one nucleotide difference within the 16S gene. Additionally, *M. ulcerans* is thought to have evolved from a *M. marinum* progenitor through acquisition of the mycolactone plasmid. *M. smegmatis* $mc^2155$ is a highly transformable, fast-growing mycobacterial strain and considered to be the "*E. coli*" of mycobacteria. Finally, *M. fortuitum* was a desirable candidate recipient for these studies because the *rep* region of the *M. ulcerans* plasmid has 68.3% amino acid identity to the *rep* of *M. fortuitum*'s plasmid, pJAZ38. The *M. fortuitum* strain 10394 was chosen specifically because it has been cured of its own native plasmids which would presumably limit molecular competition within this host.

Stinear et al cloned a 6 Kb fragment of the mycolactone plasmid into a vector, marked it with an apramycin resistance gene, and were able to successfully transform the construct into *M. marinum*. The goal was to test the heterologous stability of pMUM001-derived genes, specifically those genes predicted to be involved with replication and partitioning of the mycolactone plasmid. The *ori* site on the plasmid is where replication is initiated, specifically at the *repA* gene. *RepA* encodes a plasmid-specific, cis-acting initiation protein essential to plasmid replication (Masai, 1983). Partitioning loci, on the other hand, act independently of the plasmid replication machinery and are usually arranged as an operon that includes *parA*, *parB*, and *parS* (Stinear, 2005). *parB* encodes a DNA binding protein which specifically binds to *parS*, a cis-acting centromere-like sequence (Stinear, 2005), (Masai, 1983). The *parA* gene encodes a membrane associated ATPase that is crucial to the movement of the *parB* foci on the plasmid DNA (Masai, 1983).

Stinear et al tested the stability of the *M. ulcerans* genes in *M. marinum* by allowing late log phase cultures of the transformant to incubate for several days without antibiotic induced selective pressure. In the absence of antibiotic selection, *M. marinum* transformants subsequently lost the vector construct harboring the *M. ulcerans* plasmid genes *ori, parA,* and *repA*. In contrast, the current work did not test the stability of the 152 Kb mycolactone gene cluster in *M. marinum* transformant in the absence of antibiotic

selection. Instead, *M. marinum* 1218 (pMYCO7017::TnKm) was passaged in the presence of antibiotic selection and remained PCR positive for pMYCO7017::TnKm specific genes for several months. Both studies included back-transformation from *M. marinum* to *E. coli* as proof that the pMUM001-derived constructs were maintained as autonomous DNA elements in this heterologous host.

Stinear and colleagues offer two possible explanations as to why the *M. ulcerans* mycolactone plasmid-derived construct was not maintained in *M. marinum*. The first explanation was simply that *M. marinum* cannot stably maintain *M. ulcerans* pMUM001-derived genes and that the putative *par* locus from pMUM001 is not functional in this species (Stinear, 2005). A second possibility is that additional pMUM001 sequences may be required for plasmid maintenance that were not included in the 6 Kb pMUM001-derived DNA fragment used for their study. One pMUM001 sequence that was missing from their pMUM001-derived DNA fragment is a candidate *parS* site located 1.4 Kb upstream of *parA*. The lack of the *parS* site within the 6 Kb pMUM001 DNA fragment used for their experiment may have affected plasmid partitioning to daughter cells (Stinear, 2005).

The work described here had a similar goal, to investigate the stability of *M. ulcerans* mycolactone genes in a heterologous host. To compare and contrast the work described here with the previous work of Stinear et al, one major difference was the vectors used and the size of the pMUM001-derived DNA fragment cloned into each respective vector. To circumvent the difficulties of working with the mycolactone plasmid in its native form, we utilized a 152 Kb fragment of the 155 Kb mycolactone plasmid and cloned it into the pBeloBAC11 vector. The resulting bacterial artificial chromosome (BAC) could be carried and amplified by *E. coli* before transfer into other non-*ulcerans* mycobacteria. In Stinear's work, the small size of the pMUM001-derived construct enabled the researchers to isolate microgram amounts of the DNA and to utilize electroporation in order to transform mycobacterial recipients. In contrast, the large size of pMYCO7017::TnKm hindered the isolation of enough intact plasmid DNA to carryout electroporation experiments. Instead, electroduction was used to transfer the

mycolactone gene cluster, in the form of pMYCO7017::TnKm, directly from *E. coli* to *M. marinum*. In both Stinear's work and in the experiments reported here, attempts were made to introduce pMUM001-derived DNA into *M. marinum*, *M. smegmatis*, and *M. fortuitum*. In both cases, no *M. smegmatis* $mc^2155$ transformants were obtained. Stinear and colleagues were only successful in transforming the *M. marinum* M strain and achieved $1.3 \times 10^5 \pm 0.2$ transformants per microgram of plasmid DNA (Stinear, 2005). We were also able to successfully transfer pMUM001-derived DNA into *M. marinum* 1218, however, multiple attempts to introduce pMYCO7017::TnKm into *M. marinum* strain 1218 resulted in the creation of only one successful electroductant.

Stinear and colleagues were unable to introduce their construct into *M. fortuitum* 10394 and concluded that the pMUM001-derived *ori* did not support replication in this species (Stinear, 2005). In contrast, the present work describes the first examples of the successful manipulation of the entire mycolactone gene cluster, in the form of a BAC designated pMYCO7017::TnKm, and its introduction into faster growing mycobacterial species, *M. fortuitum* 10394. We have shown that the pMUM001-derived construct, pMYCO7017::TnKm, was stably maintained in *M. fortuitum* 10394 under the influence of antibiotic selection. Furthermore, the *M. ulcerans* mycolactone genes harbored by pMYCO7017::TnKm were at least partially expressed in *M. fortuitum* 10394 leading to the heterologous production of the mycolactone core. It would be advantageous to explore why *M. fortuitum* 10394.6 (pMYCO7017::TnKm) was only able to produce the core of the mycolactone toxin.

There may be basic difference in the biosynthetic requirements for synthesis of the mycolactone side chain versus the toxin core. Alternatively, there may be unique, trans-acting chromosomal factors present in the *M. ulcerans* genome required for mycolactone production that are absent or non-functional in *M. fortuitum* and *M. marinum*. Once available, the complete genome sequence of *M. fortuitum* will allow further investigation into the intricacies of mycolactone production in this heterologous host. However, the genome sequences of both *M. ulcerans* and *M. marinum* are available for comparison.

Dr. Armand Ove-Obiang, a post-doc in Pam Small's lab at the University of Tennessee, used random transposon mutagenesis to create a library of *M. ulcerans* mutants. Two of the *M. ulcerans* mutants were only able to produce the mycolactone core and had transposon insertions interrupting chromosomal genes; two were annotated as magnesium chelatase and one as a succinate dehydrogenase. A search of the *M. marinum* genome showed that these two genes are present in this close relative of *M. ulcerans.* The Burulist and Marinolist websites were used to obtain the protein sequence for both the magnesium chelatase and succinate dehydrogenase genes in *M. ulcerans* and *M. marinum* respectively. MultAlin (http://bioinfo.genotoul.fr/multalin/multalin.html) was used to compare the protein sequences and both conserved and non-conserved amino acid changes were observed. The ramifications of the amino acid changes are unclear because the exact role of magnesium chelatase and succinate dehydrogenase in mycolactone biosynthesis is not known. It will be interesting for future research to explore the potential role of trans-acting genes and their involvement in the production of mycolactone.

Those that continue work on this project many endeavor to more closely investigate the heterologous expression of *M. ulcerans* pMUM001-derived genes through reverse transcriptase – real time PCR (RT-RT PCR). With this type of PCR, mRNA is isolated from bacterial cells and subjected to a reverse transcriptase reaction to create a complimentary DNA (cDNA) library of total cell mRNA transcripts. Next, the cDNA is used a template for a real time – PCR reaction using fluorescently labeled probes specific for the genes of interest. This method would enable expression-level evaluation of pMUM001-derived genes in heterologous host species.

The ability to transfer the mycolactone gene cluster, in the form of pMYCO7017::TnKm, into various non-*ulcerans* mycobacterial species in the lab is certainly a noteworthy achievement and will prove useful in future studies. However, electroduction as a form of horizontal gene transfer does not generally occur in nature and therefore, does not offer a realistic explanation for how *M. ulcerans* evolved from an *M. marinum* progenitor through acquisition of the mycolactone plasmid. Horizontal gene transfer of plasmid

DNA is well documented in gram negative bacteria and gram negative mechanisms of conjugative transfer have been described in detail. This is not to say that horizontal gene transfer as a result of conjugation does not exist in gram positive or acid fast positive bacteria, but merely that these systems are not well understood.

Genetics studies of pathogenic mycobacteria, such as *M. tuberculosis* and *M. ulcerans*, have been specifically hindered by the genera's slow growth, thick cell wall, and G+C rich genomes. Moreover, the search for conjugative systems in mycobacteria has been based upon the discovery of homologous genes that have already been described in gram negative conjugative systems. The inherent problem with this strategy is that mycobacteria may possess unique conjugative systems that have not been previously described. Such is the case with the basis of mobility (*bom*) system described in *M. smegmatis*, the first mycobacterial conjugation system ever described (Parsons, 1998). *M. smegmatis* donor strains are able to participate in a conjugation-like event in which sections of the chromosome are mobilized by *bom* sequences and transferred to distinct recipient strains of *M. smegmatis*. Although this newly described system is similar to the phenomenon observed in Hfr strains of *E. coli*, this conjugative system is completely unique and unprecedented in *Mycobacteria.*

Much work remains in order to figure out the details of the exact mechanisms responsible for conjugative transfer of chromosomal DNA via the *bom* system. However, discovery of this novel system does illustrate the potential for the discovery of other conjugative systems in mycobacteria that have not yet been discovered through genomic analysis. Research has suggested that *M. ulcerans* acquired the mycolactone plasmid through horizontal gene transfer and plasmid DNA transfer through the process of conjugation cannot be ruled out as a possibility. Therefore, conjugation filter mating experiments were carried out between *M. ulcerans* 1615::Tn118 and *M. fortuitum* 10394 as a preliminary investigation of this possibility. *M. fortuitum* 10394 is a strain that has been cured of its own native plasmids and should experience less molecular competition upon introduction of a foreign plasmid. Additionally, the *M. ulcerans* mycolactone gene cluster (in the form of pMYCO7017::TnKm) was successfully introduced into and

replicated by *M. fortuitum* 10394. *M. fortuitum* 10394 (pMYCO::TnKm) electroductants were able to stably replicate this plasmid. One particular electroductant, *M. fortuitum* 10394.6 (pMYCO7017::TnKm) was subsequently shown to heterologously express the *M. ulcerans* mycolactone genes leading to the production of the mycolactone core.

The conjugation experiments between *M. ulcerans* 1615::Tn118 and *M. fortuitum* 10394 were carried out under the conditions described as optimal for conjugation between *M. smegmatis* donor and recipient strains (Parsons, 1998). Filter mating is ideal because it allows close contact of cells at high density and allows ease of recovery of potential transconjugants (Parsons, 1998). Conjugation experiments performed on solid media have been shown to be drastically more efficient that conjugation attempts in liquid media. Despite all attempts to maximize mycobacterial conjugation conditions, no transconjugants were obtained after filter mating experiments. This is not discouraging, as these are only preliminary experiments. Additionally, mycobacterial conjugative systems have only recently been discovered and most of the details remain unknown. Certainly, future investigations into mycobacterial conjugation may shed light on the potential for *M. ulcerans* to participate in this type of horizontal gene transfer.

This work describes the first successful transfer of the mycolactone gene cluster and subsequent expression of *M. ulcerans* mycolactone genes in a heterologous host. The ability of *M. fortuitum* 10394 to replicate pMYCO7017::TnKm and express the mycolactone polyketide synthase genes has important implications. Importantly, the BAC construct pMYCO7017::TnKm may be used in future research to learn more about the mycolactone gene cluster and the biology of the mycolactone plasmid.

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## VITA

Samantha Elizabeth Wirth was born on October 25, 1980 in Marlton, NJ. She spent most of her life in New Jersey and graduated with a B.S. in Animal Science from Rutgers University. Life then led her to Knoxville, Tennessee where she worked for two years before applying to graduate school at the University of Tennessee in 2005. She joined the laboratory of Dr. Pamela Small where she focused her graduate education on the pathogenesis of *Mycobacterium ulcerans*. In August of 2008, she was awarded an M.S. in Microbiology. Currently, Samantha is a fellow in Emerging Infectious Disease Laboratory Training Program co-sponsored by the Centers for Disease Control and the Association of Public Health Laboratories. After completing the one year fellowship, she plans to begin a career in infectious disease research.