8-2004

Studies on *Mycobacterium ulcerans*

Terri Alford
University of Tennessee, Knoxville

To the Graduate Council:

I am submitting herewith a thesis written by Terri Alford entitled "Studies on *Mycobacterium ulcerans*." 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 L. C. Small, Major Professor

We have read this thesis and recommend its acceptance:

Steven W. Wilhelm, Robert N. Moore

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

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

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Studies on *Mycobacterium ulcerans*

A Thesis
Presented for the
Master of Science Degree
The University of Tennessee, Knoxville

Terri Alford
August 2004

Acknowledgments

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Abstract

Efforts to create a mycolactone-negative mutant of *Mycobacterium ulcerans* via transposon mutagenesis were undertaken to elucidate the genes involved in the production of this toxin. Though only five PCR-confirmed insertion mutants were produced, one of these, 1615:tnp6w, was confirmed to be deficient in mycolactone production via thin-layer chromatography (TLC) of acetone soluble lipids (ASLs), cytopathicity assay of culture filtrate on L929 fibroblasts, and mass spectroscopy of ASLs. Using nested-PCR, it was determined that the transposon insertion of 1615:tnp6w had occurred in a region of DNA containing a gene 97% identical to the *M. marinum* ferric uptake regulator homolog (FurA) gene and 89% identical to the *fur*A gene of *M. tuberculosis*. The region of *M. ulcerans* homologous to the already characterized *M. marinum fur*A gene was cloned; and, the construct was used in complementation studies in an attempt to confirm the role of FurA in mycolactone production. Transformation of the 1615:tnp6w mutant with the construct containing *fur*A failed to restore mycolactone production. The failure of the complementation of the *fur*A gene to restore mycolactone production was explained via PCR studies which revealed a spontaneous deletion in the thioesterase II gene recently identified to be required for mycolactone production. Secondly, experiments aimed at developing an efficient transformation protocol for *M. ulcerans* were undertaken. In an effort to determine the effect of temperature on the transformation efficiency of *M. ulcerans* via electroporation, electroporation experiments were performed on *M. ulcerans* 1615 grown in Middlebrook 7H9 with 10% oleic acid-albumin-dextrose-complex supplement (M7H9 + OADC). Log phase cultures were

harvested, washed with 10% glycerol, and apportioned into 60 μL aliquots. Aliquots were briefly held at room temperature, 37℃, 40℃, or 45℃ before electroporation in the presence of 1 μg of pPR27H1, a hygromycin B resistance conveying plasmid. After 8 weeks, colonies were counted to calculate transformation efficiencies. To determine if the presence of mycolactone affects electroporation efficiency, this experiment was repeated with *M. ulcerans* grown in Modified Reid's synthetic medium and *M. ulcerans* grown in Sauton's synthetic medium, both media which are thought to promote a decreased production of mycolactone; *M. ulcerans* grown in Middlebrook 7H9 with 20% oleic acid-albumin-dextrose-complex supplement (M7H9 + 2[OADC]), which is believed to foster an increased production of mycolactone; and, a polyketide synthase (PKS) knock-out mutant of *M. ulcerans* (mutant 115) which does not produce mycolactone grown in M7H9 + OADC. Though electroporation of *M. ulcerans* grown in M7H9 + OADC produced transformants reliably (regardless of the temperature of the cell at the time of electroporation); the transformation efficiencies were not high and, there was no significant difference in the transformation efficiencies obtained by cells exposed to different temperatures before electroporation. However, an additional experiment, demonstrated that these cells exposed to 55℃ for the same amount of time prior to electroporation did have a modest but significant increase in transformation efficiency. A similar increase in transformation efficiency was obtained when *M. ulcerans* grown in M7H9 + 2[OADC] was electroporated after incubation at the lower temperature of 45℃. In an additional experiment, the fast growing *M. fortuitum* grown in M7H9 + OADC had the highest transformation efficiency when electroporated after incubation at 45℃. Finally, though the natural reservoir of *M. ulcerans* is unknown, Buruli ulcer disease

seems to be associated with water. For this reason, experiments aimed at investigating aquatic snails, also associated with water in Buruli ulcer endemic regions, as a component of the ecological niche of *M. ulcerans* were undertaken. Snails of the species *Biomphalaria glabrata* are intermediate hosts of the schistosome species *Schistosoma mansoni*; and, it is feasible that *M. ulcerans* could associate with cercariae, the schistosomal life stage infectious to humans, infecting humans through the wound caused by the cercaria entering its human host. Through various experiments, both healthy *Biomphalaria glabrata* and *Schistosoma mansoni*-infected *Biomphalaria glabrata* were exposed to *M. ulcerans* and examined for *M. ulcerans* infection via PCR and plating of ground tissue. While PCR analysis demonstrated a possible association between *M. ulcerans* and healthy snails; *M. ulcerans* was not recovered by plating of the tissue of snails exposed to the bacteria during any of three infection experiments. Hystological sectioning of snail tissue yielded no reliable data.

Table of Contents

List of Tables

Table **Page**

List of Figures

Figure **Page**

List of Abbreviations

ASLs—acetone soluble lipids

ATCC—American Type Culture Collection

BLAST—Basic Local Alignment Search Tool

BSA—bovine serum albumin

bp—basepair

DNA—deoxyribonucleic acid

g—gram

× g—× gravitational force

HIV—human immunodeficiency virus

IS2404—insertion element 2404

IS2606—insertion element 2606

kV—kilovolt

μg—microgram

μL—microliter

mg—milligram

mL—milliliter

mm—millimeter

mM—millimolar

M—molar

ng—nanogram

Ω—ohm, unit of resistance

μF—microfarad, unit of capacitance

m/z—mass/charge

M7H9—Middlebrook 7H9 broth

M7H10—Middlebrook 7H10 agar

NMR—nuclear magnetic resonance

OADC—oleid acid-albumin-dextrose-complex

PCR—polymerase chain reaction

PKS—polyketide synthase

pmol—picomol

R_f—ratio to front

rpm—rounds per minute

SDS—sodium dodecyl sulfate

TLC—thin-layer chromatography

TMC—Trudeau Mycobacterial Culture Collection

U—unit

WHO—World Health Organization

I. Literature Review

Clinical Presentation of Buruli Ulcer Disease

Mycobacterium ulcerans is the causative agent of the necrotizing skin disease Buruli ulcer, the third most common mycobacterial disease of non-immunocompromised people (81). For reasons not fully understood, Buruli ulcer can present in ways unique to different endemic regions. In Africa, the disease usually begins as a small subcutaneous mobile nodule measuring 1-2 cm in diameter (19, 31). In Australia, the disease most commonly begins as an intradermal papule but may also be first noticed as an edematous limb, a characteristic of necrotizing panniculitis (31, 35). Other preulcerative lesions that may present include more localized edema and plaques—irregularly shaped indurated patches of skin (81). These nonulcerative stages of the disease are generally painless; however, though not common, they may itch (19). Subcutaneous calcification may also occur (12, 31, 76). The first (nonulcerative) stage of Buruli ulcer may persist for 1 or 2 months, after which the ulcer characteristic of this disease begins to form, extending 15 cm or more (19) (Figure 1).

After the second (ulcerative) stage of the disease ensues, necrosis of the subcutaneous fat along with vascular damage causes the skin overlying the ulcerative area to die and slough off, producing secondary ulceration of the skin with a distinct undermined edge (81) (Figure 2). If untreated, the ulceration may progress to involve an entire limb or up to 15% of the body surface (23). Though rare, extensive ulceration can result in osteomyelitis and even autoamputation. Ulcers that begin to heal contain granulomatous tissue at their edges and may be said to have progressed to stage 3 of the

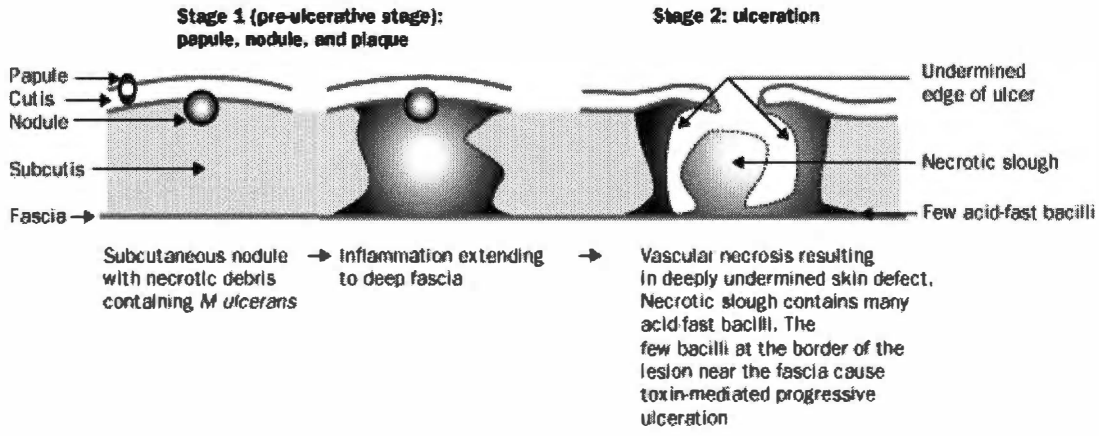


Figure 1. The progression of the Buruli ulcer lesion to the ulcerative stage. (Adapted from van der Werff, et al., 1999.)

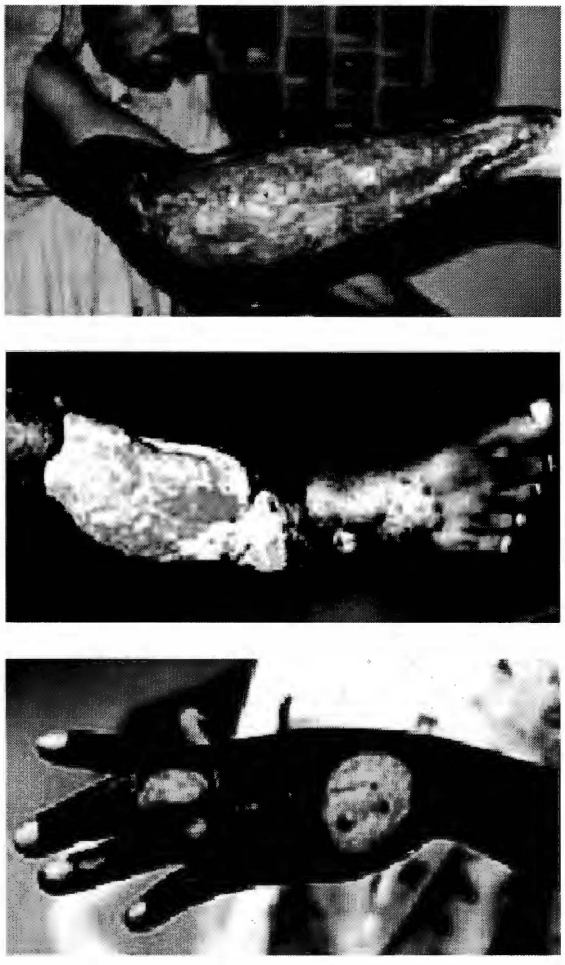


Figure 2. Ulcerative lesion of Buruli ulcer (stage 2) on an arm, leg, and hand. (Adapted from *Buruli ulcer: Diagnosis of Mycobacterium ulcerans Disease*, WHO 2001.)

disease; while, those who have been debilitated by the disease even after the disease has healed may be said to be in stage 4 of the disease (70). Though it is thought that massive ulcers may cause systemic effects, ordinarily there are no effects beyond the region of the ulcer (19). It is rare that Buruli ulcer causes pain or occurs in more than one site on the body. Some ulcers may heal spontaneously, doing so slowly over months or years. There is anecdotal evidence that patients with such spontaneously healing ulcers develop a positive tuberculin skin test; while, those with ulcers which do not spontaneously heal remain negative with regard to the tuberculin skin test (81). However, upon histopathological examination, there appears to be a lack of a host inflammatory response or granuloma formation at the site of the ulcer (12, 33, 76). Unlike other mycobacteria, *M. ulcerans* is rarely found inside macrophages. *M. ulcerans* can be found in the ulcer only in extracellular foci from which the necrosis extends well into areas from which bacteria can not be isolated.

One study has shown that of the Buruli ulcer patients treated at hospitals, up to 10% require amputation of a limb (6). Common sequelae include ankylosis, contracture, and lymphadema, all the result of massive scaring during the healing process (Figure 3). The loss of important structures on the face (e.g., the eye), genitalia, and breasts may also occur as a result of the disease or may be required to treat it successfully. People who recover from Buruli ulcer are often left with permanent disabilities and unable to care for themselves. This is particularly devastating for people who live in regions of the world where life is difficult for even healthy individuals.

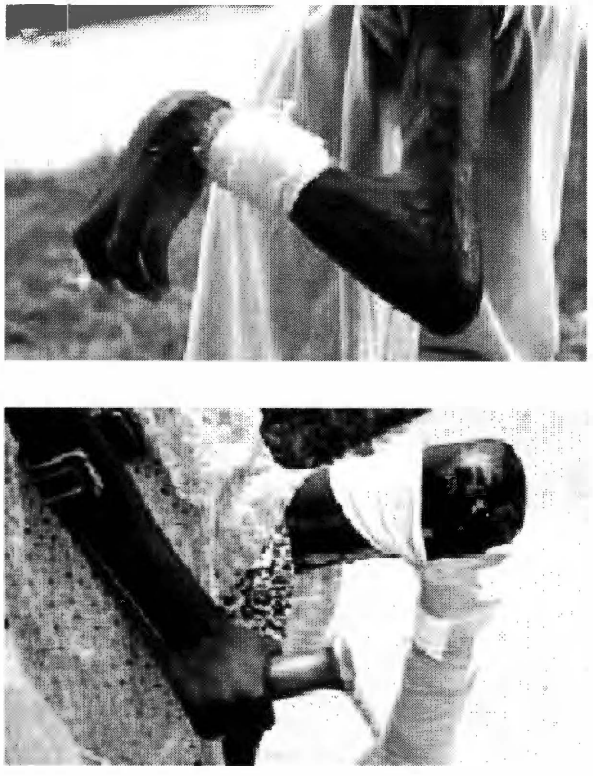


Figure 3. Contracture in an arm and leg after healing of Buruli ulcer.
(Adapted from *Buruli ulcer: Diagnosis of Mycobacterium ulcerans Disease*, WHO 2001.)

Treatment

The most effective treatment for Buruli ulcer is surgical excision. Excision of the early, non-ulcerative stages of the disease is usually curative (76). Because such medical care is not readily available for many victims of Buruli ulcer, many do not seek medical care until after the disease has progressed to a large, disfiguring ulcer. For the ulcerative forms of the disease, surgical excision of the ulcer and a margin of healthy tissue followed by skin grafting is relatively effective; though, some reports indicate that the disease recurs, requiring further surgery, in more than 15% of cases (76).

Although many drugs such as dapsone, streptomycin, clarithromycin, clofazimine, and rifampicin are effective against *M. ulcerans* in vitro, none have been found to be effective in vivo on patients with Buruli ulcer disease (76). Studies which have shown drugs, such as clofazimine, which are greatly effective in vitro to have no clinical value for the treatment of Buruli ulcer, suggest that drugs taken systemically may be of no benefit due to the nature of the lesion, particularly the poor perfusion to it (67, 76). The anti-convulsant phenytoin, applied topically, has been reported to promote healing of the ulcerative form of the disease with a reduction in scarring and contractures, but results have not been substantiated (3).

Several reports have shown treating Buruli ulcer with heat to be a promising treatment (25, 52). This takes advantage of the fact that *M. ulcerans* is heat sensitive. However, this method of treatment is not widely utilized and has not been extensively evaluated. To be effective, the heat must be applied constantly and requires equipment, such as an electric heating pad or other source with thermostatic control, which is not readily available in many regions in which Buruli ulcer is endemic. This type of

treatment confines patients who are otherwise healthy for long periods of time and is most likely not suitable for lesions on the face and other body parts.

Geographical Distribution and Epidemiology of Buruli Ulcer

It is believed by many that Buruli ulcer disease was described as early as 1897 by Sir Albert Cook in his Mengo Hospital Notes which describe the characteristic ulcers among his patients in Kampala, Uganda (1, 13, 76). Aslop, an Australian surgeon, also described treating patients with similar ulcers as early as 1937 (5). However, it was not until 1948 that McCallum and colleagues described the ulcers in correlation with their causative agent, which they tentatively named the Bairnsdale bacillus after the Bairnsdale region of Australia in which most of their patients with this disease lived (48). This bacterium was later named *Mycobacterium ulcerans*; however, the disease is still known as Bairnsdale ulcer in Australia (82). Though it bares various monikers in different regions of the world—for example, Kumusi ulcer in Papua New Guinea, Kasongo ulcer in Zaire, and Bairnsdale ulcer in Australia, the necrotic lesions caused by *M. ulcerans* are most widely known as Buruli ulcer after the Buruli region of Uganda (2, 11, 38). This region was the site of several large outbreaks of Buruli ulcer in the 1960's and 1970's which provided the arena for a more thorough investigation and publication of the disease (11, 16).

In addition to Uganda and Australia, Buruli ulcer has been found endemic to the Democratic Republic of Congo, Nigeria, Cameroon, Ghana, Papua New Guinea, Malaysia, French Guiana, Mexico, Bolivia, Sri Lanka, Sumatra, and others totaling over 30 countries (4, 8, 29, 32, 34, 41, 51, 63, 85) (Figure 4). It is interesting to note that

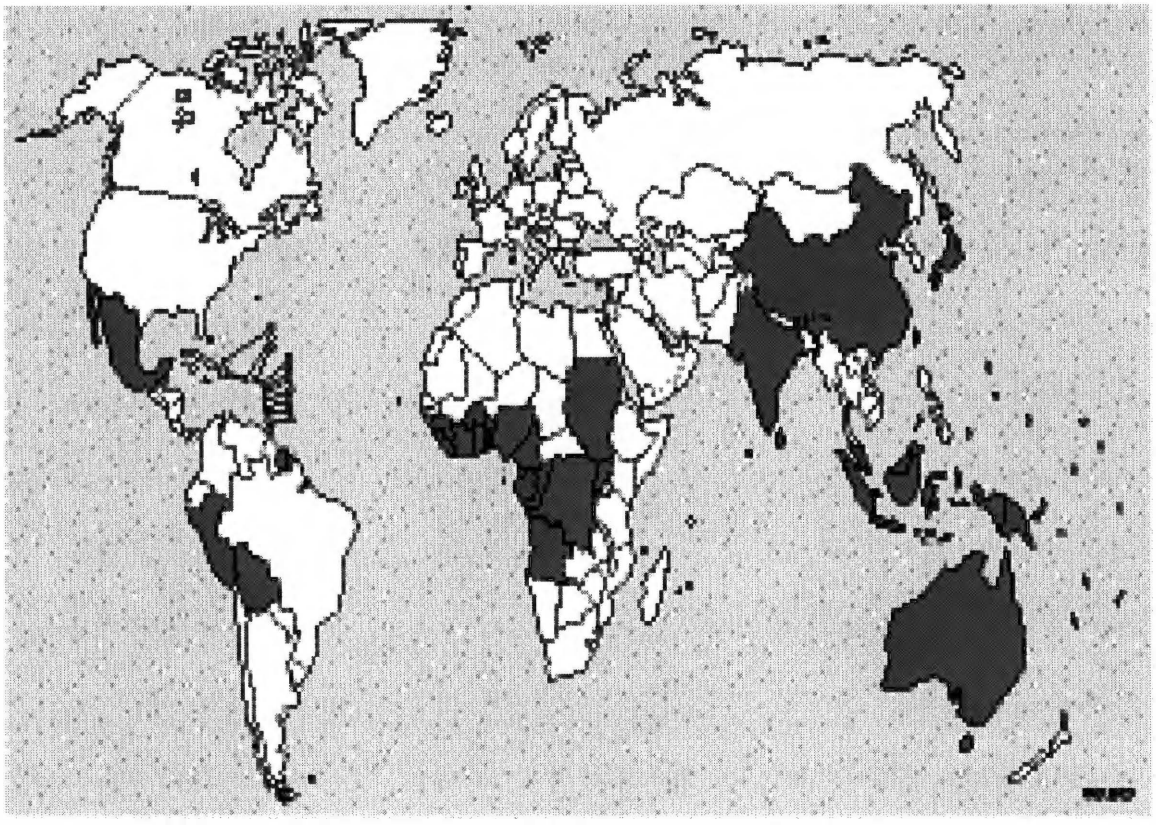


Figure 4. Countries in which Buruli ulcer disease has been reported.
Countries shown in black are regions of endemicity. (Adapted from WHO Buruli ulcer website: http://www.who.int/gtb- buruli/global_situation/index.html.)

based on the broad distribution of the endemic areas of Buruli ulcer, it has been suggested that *M. ulcerans* evolved before or during the Jurassic period—before the breakup of the continents (32). Though Buruli ulcer disease has been found in non-tropical regions such as southern Australia, Japan, and the People's Republic of China, it is most prevalent in tropical regions (20, 33, 79, 80). While the disease has probably always been most prevalent in the rural communities of equatorial Africa, there seems to have been a great increase in the occurrence of Buruli ulcer in East Africa in the early 1970's and late 1960's (2, 41). At present, in West Africa a similar epidemic is transpiring, contributing to the momentously increasing overall incidence of Buruli ulcer disease (41, 43, 50, 54). Buruli ulcer was recognized as an emerging infectious disease in 1998 with the establishment of the Global Buruli Ulcer Initiative by the World Health Organization (WHO) (85).

Within the many regions and countries of endemicity, Buruli ulcer disease occurs in well defined areas, often called foci, which can be as small as 2 square kilometers in size (41, 42, 82). These foci are typically in close proximity to standing or slow moving water (e.g., a lake, stream, or swampy area) (41, 64). For this reason, it is believed that the organism is in some way associated with the water or colonizes some organism associated with the water. While many different organisms have been experimentally infected with *M. ulcerans*, until recently, the only organisms found to acquire natural *M. ulcerans* infections were koalas, ringtail possums, and an alpaca (19). In 1999, Portaels, et al., reported the finding of five water bugs from the families Naucoridae and Belostomatidae collected in Buruli ulcer-endemic regions of Benin and Ghana to be PCR-positive for *M. ulcerans* (65). Recently, the aquatic, carnivorous insects of the

family Naucoridae were found able to be experimentally infected with *M. ulcerans* as a carrier and transmit this infection to mice upon biting them (49). The group conducting these studies was then able to isolate *M. ulcerans* from species of this family of insects collected from the wild (49). Based on this information, it is likely that insects in the family Naucoridae do play a role in the transmission of *M. ulcerans*. It is not clear how important this role is and what other organisms and environmental factors may be involved in the natural reservoir of *M. ulcerans*.

There have been very few reports of people acquiring Buruli ulcer from those already infected. The two most common incidences were infection after a human bite and the infection of a plastic surgeon who had excised a Buruli ulcer lesion (15, 18). These are considered isolated and possibly unsubstantiated incidences. Buruli ulcer is not passed from person to person, and infects people of all ages—both male and female. However, it does occur more often in children (17). There appears to be no link between the human immunodeficiency virus (HIV) epidemic in Africa and the incidence of Buruli ulcer disease; and, co-infection with HIV does not appear to alter the course of an *M. ulcerans* infection (17).

Diagnosis of Buruli Ulcer and Identification of *Mycobacterium ulcerans*

In regions in which Buruli ulcer is endemic, it is readily diagnosed in the ulcerative form. A painless ulcer with undermined edges in the absence of the lymphadenitis, fever, or other inflammation present in infections caused by other bacteria is unique to the disease caused by *M. ulcerans*. Because the lesions are usually found on the limbs or trunk, the ones which appear on the face may be mistaken for cancrum oris,

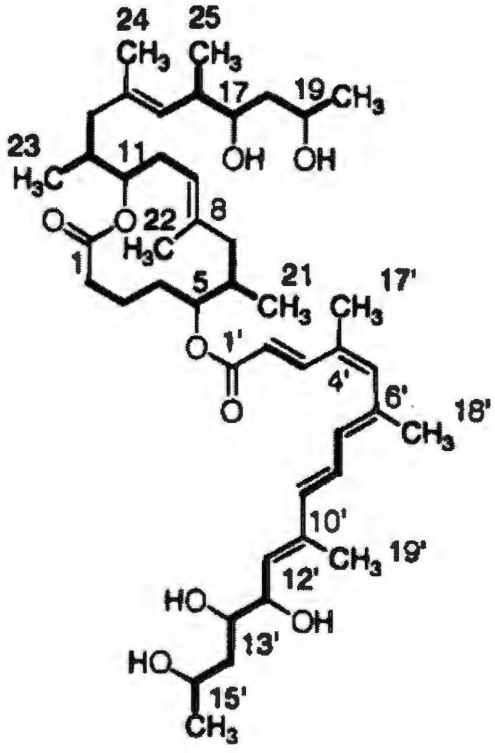
or noma, a disease characterized by necrotic facial lesions which unlike *M. ulcerans* exhibit signs of inflammation (81). Commonly, acid-fast staining is performed on suspected Buruli ulcers; however, neither the presence nor absence of acid-fast bacilli in the ulcer can definitively confirm or refute a preliminary diagnosis of Buruli ulcer. In most cases the organism can be cultured from the lesion, but culturing requires that the appropriate media and incubation apparatus be available and may take three or more months (48).

Though generally limited to research and clinical use in industrialized countries, PCR can be used to definitively identify *M. ulcerans*. The *M. ulcerans* genome contains two insertion sequences, IS2404 and IS2606, which have not been found together in any other organism. IS2606 has been found to be present in the genome in up to 40 copies; while, IS2404 has been found to be present within the genome in over 50 copies (72). IS2606 has been detected in only one other species—*M. lentiflavum*; while, IS2404 to date has been detected only in *M. ulcerans* (72). Concurrent detection of both IS2606 and IS2404 in a single isolate is indicative of *M. ulcerans*.

Mycolactone

Due to the presence of only small foci of *M. ulcerans* in even large ulcers it was suggested as early as 1965 that Buruli ulcer disease was mediated by a diffusible toxin (12). Several studies have demonstrated that the sterile filtrates of *M. ulcerans* have a cytopathic effect on L929 murine fibroblasts, causing rounding and detaching from the monolayer (22, 36, 46). In 1998, George, et al. was able to partially purify the toxin causing this effect and identify the mechanism by which it functions (22). They

demonstrated with flow cytometric analysis that the toxin causes cell cycle arrest in the G_0/G_1 phase of the growth cycle accompanied by cytoskeletal rearrangement (22). One year later, the toxin was completely purified, and its structure was determined to be that of a 12-membered lactone ring to which two different polyketides were attached (23). This molecule, designated mycolactone, as a polyketide-derived macrolide is a member of a class of molecules which are often biologically active. This is the first such molecule identified in mycobacteria and any human pathogen. When the biologically active molecule, designated mycolactone, was subjected to ^{1}H and ^{13}C NMR, it was revealed that the compound actually exists as a mixture of two *cis/trans* isomers in equilibrium at approximately a 3:2 ratio (21). The predominant molecule has been designated mycolactone A (Figure 5). Mycolactone A/B is represented by a single peak at *m/z* 765.5 upon mass spectroscopic analysis. It has been found that *M. ulcerans* produces not just two isomers of mycolactone, but multiple heterogeneous congeners which primarily differ in their number of double bonds and hydroxyl groups (9). In 2003, Mve-Obiang, et al. reported that while mycolactone A/B was originally isolated from *M. ulcerans* 1615, a Malaysian isolate; it has been found in all cytotoxic strains of *M. ulcerans* tested except two isolates from Mexico (56). They further reported that a mycolactone C, represented by a peak at *m/z* 726.3 upon mass spectroscopic analysis, was the predominant mycolactone in eight Australian strains analyzed; while, a mycolactone D (*m/z* 781.2) was present in two Asian strains of *M. ulcerans* (56). Though the congeners have the same biological activity, they differ in potency (56). The fact that strains of *M. ulcerans* of the same geographic region appear to produce the same repertoire of mycolactones may explain the variation in presentation and severity of



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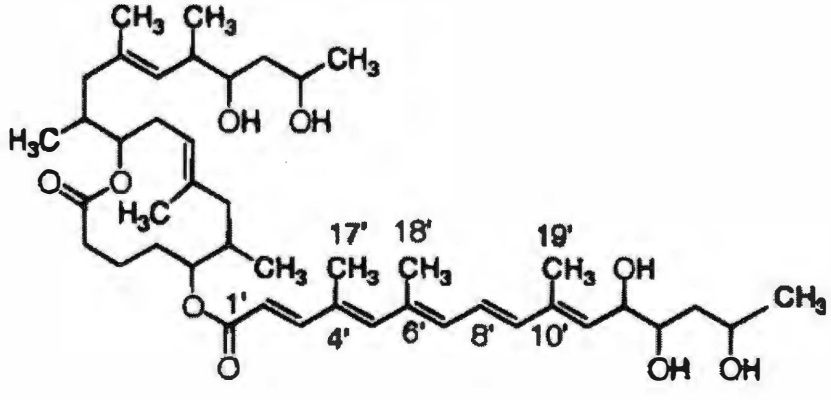

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Figure 5. Structure of 1) mycolactone A and 2) mycolactone B.
(Adapted from Gunawardana, et al., 1999.)

Buruli ulcer among these different regions.

II. Materials and Methods

Bacterial Culture

Mycobacterium ulcerans 1615, originally isolated from a human patient in Malaysia, is a part of the Trudeau Mycobacterial Culture Collection (TMC) and was obtained from the American Type Culture Collection (ATCC 35840) (Manassas, Virginia). Mycolactone-negative mutants of *M. ulcerans* 1615 not constructed as a part of this work, mutants 97 and 115, were obtained from Dr. Armand Mve-Obiang and were constructed by him via transposon mutagenesis via a phage delivery system while working at the University of Tennessee in Knoxville. All *M. ulcerans* cultures were grown in stir flasks at 32℃ in Middlebrook 7H9 broth (M7H9) (Difco, Detroit, Michigan) with 10% oleic acid-albumin-dextrose-complex (OADC) unless otherwise stated. OADC was prepared with bovine serum albumin (BSA) from Sigma (Saint Louis, Missouri) and all other reagents from Fisher Scientific (Pittsburgh, Pennsylvania). Both Reid's synthetic medium and Sauton's synthetic medium were prepared as described in The Mycobacteria: A Sourcebook Part A using reagents purchased from Fisher Scientific (84). The *M. fortuitum* strain used, *M. fortuitum fortuitum* da Costa Cruz, is a Spanish isolate and was obtained from ATCC (ATCC 6841) and is a part of the TMC. It was grown in M7H9 with 10% OADC (M7H9 + OADC) while shaking at 37℃.

Transposon Mutagenesis

Transposon mutagenesis was performed using the commercially available EZ::TN™ <KAN-2>Tnp Transposome™ Kit from Epicentre (Madison, Wisconsin). The

transposome is constructed by mixing the transposon, a segment of linear DNA containing the kanamycin resistance gene Tn903 flanked by 19 bp transposase recognition sequences, and the transposase in the absence of magnesium (Figure 6). This transposome construct was introduced into *M. ulcerans* through electroporation as described below. Upon entry into the cell, the transposome complex is activated by Mg^{2+} present in the cell, and the transposon randomly inserts into the genomic DNA of the host. Insertion mutants were selected on Middlebrook 7H10 agar (Difco) supplemented with 10% OADC (M7H10 + OADC) with kanamycin at a concentration of 30 μg/mL, because the transposon includes the Tn903 selectable marker, conveying kanamycin resistance (Figure 7).

Electroporation of *M. ulcerans*

Electrocompetent cells were prepared in the following manner. All steps were carried out in and all reagents were used at room temperature unless otherwise stated. Log-phase cultures were harvested by centrifugation at 5,509 × g for 10 min without brake. The supernatant was carefully discarded, and the pellet was gradually resuspended in a volume of 10% glycerol (Fischer Scientific) equal to one half the volume of the original culture. The cells were centrifuged as above, and the pellet was gradually resuspended in a volume of 10% glycerol equal to one fifth the volume of the original culture. The cells were again centrifuged as above, and the supernatant was carefully discarded. The cells were finally resuspended in a volume of 10% glycerol equal to one one thousandth the volume of the original culture. The cells were then aliquoted in 60 μL volumes into sterile 1.5 mL microcentrifuge tubes. Because mycobacteria have

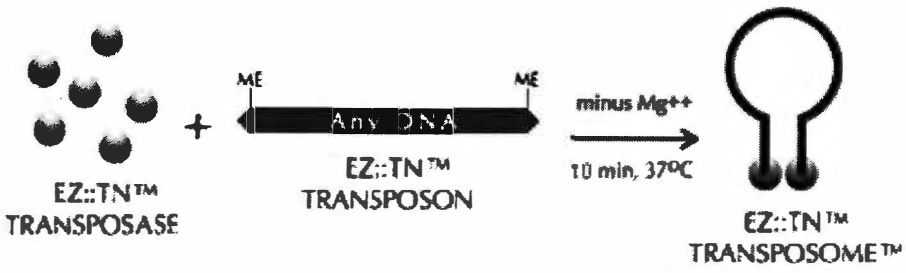

Figure 6. Formation of the EZ::TN™ Transposome™.
The EZ::TN™ Transposome™ is formed by incubating the EZ::TN™ Transposon™ with EZ::TN™ Transposase™ in the absence of Mg^{2+}. (Adapted from 2003 Epicentre catalogue.)

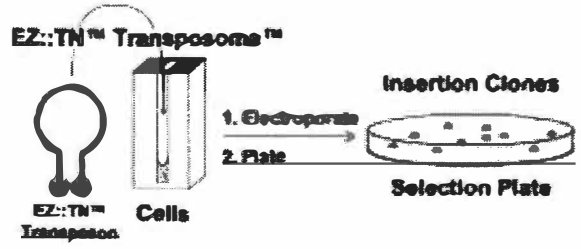

Figure 7. Activation of the EZ::TN™ Transposome™.
Upon electroporation into electrocompetent cells, the transposome is activated by cellular Mg^{2+}; and, the transposon is randomly inserted into the host genomic DNA. Cells in which insertion has occurred are selected for on selective media. (Adapted from 2003 Epicentre catalogue.)

extremely low transformation efficiencies when compared to other bacteria and transformation efficiencies of competent cells decrease after storage, electrocompetent cells were used immediately.

Immediately before electroporation, the 60 µL volume of electrocompetent cells was loaded into a BTX® 2 mm gap cuvette (Fisher Scientific) along with 1 µL of DNA (at a concentration of 1 µg/µL unless otherwise stated). The cuvette was placed in the electroporation chamber and pulsed with a Bio-Rad Gene Pulser® II with the following parameters: 2.5 kV voltage, 25 µF capacitance, and 800 Ω resistance. Immediately after electroporation, the content of the cuvette was transferred to 2 mL of warm M7H9 + OADC, unless otherwise stated, and placed at 32℃ in a rollerdrum for 72 hours. The cells were then plated on M7H10 + OADC plates, unless otherwise stated, with the appropriate antibiotic added. The plates were double wrapped in Parafilm M® (Pechiney Plastic Packaging, Chicago, Illinois) to prevent dehydration and incubated at 32℃. Plates were checked weekly.

Electroporation of *M. fortuitum*

Electrocompetent cells were harvested, washed, aliquoted, and electroporated as described for *M. ulcerans*. Immediately after electroporation, cells were transferred to 2 mL M7H9 + OADC and shaken at 37℃ for 5 hours before being plated on M7H10 + OADC with 30 µg/mL aparamycin. Plates were incubated at 37℃ and checked daily.

PCR

To confirm the presence of a transposon insertion in kanamycin resistant colonies after transposon mutagenesis, a 1,108 bp fragment of the transposon was amplified with primers Tnp1 (5'-CTC AAA ATC TCT GAT GTT ACA TTG C-3') and Tnp2 (5'-GGT TGA TGA GAG CTT TGT TGT AGG T-3'). To confirm the presence of *M. ulcerans*, a 492 bp fragment of IS2404, an insertion element unique to *M. ulcerans*, was amplified as described by T. Stinear, et al. with primers MU5 (5'-AGC GAC CCC AGT GGA TTG GT-3') and MU6 (5'-CGG TGA TCA AGC GTT CAC GA-3') (72). A small portion of each colony to be tested was placed in 200 µL of sterile water and boiled for 20 minutes. The bacterial cells were removed by centrifugation. Each 50 µL reaction contained 10 µL of the boil as template, 250 pmol of each primer, 2.5 units of enzyme mix from the FailSafe™ PCR Kit (Epicentre), and 25 µL of 2 × FailSafe™ PCR enzyme Buffer B which contains dNTPs. As positive controls for the presense of the transposon or IS2404, a reaction was prepared with a template of either 1 ng of the transposome in 10 µL of sterile water or 200 ng of *M. ulcerans* genomic DNA 10 µL of sterile water, respectively. For the detection of *M.* ulcerans associated with snails, 10 µL of total genomic DNA isolated from snail tissue was used as template. The reactions were carried out in a thermocycler (Eppendorf, Hamburg, Germany) with an initial denaturation period of 5 minutes at 94℃ followed by 30 cycles of 1) 94℃ for 1 minute, 2) 55℃ for 1.5 minutes, and 3) 72℃ for 1.5 minutes followed by a final elongation period of 10 minutes at 72℃.

To obtain the sequences of the genomic DNA flanking the transposon of *M. ulcerans* 1615:tnp6w (i.e., to determine which gene was interrupted to give the mycolactone-negative phenotype), an arbitrarily primed PCR method was performed.

The protocol was obtained from Dr. Eric Rubin at Harvard School of Public Health (Boston, Massachusetts) in whose lab the protocol had been adapted from an original protocol by O'Toole (59). A separate first round PCR reaction was prepared for each side of the transposon. For this reaction, 50 μL reactions were prepared with 1 μL mutant 1615:tnp6w genomic DNA as template, 1 unit of *Taq* DNA polymerase, 1 mM dNTPs, 1.5 mM $MgCl_2$, 1 mM of ARB1 random primer (5'-GGC CAC GCG TCG ACT AGT NNN NNN NNN GAG GG-3'), and 1 mM of either ARB1TNP1 (5'-GCG GCT TTG TTG AAT AAA TCG A-3') to amplify genomic DNA downstream of the transposon or ARB1TNP2 (5'-TCC ATG TTG GAA TTT AAT CGC G-3') to amplify genomic DNA upstream of the transposon. Both ARB1TNP1 and ARB1TNP2 are complementary to regions on either side of the transposon approximately 150 bp within the transposon. The reactions were carried out in a thermocycler with an initial denaturation period of 3 minutes at 95℃ followed by 10 cycles of 1) 95℃ for 30 seconds, 2) 35℃ for 30 seconds, and 3) 72℃ for 1.5 minutes followed by an additional 30 cycles of 1) 95℃ for 30 seconds, 2) 50℃ for 30 seconds, and 3) 72℃ for 2 minutes plus 5 seconds per cycle followed by a final elongation period of 7 minutes at 72℃.

For the second round of the arbitrary primed PCR protocol, 50 μL reactions were prepared using 1 μL of the first round products as template, 1 unit of *Taq* DNA polymerase, 1 mM dNTPs, 1.5 mM $MgCl_2$, 1 mM of ARB2 arbitrary primer (5'-GGC CAC GCG TCG ACT AGT AC-3') and 1 mM of either ARB2TNP1 (5'-AGT TGA AGG ATC AGA TCA CGC-3') or ARB2TNP2 (5'-TTC CCG TTG AAT ATG GCT CAT AAC-3'). Both ARB2TNP1 and ARB2TNP2 are complementary to regions on either side of the transposon between the first round primers complementary to the

transposon (ARB1TNP1 and ARB1TNP2) and the primers to be used for sequencing. The reactions were carried out in a thermocycler with an initial denaturation period of 3 minutes at 95℃ followed by 30 cycles of 1) 95℃ for 30 seconds, 2) 62℃ for 30 seconds, and 3) 72℃ for 1 minute plus 5 seconds per cycle followed by a final elongation period of 7 minutes at 72℃. The products were sequenced using the sequencing primers included in the EZ::TN™ <KAN-2>Tnp Transposome™ Kit by the University of Tennessee Molecular Biology Sequencing Facility (Knoxville, Tennessee).

To confirm transformation with pPR27HI and pPRFURA, an 822 bp fragment of the hygromycin phosphotransferase gene (*hph*) which conveys hygromycin resistance was amplified using the primers HPH1 (5'-CGA TGT AGG AGG GCG TGG ATA-3') and HPH2 (5'-GCT TCT GCG GGC GAT TTG TGT-3'). To confirm transformation with pYUB178, a 476 bp fragment of the aparamycin resistance gene (*aac*(3)-IVa) contained on it was amplified using the primers APRA1 (5'-GTC CAC AGC TCC TTC CGT AGC-3') and APRA2 (5'-CTC GAG ATA ATC GAC GCG TAC C-3'). A small portion of each colony to be tested was placed in 200 µL of sterile water and boiled for 20 minutes. The bacterial cells were removed by centrifugation. Each 50 µL reaction contained 10 µL of the boil as template, 1mM of each primer, 1 unit of *Taq* DNA polymerase, 1 mM dNTPs, and 1.5 mM $MgCl_2$. For positive controls, a reaction was prepared with a template of 100 ng of the respective plasmid in 10 µL of sterile water. The reactions were carried out in a thermocycler with an initial denaturation period of 5 minutes at 94℃ followed by 30 cycles of 1) 94℃ for 1 minute, 2) 55℃ for 1 minute, and 3) 72℃ for 1 minutes followed by a final elongation period of 10 minutes at 72℃.

Cytopathicity Assay

L929 murine fibroblast cells were obtained from ATCC (ATCC CCL1) and were maintained by passaging in Dulbecco modified Eagle medium (DMEM) supplemented with 5% heat-inactivated calf serum (Gibco BRL, Grand Island, New York) at 37℃ with 5% carbon dioxide. To initially determine if the six insertion mutants obtained via transposon mutagenesis were cytopathic, a qualitative cytopathicity assay was performed. For this assay, the cells were plated at 6×10^4 cells/well (after dilution to 6×10^4 cells/mL using a hemocytometer) in a 24-well tissue culture plate and allowed to form a confluent monolayer for 24 hours. At this time, 50 μL of sterile filtrate of each of the mutants was added to a well. Sterile filtrate was obtained by passing 1 mL of culture through a Steritop™ 0.22 μm pore filter (Millipore, Billerica, Massachusetts). After 24 and 48 hours, the cells in each well were observed for rounding and detachment, which both indicate cytopathicity.

Mass Spectroscopy

All mass spectroscopy (MS) analysis was performed by Dr. Richard E. Lee of the College of Pharmacy, Department of Pharmaceutical Sciences, University of Tennessee Health Science Center (Memphis, Tennessee).

Isolation of ASLs

Acetone soluble lipids (ASLs) were extracted as described by George, et al. (22). The cells of 250 mL of bacterial culture were harvested by centrifugation at 5,509 × g for 10 min at room temperature without brake as described above for harvesting cells for

electroporation. Most of the supernatant was discarded; while, a small amount was used to resuspend the pellet for transferring the cells to a preweighed 30 mL glass Corex® tube (Fisher Scientific). The cells were then centrifuged at 4,000 × g for 10 min at room temperature (Fisher Marathon™ 21000R benchtop centrifuge; Fisher Scientific). The supernatant was discarded, and the pellet was dispersed around the walls of the tube with a small spatula for faster drying in a vacuum oven (Fisher Isotemp™ vacuum oven model 280A; Fisher Scientific) at approximately 55℃ and approximately -15 in.Hg. It took 8-10 hours for the pellets to dry completely. During this process, the tube was wrapped in foil to prevent excess light exposure. For quantitative purposes, the dried cell matter was massed (analytical balance model TR-204; Denver Instrument Company, Denver, Colorado). Then, 15 mL of chloroform:methanol (2:1, vol/vol) (Fisher Scientific) was added to the pellet and stirred for 2 hours. After this time, the tube was centrifuged at 4,000 × g at 4℃ for 15 min to separate the organic layer from the cell debris. Organic layer (top layer) was carefully removed by Pasteur pipette to a vial for evaporation under nitrogen using a heating module (Pierce Reacti-Therm™ heating module; Rockford, Illinois) at approximately 55℃. After the chloroform:methanol solvent was completely evaporated, the vial was placed on ice; and, 2 mL of ice-cold acetone was added to resuspend the ASLs present in the isolated organic fraction (total lipid content of cells). This suspension was then transferred to a small pre-weighed vial, and the acetone was evaporated off as described for the chloroform:methanol. For quantitative purposes, the dry ASLs were massed (Metler Toledo AT20 balance;Metler Toledo, Columbus, Ohio).

Analysis of ASLs by TLC

ASL analysis was performed by thin-layer chromatography (TLC) using silica-coated aluminum TLC plates (Alltech, Deerfield, Illinois) and a solvent system consisting of chloroform:methanol:water (90:10:1, vol:vol:vol) (Fisher Scientific). After the TLC plate was run, it was dried with hot air from a common hair dryer. The TLC plate was visualized under ultraviolet light, and all visible bands were noted. For further visualization of the ASLs, the TLC plate was developed by dipping in a para-anisaldehyde stain solution, then drying with hot air. The stain was prepared by combining the following reagents in the following order: 6 mL glacial acetic acid (Mallinckrodt Baker, Inc., Phillipsburg, New Jersey), 30 mL para-anisaldehyde (Acros Organics, a division of Fisher Scientific), 540 mL ice-cold ethanol (Aaper Alcohol And Chemical Company, Shelbyville, Kentucky), and 30 mL concentrated sulfuric acid (Mallinckrodt Baker, Inc.).

Isolation of *M. ulcerans* Genomic DNA

The isolation of genomic DNA from *M. ulcerans* is very difficult due to its polyketide-enriched cell wall. There is no published reference for a consistently high-yielding genomic DNA isolation protocol for *M. ulcerans*. The technique used in this work was adapted from protocols used to isolate genomic DNA from *M. tuberculosis* and other mycobacteria. The cells of 100 mL of log-phase culture were harvested by centrifugation at 7,500 × g for 15 min without brake. The supernatant was decanted; and, the pellet was resuspended in 5 mL TE (pH 8.0) (Fisher Scientific) and transferred to a 50 ml Nalgene™ Oak Ridge Teflon™ FEP tube (Fisher Scientific). To assist in cell lysis, 0.5

mL of a 10 mg/mL solution of lysozyme (Sigma) was then added, and the cells were incubated at 37℃ for 1 hour. To further lyse cells, 30 µL of 10% SDS (Fisher Scientific) and 700 µL of 20 mg/mL proteinase K (Sigma) were added and incubated at 65℃ overnight. Proteinase K was heat-inactivated at 80℃ for 10 min, and the cell mixture was cooled to room temperature before 30 µL of 4 mg/mL RNaseA (Sigma) was added and the mixture incubated at 37℃ for 45 min. 1 mL of 5M NaCl and 800 µL of 10% CTAB/0.7M NaCl (Fisher Scientific) was added to the mixture which was then incubated at 65˚C for 20 min. The mixture was then cooled to room temperature before the genomic DNA was extracted. Genomic DNA was extracted by adding 8 mL chloroform:isoamyl alcohol (24:1, vol:vol) (Fisher Scientific) and vigorously shaking for 30 seconds. After phase separation was apparent, the tube was centrifuged at 23,500 × g for 20 min at 4℃. The aqueous layer was removed and genomic DNA was extracted a second time in an identical manner using 8 mL phenol: chloroform: isoamyl alcohol (25:24:1, vol:vol:vol) (Sigma). The genomic DNA was then precipitated with 800 µL 3M sodium acetate (pH 5.2) (Fisher Scientific) and 8 mL isopropanol (Sigma) and centrifugation at 23,500 × g for 20 min at 4℃. The DNA was washed twice with 8 mL 70% ice-cold ethanol then allowed to dry. It was resuspended in 0.5 mL water at 65℃ for 30 min then overnight at 4℃. The DNA was quantitated and analyzed for purity via spectrophotometry (Eppendorf Biophotometer; Eppendorf).

Southern Blot

Approximately 2 µg of genomic DNA of mutant 1615:tnp6w was digested overnight at 37℃ in each of three digestions with *Sac*I, *Pvu*II, and *Eco*RI (New England

Biolabs, Beverly, Massachusetts). Genomic DNA from *M. ulcerans* 1615 and an *Escherichia coli* strain known to have an insertion of the EZ::TN™ <KAN-2>Tnp transposon were also digested with *Eco*RI to be used as a negative and positive control, respectively. All digestions along with *Hin*dIII cut λ DNA ladder (Promega) and 1 kb DNA ladder (Promega) were electrophoresed using a .8% agarose gel prepared with ethidium bromide. After electrophoresis, the gel was photographed using UV illumination to aid in interpretation of the blot.

The gel was prepared for the Southern blot as prescribed by the ECL™ Direct Nucleic Acid Labelling and Detection Systems kit (Amhersham Pharmacia Biotech, Inc., Piscataway, New Jersey). Briefly, the gel was subjected to depurination by agitation for 10 minutes in a depurination solution of 250 mM HCl (Fisher Scientific). After rinsing with distilled water, the gel was subjected to denaturation by agitation for 25 minutes in a denaturation solution of 1.5 M NaCl and 0.5 M NaOH (Fisher Scientific). After rinsing with distilled water, the gel was subjected to neutralization by agitation for 30 minutes in a neutralization solution of 1.5 M NaCl and 0.5 M Tris HCL (Fisher Scientific) at pH 7.5. After the initial 30 minute period, the neutralization solution was replaced with fresh solution and the gel was agitated in this solution for an additional 15 minutes. The capillary blot was constructed in the traditional manner as prescribed by the ECL™ Direct Nucleic Acid Labelling and Detection Systems kit using 20 × SSC (0.3 M Na_3 citrate and 3 M NaCl at pH 7.0 (Fisher Scientific)) as the transfer buffer, Whatman 3 MM paper (Whatman, Inc., Clifton, New Jersey) as a wick, and a Hybond-N+ membrane (Amersham).

After overnight blotting, the blot was removed, rinsed with 6 × SSC, allowed to air dry, and baked at 80℃ for 2 hours. The blot was pre-hybridized for 1 hour at 42℃ and then hybridized with labeled probe under the same conditions overnight. The probe was prepared from 100 ng of a 1,108 bp fragment of the transposon amplified with primers Tnp1 (5'-CTC AAA ATC TCT GAT GTT ACA TTG C-3') and Tnp2 (5'-GGT TGA TGA GAG CTT TGT TGT AGG T-3'). The probe was labeled as directed with a proprietary labeling reagent followed by a glutaraldehyde solution both included in the ECL™ Direct Nucleic Acid Labelling and Detection Systems kit. After hybridization, the blot was washed twice for 20 minutes each time at 42℃ with primary wash buffer (6 M urea, 0.4% SDS, and 0.5 × SSC (Fisher Scientific)). It was then washed twice for 5 minutes each time at room temperature in secondary wash buffer (2 × SSC). The blot was drained of secondary wash buffer before being developed for 1 minute with equal parts of detection reagents 1 and detection reagents 2 included in the kit. The blot was drained, wrapped in plastic film, and placed in a film cassette. The film was exposed for several minutes.

Cultivation of Snails

Schistosome-infected and uninfected snails of the species *Biomphalaria glabrata* were obtained from Dr. Fred Lewis, Schistosomiasis Laboratory, Biomedical Research Institute (BRI) (Rockville, Maryland). Those which were infected had been exposed to the miracidiae of *Schistosoma mansoni* strain NMRI, a Puerto Rican strain, en masse at eight miracidiae per snail.

The snails were maintained as instructed by Dr. Lewis in shallow translucent plastic containers (12 in × 7 in × 4 in) with non-airtight lids made of the same material. The containers were filled to a depth of two inches with water (1.5 L) which had been filtered using a commercially available water filtration system (Pur® Select Faucet Mounted Filtration System; Pur, available at most major retail stores) and then aerated for two to three days before use. Such conditioning of the water is necessary to insure that the snails do not come in contact with substances such as chlorine and copper which are deadly to them. Cercariae release greatly decreases the lifespan of the schistosome infected snail. Because a change in light intensity and temperature are stimuli which cause cercariae release, snails were maintained at room temperature and in constant light. The snails of each container were fed a single segment of carefully washed Romaine lettuce with an area of approximately two square inches every day. Any uneaten food was removed the following day. The snails were placed in common glass 500 mL beakers filled with conditioned water every third day for the snail containers to be disinfected, washed, and refilled with conditioned water. Once weekly, groups of snails were fed approximately .5 in^2 of a snail food supplement.

Prepared Snail Food Supplement

The snail food supplement was prepared as follows: 250 mL of water was stirred and heated. To this was added 2.5 g wheat germ (ICN Biomedicals, Inc., Aurora, Ohio), 1.25 g nonfat dried milk (Carnation®, available at most major retail stores), 2.5 g Ward's Cereal Grass Media (Ward's, Saint Catharines, Ontario, Canada), 2.5 g fish flakes (Tetra, Blacksburg, Virginia), and 2.5 g sodium alginate (Fisher Scientific). After mixing, the

mixture was poured into a glass pan and autoclaved for 20 minutes, then cooled overnight. After cooling, 2% $CaCl_2$ was poured on the surface and allowed to sit for 24 hours. The $CaCl_2$ was poured off and replaced with sterile water. The supplement was stored at 4℃.

Infection of Snails with *M. ulcerans*

A group of 6 uninfected snails were placed in a 50 mL conical tube with 25 mL of conditioned water to which 2.5 mL of *M. ulcerans* 1615 culture was added. A control group of 6 snails was placed in a 50 mL conical tube to which 2.5 mL of sterile M7H9 + OADC was added. After 24 hours, all snails had died. They were washed repeatedly with sterile water. Each group was then placed in 25 mL of a 10 μg/mL solution of amikacin, an antibiotic used to kill extracellular *M. ulcerans* during phagocytosis assays. After 36 hours, the snails were rinsed with sterile water, frozen at -80℃, and ground over dry-ice in a sterile tissue grinder. Approximately 20 mg of tissue was placed in a microcentrifuge tube for total genomic DNA extraction. The remaining tissue was decontaminated and plated as described below.

Isolation of Total DNA from Snail Tissue

The Wizard® Genomic DNA Purification Kit (Promega, Madison, Wisconsin) was used to isolate total DNA from 20 mg samples of ground snail tissue. The protocol was modified to include boiling at 100℃ for 20 minutes after the addition of the Nuclei Lysis Solution. After which, the sample was allowed to cool to room temperature before

continuing with the protocol provided in the kit. All reagents used were included in the kit.

Environmental Infection of Schistosome-Infected Snails with *M. ulcerans*

Two groups of 10 schistosome-infected snails each were maintained for 4 weeks to allow time for the schistosome infection to develop. 2 mL of *M. ulcerans* 1615 culture was washed twice by centrifugation (4,000 rpm, 25℃, 10 min) and resuspension in sterile water to reduce its quantity of mycolactone and remove elements of the M7H9 + OADC medium in which it was grown which would be harmful to the snails. The final pellet was resuspended in 2 mL of sterile water and added to a 500 mL beaker containing 250 mL conditioned water and one group of 10 snails. The other group of 10 snails served as a control and was held in a 500 mL beaker containing 252 mL conditioned water. Both beakers were covered in plastic wrap. After 24 hours, each group of snails was returned to its respective container and maintained as usual for 8 weeks.

Infection of Schistosome-Infected Snails with *M. ulcerans* through Foodborne Inoculation

Two groups of 10 schistosome-infected snails each were maintained for 4 weeks to allow time for the schistosome infection to develop. 2 mL of *M. ulcerans* 1615 culture was washed twice by centrifugation and resuspension in sterile water as described for the environmental infection of snails. The final pellet was resuspended in a very small volume of water (100 μL) and was injected into 1 g of the prepared snail food supplement. This food source is very dense and provided a good matrix for delivery of the bacteria to one group of 10 snails held in a 500 mL beaker containing 250 mL

conditioned water. 1 g of sterile food supplement was given to the control group of 10 snails held in another 500 mL beaker containing 250 mL conditioned water. Both beakers were covered with plastic wrap. After 24 hours, each group of snails was returned to their respective containers and maintained as usual for 8 weeks.

Preservation of Snails

Snails of both the environmental and food infection studies were checked daily. When dead snails were not cannibalized, they were washed with sterile water before being frozen to be decontaminated and plated later or before being removed from their shells using sterile forceps to be fixed. Snail tissue for slide preparation was preserved by immediate emersion in Bouin's fixative (LabChem, Inc., Pittsburgh, Pennsylvania) for 4 days. The snail was then removed and immersed in the following solutions for 24 hours each: sterile water, 10% ethanol, 20% ethanol, 30% ethanol, 50% ethanol, and 70% ethanol.

Preparation of Slides

Histological sectioning and preparation of slides was performed by Histo-Path of America (Millersville, Maryland). Sections were stained alternately by hematoxylin-and-eosin and acid-fast staining. These two staining procedures were chosen, because hematoxylin-and-eosin staining is the procedure commonly used to stain snail sections; while, acid-fast staining is the most commonly used staining procedure for identifying mycobacteria. There are several references which are helpful in orienting yourself while viewing histological snail sections stained with hematoxylin and eosin.

Plating Snail Tissue

Before plating, snail tissue was frozen at -80℃ and ground over dry-ice using a sterile tissue grinder. The decontamination protocols used were adapted from procedures used to decontaminate mammalian tissue for the isolation of *M. ulcerans*. For the decontamination of snails from the preliminary infection, most homogenates had volumes of 100-200 µL. To this sample, .5 mL 2 M HCl was added. The sample was vortexed and incubated at room temperature for 15 minutes. The tubes were then centrifuged at 6,000 × g for 15 min and the supernatant decanted. Attempts to neutralize the pellet were made by adding 2% NaOH drop-by-drop while monitoring the pH with pH test strips. Because this was not a very effective method, a new method was adapted for the subsequent infection studies. For the infection of snails with *M. ulcerans* through 1) environmental inoculation and 2) foodborne infection, the ground snail tissue along with 5 mL of sterile water used to wash out the tissue grinder was placed in a 50 mL conical tube. To the 5mL volume, an equal volume of 2% NaOH was added. The sample was vortexed and incubated at room temperature for 15 minutes. After 15 minutes, the samples were centrifuged at 3,000 × g for 15 min to collect debris and bacteria as a pellet. After centrifugation, the supernatant was decanted and the sediment was resuspended in 1 mL of sterile water. 1 drop of a 0.4% phenol red powder in 2% NaOH was added to the solution. The sample was neutralized using a 2 N HCl solution added drop-by-drop. The phenol red indicator allowed visual estimation of neutralization. 100 µL, 50 µL, and the remaining sample concentrated to 100 µL were plated on M7H9 + 50 µg/mL cycloheximide. Plates were wrapped in parafilm and incubated at 32℃ for 4 months.

III. The Identification of a Mycolactone-Negative Mutant of *Mycobacterium ulcerans*

Introduction

Mycobacterium ulcerans causes the third most common mycobacterial infection in immunocompetent humans world-wide. This infection causes a condition commonly referred to as Buruli ulcer disease, which is characterized by necrotizing skin lesions. Buruli ulcer disease is considered by many to be the most important mycobacterial infection in West Africa and possibly other parts of the world. Poor health care availability and substandard infrastructures in the regions endemic to *M. ulcerans* infection make it difficult to determine the true number of people affected by this disease or even how the disease is transmitted. At present, the only effective means of combating this disease is surgical excision followed by skin grafting, measures which are not readily available in endemic regions. Any knowledge that could lead to an effective treatment or to the prevention of transmission is greatly needed.

In 1999, a toxin produced by *M. ulcerans* was isolated and its structure was determined to be that of a 12-membered lactone ring to which two polyketide chains are attached. This molecule was designated mycolactone and was shown upon injection into Guinea pigs to produce ulcers that are nearly identical to those produced by the injection of *M. ulcerans* itself (23). This and the absence of ulcer formation in Guinea pigs upon injection with a spontaneous mycolactone-free mutant of *M. ulcerans* suggests that mycolactone could be solely responsible for the development of Buruli ulcer disease (23).

It has been suggested that an antitoxin targeted against mycolactone could be an effective treatment for Buruli ulcer disease (78).

However, the development of an antitoxin or other treatment for Buruli ulcer is not the principal motivation for studying mycolactone. As a polyketide-derived macrolide, mycolactone is a member of a class of compounds which are often biologically active and pharmaceutically useful (e.g., the antifungal amphotericin B, the immunosuppressant rapamycin, the antihypercholesterolemic lovastatin, etc.). Some polyketides have even multiple pharmacological activities (44). The polyketide antibiotic erythromycin is also a gastrointestinal stimulant; and, the immunosuppressant FK506 is also an antifungal (39, 45). Mycolactone itself has been demonstrated to be biologically active in that in an animal model it causes cell death and lack of an acute inflammatory response, mimicking the appearance of Buruli ulcer (24). Mycolactone has also been demonstrated to cause arrest of the cell cycle in the G_0/G_1 phase in both L929 murine fibroblasts and J774 murine macrophages (23, 24). Based on these properties, it is plausible that mycolactone could be of some pharmaceutical value as an antineoplastic agent, antibiotic, immunosuppressant or other drug either as it occurs in culture or in a modified state.

Further studies will be required for either the development of an antitoxin or the evaluation of mycolactone as a biologically active compound. Research aimed at these and any other findings with regard to mycolactone would be greatly facilitated by the ability to produce mycolactone in a fast growing mycobacterium (e.g., *M. smegmatis* or *M. fortuitum*). For these reasons, the identification of the molecular machinery required to produce this mycolactone is imperative. Based on structural analysis, it was

hypothesized that the biosynthesis of mycolactone requires at least 100 kb of DNA comprising two polyketide synthase (PKS) genes, an acyl transferase, and a p450 hydroxylase. The large size of the biosynthetic pathway for mycolactone precluded ready cloning but made transposon mutagenesis a possible strategy for identifying these genes.

The purpose of the following experiments was to create a mycolactone-negative mutant of *M. ulcerans* through transposon mutagenesis and characterize this mutant with the intention of identifying a gene involved in mycolactone production. At the time of this study, no defined mutant of *M. ulcerans* had been created, and there was no established method for making such mutants.

Results

In an effort to identify genes responsible for the production of mycolactone, efforts to produce a mycolactone-negative mutant of *M. ulcerans* via transposon mutagenesis were undertaken. Many attempts to create insertion mutants of *M. ulcerans* using the EZ::TN™ <KAN-2>Tnp Transposome™ Kit from Epicentre were made by delivering the transposon via electroporation. Electrocompetent cells were prepared by washing log-phase cultures (grown in stir flasks at 32°C in Middlebrook 7H9 with 10% oleic acid-albumin-dextrose-complex (M7H9 + OADC)) with successively smaller volumes of ice-cold 10% glycerol and apportioning the final volume of cells into 60 µL aliquots. Each aliquot of cells was electroporated with 1 µL (20 ng/µL) of the transposome in a 2 mm gap cuvette with a pulse of parameters 2.5 kV, 25 µF, and 800 Ω. The cells were immediately transferred to M7H9 + OADC and incubated in a rollerdrum

for 72 hours; after which, the cells were plated on selective media--M7H9 + OADC with 10 μg/μL kanamycin—and incubated at 32°C. After eight weeks of incubation, eight colonies had appeared on various selective plates. Five of these colonies were verified via PCR to contain the Tn903 kanamycin resistance gene contained on the transposon (Figure 8).

One of these five colonies (mutant 1615:tnp6w), unlike wild-type *M. ulcerans* which is yellow, remained white after several months incubation. Because it was known that mycolactone imparts the yellow color to wild-type *M. ulcerans* and spontaneously arising mycolactone deficient mutants of *M. ulcerans* are white, it was suspected that this white colony was mycolactone-negative. Having the same morphology and growth rate as wild-type *M. ulcerans*, it was unlikely that any of the colonies were not *M. ulcerans*; however, to verify their identity as such, mutants 1615:tnp6w and 1615:tnp3, an insertion mutant with a wild-type phenotype, were subjected to PCR analysis for the presence of the insertion element IS2404 unique to *M. ulcerans*. Both mutants were PCR-positive for the presence of IS2404, confirming them to be *M. ulcerans* (Figure 9).

Both mutants 1615:tnp6w and 1615:tnp3, which was used as transposon-containing, phenotypical normal control, were inoculated into M7H9 + OADC for expansion. ASLs were isolated from log-phase cultures via methanol:chloroform extraction as described by George, et al. (22). Both are PCR-positive for the presence of insertion element IS2404 unique to *M. ulcerans*.

ASLs of 1615:tnp6w were analyzed for the presence of mycolactone via thin-layer chromatography (TLC) on silica-coated aluminum plates. The ASLs of both *M.*

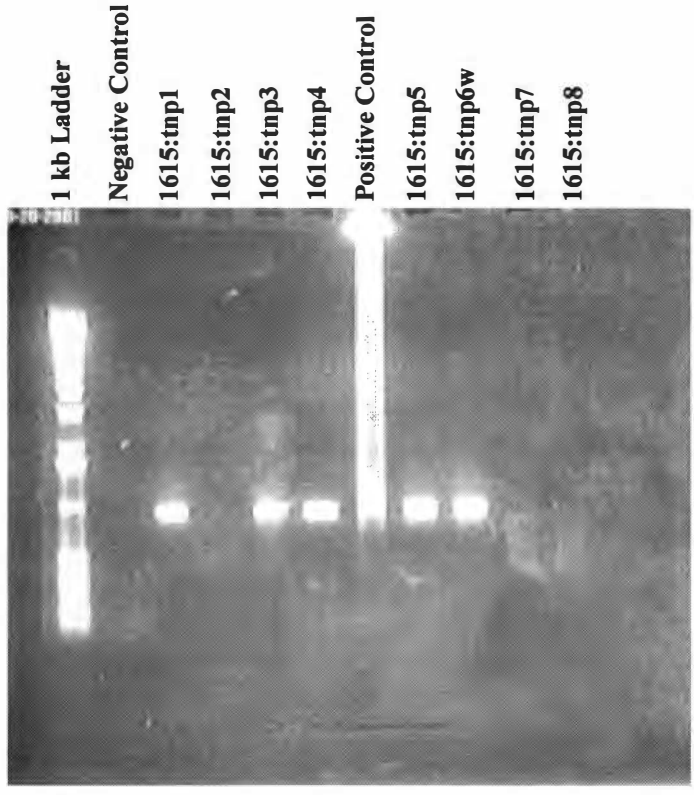

Figure 8. Mutants 1615:tnp1 and 1615:tnp3-6 are PCR-positive for the EZ::TN™ <KAN-2> Tnp Transposome™.

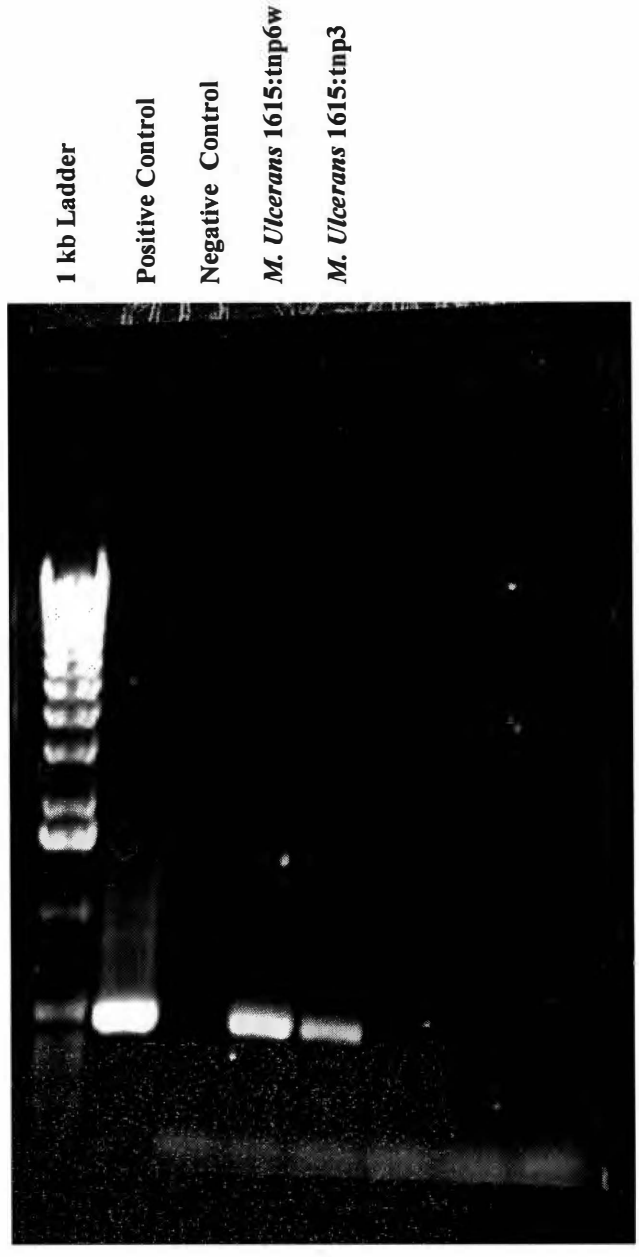

Figure 9. PCR analysis of suspected *M. ulcerans* transposon insertion mutants 1615:tnp6w and 1615:tnp3 for the presence of IS2404.

ulcerans 1615 and mutant 1615:tnp3 were included as controls. Approximately equal volumes (approximately 16 μL) and masses (approximately 825 μg) of ASLs were loaded on the plate for each of the three samples, and the TLC was run in a chloroform:methanol:water (90:10:1, vol:vol:vol) solvent before being stained with para-anisaldehyde staining solution. While mycolactone was present in both the ASLs isolated from *M. ulcerans* 1615 and the mutant 1615:tnp3 as a band with a ratio to front (R_f) value of approximately 0.23, mycolactone was not present in the ASLs of mutant 1615:tnp6w (Figure 10). On an additional TLC analysis (not shown) for which nearly twice the mass of ASLs was loaded (more than 1.5 mg), there was still no mycolactone detected in the ASLs isolated from mutant 1615:6w.

To further characterize the 1615:tnp6w mutant, a cytopathicity assay was performed on sterile filtrate of the mutant. Sterile filtrate was obtained by passing 1 mL of log-phase culture through a .22 μm pore polyethersulfone filter. 50 μL of the sterile filtrate was added to a well of a 24-well tissue culture plate containing a confluent monolayer of L292 murine fibroblasts plated at 6×10^4 cells/well. The sterile filtrates of wild-type *M. ulcerans* 1615 and mutant 1615:tnp3 as well as sterile M7H9 + OADC were used as controls. At 24 and 48 hours, the cells were examined for rounding and detachment, which are both indicative of cytopathicity. At 48 hours, as expected, unlike those exposed to sterile M7H9 + OADC, which remained a healthy confluent monolayer of rhomboid shaped cells (Figure 11), the cells exposed to the sterile filtrate of wild-type *M. ulcerans* 1615 exhibited a great degree of rounding and detachment (Figure 12).

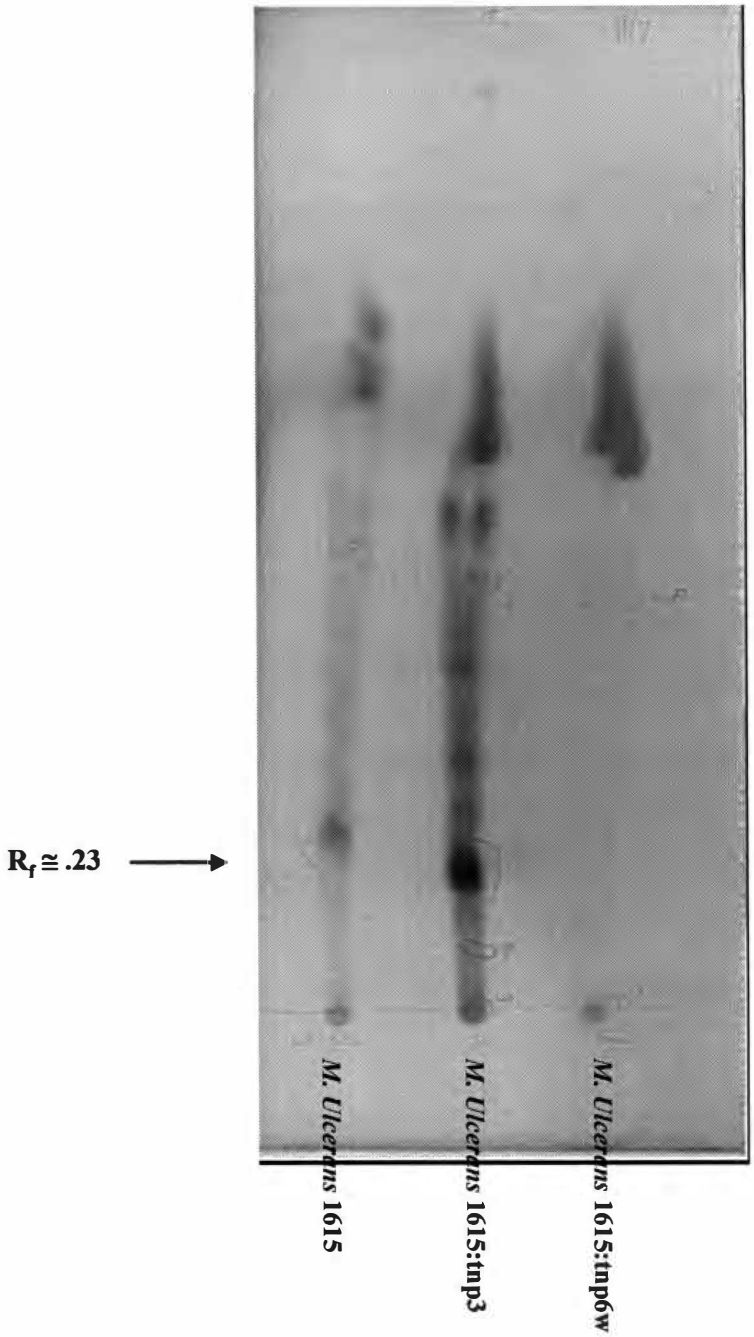

Figure 10. TLC analysis of ASLs isolated from *M. ulcerans* mutant 1615:tnp6w.
While mycolactone is present as a band with an R_f value of approximately .23 in the ASLs of *M. ulcerans* 1615 and *M. ulcerans* mutant 1615:tnp3, mycolactone is not present in the ASLs of *M. ulcerans* 1615:tnp6w.

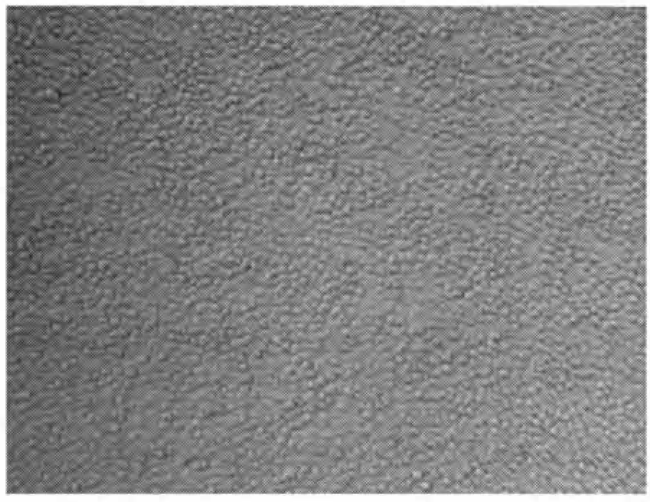

Figure 11. L929 murine fibroblasts after exposure to M7H9 + OADC.
Picture was taken at 48 hours and 100 × magnification. Cells are phenotypically undisturbed.

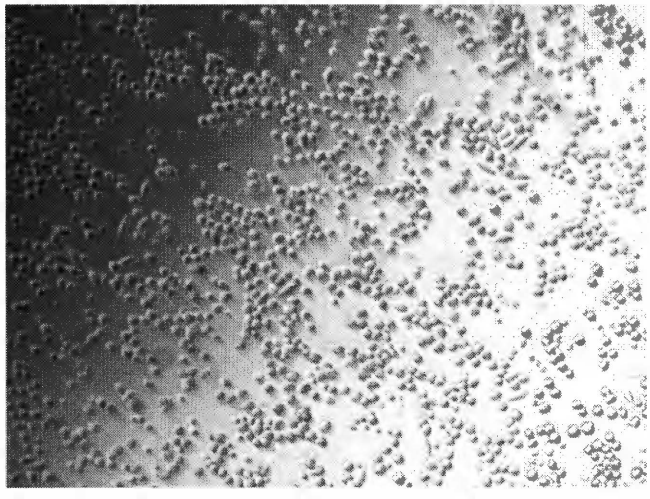

Figure 12. L929 murine fibroblasts after exposure to sterile filtrate of *M. ulcerans* 1615.
Picture was taken at 48 hours and 100 × magnification.

The sterile filtrate of the mutant 1615:tnp6w had no apparent cytopathic effect. After 48 hours, fibroblasts exposed to the sterile filtrate of mutant 1615:tnp6w appeared identical to those exposed only to sterile M7H9 + OADC (Figure 13). The sterile filtrate of mutant 1615:tnp3 had a cytopathic effect on the fibroblasts identical to that of the wild-type *M. uclerans* (Figure 14).

In order to more definitively determine if mutant 1615:tnp6w was producing mycolactone, its ASLs were subjected to mass spectral analysis. The mass spectrum of ASLs of wild-type *M. ulcerans* 1615 have a sharp peak at *m/z* 765.5, which represents mycolactone, and a peak at *m*/z 445.3, which represents the core lactone (Figure 15). There are no comparable peaks present in the mass spectrum of the ASLs of mutant 1615:tnp6w (Figure 16). The absence of peaks at *m/z* 765.5 or at *m*/z 445.3 is indicative that neither mycolactone nor core lactone is produced by this mutant.

Having established mutant 1615:tnp6w to be negative for mycolactone production, efforts to determine the location of the transposon were undertaken. A .8% agarose gel for Southern blot was prepared with overnight *Sac*I, *Pvu*II, and *Eco*RI digestions of mutant 1615:tnp6w genomic DNA. As a negative and positive control (respectively), *Eco*RI digestions of genomic DNA from *M. ulcerans* 1615 and an *Escherichia coli* strain known to have an insertion of the EZ::TN™ <KAN-2>Tnp transposon were also included. *Hin*dIII cut λ DNA ladder and 1 kb DNA ladder were included in the gel. The Southern blot was prepared as instructed by the ECL™ Direct Nucleic Acid Labelling and Detection Systems kit to be used to probe and develop the blot. The blot was washed, pre-hybridized, and hybridized as instructed by the kit. 100 ng of a 1,108 bp region of the transposon was labeled using a proprietary labeling reagent

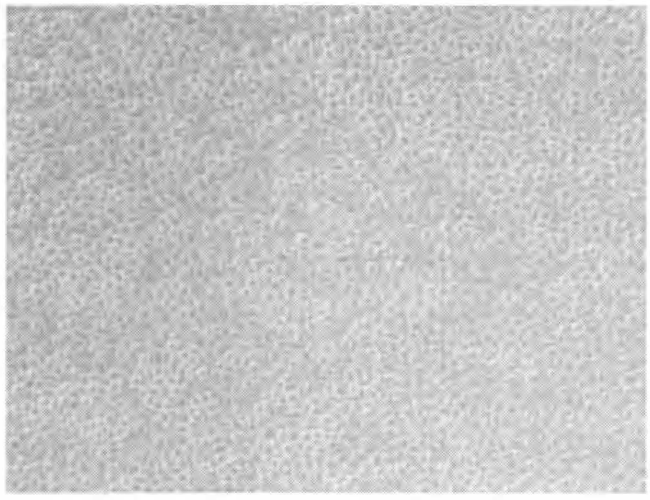

Figure 13. L929 murine fibroblasts after exposure to sterile filtrate of mutant 1615:tnp6w.
Picture was taken at 48 hours and 100 × magnification. Cells appear phenotypically undisturbed.

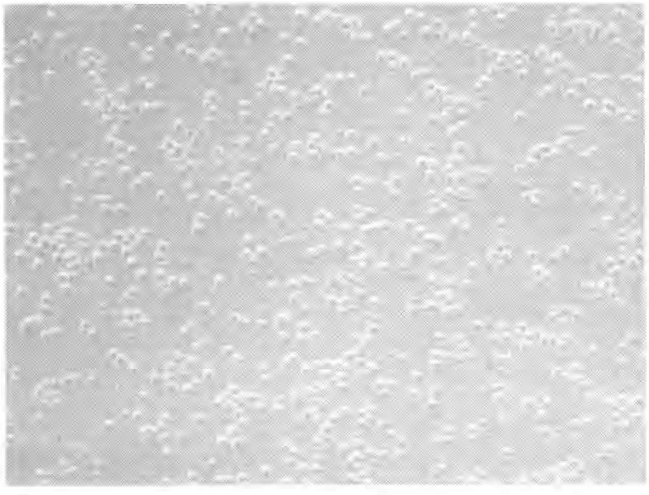

Figure 14. L929 murine fibroblasts after exposure to sterile filtrate of mutant 1615:tnp3.
Picture was taken at 48 hours and 100 × magnification.

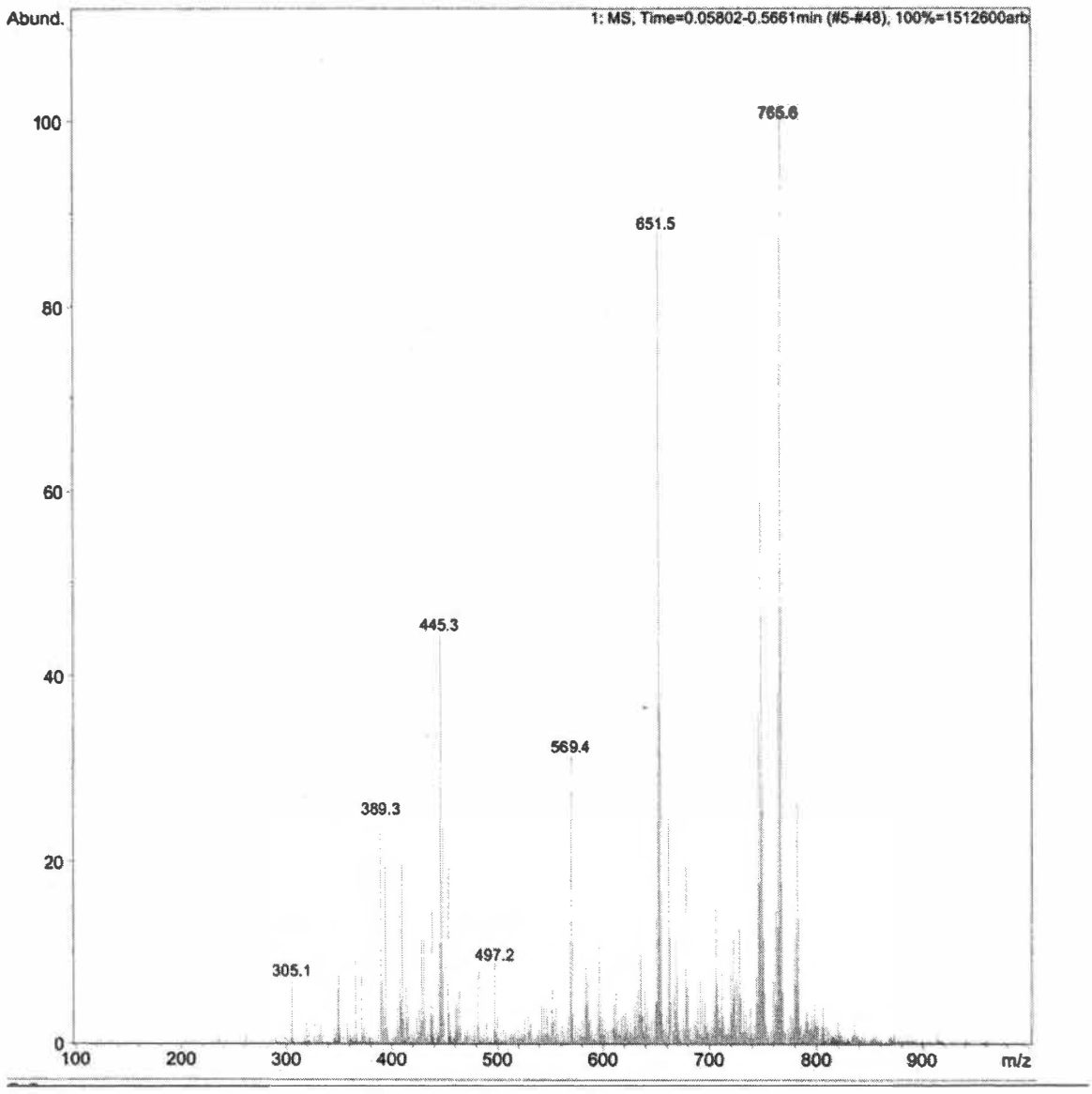

Figure 15. Mass spectrum of *M. ulcerans* 1615 ASLs.
The large, sharp peak at *m/z* 765.5 is known to represent mycolactone, the most abundant species present; while, the peak at *m/z* 445.3 is known to represent the core lactone.

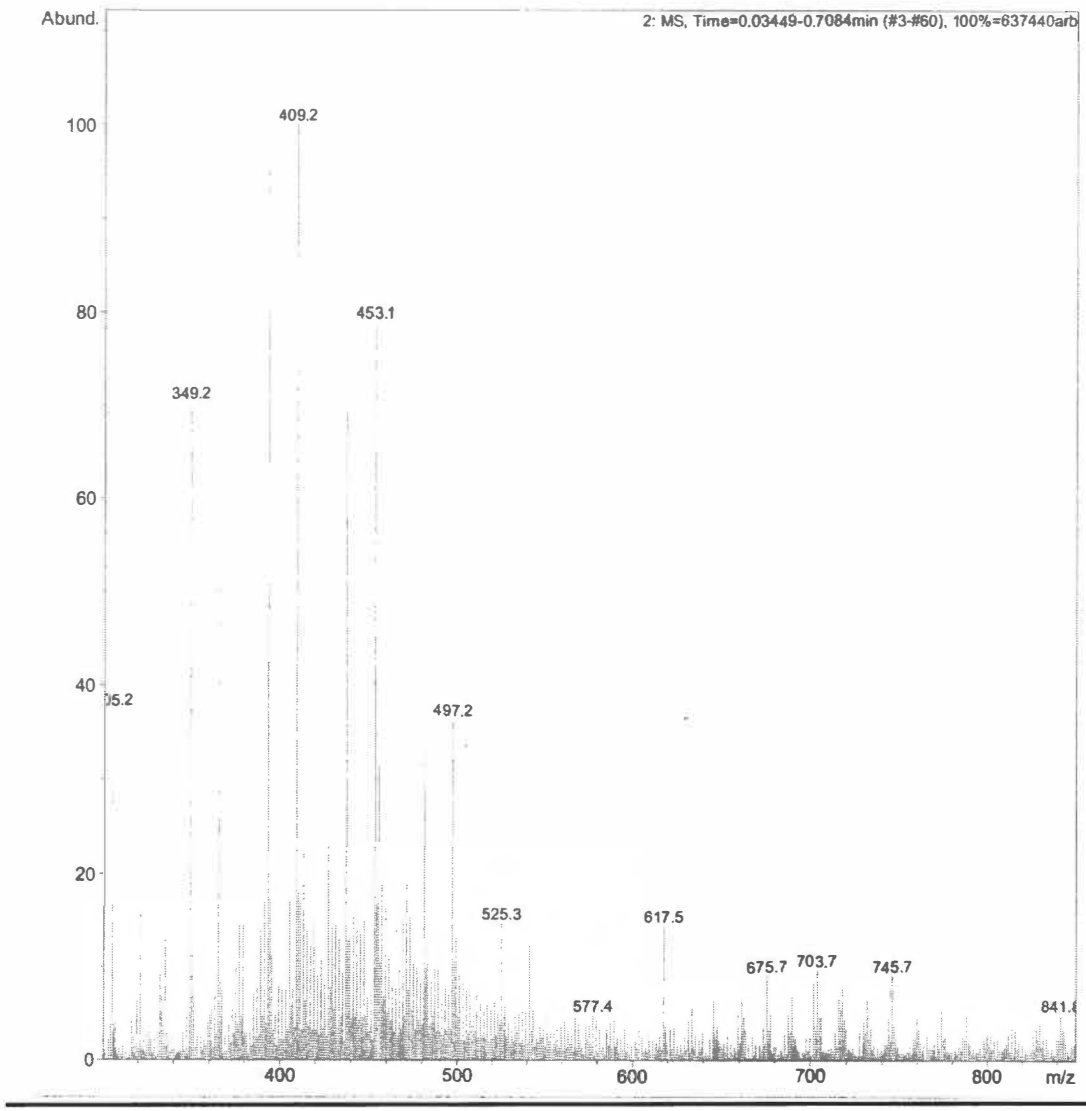

Figure 16. Mass spectrum of *M. ulcerans* 1615:tnp6w ASLs.
The absence of a peak at *m/z* 765.5 indicates that mycolactone is not produced by this organism. In addition, there is no peak at *m/z* 445.3, the peak which is known to represent the core lactone.

followed by a glutaraldehyde solution as described by the kit and was used to probe the blot. The blot was developed chemically using proprietary developing reagents included in the kit. Hybridization by the probe was detected by exposing the blot to film for several minutes. Single, distinct bands were hybridized by the transposon-complementary probe for both the genomic mutant 1615:tnp6w genomic DNA digested with *Pvu*II and that digested with *Eco*RI, indicating that the transposon is present in only one copy (i.e., one insertion) in the genome of the mutant (Figure 17). These bands migrated from the well 22 mm and 11 mm, respectively. There was no clear band hybridized to the probe in the lane corresponding to the mutant genomic DNA digested with *Sac*I—only a high molecular weight smear. This can be attributed to incomplete digestion as was also evidenced by the picture of the gel taken before blotting (not shown). As expected, the probe hybridized to a distinct area corresponding to the genomic DNA of the *E. coli* transposon insertion mutant—the positive control—but did not hybridize to that of *M. ulcerans* 1615—the negative control. Linear regression analysis revealed that the transposon is located in the mutant 1615:6w genomic DNA on a fragment of DNA of approximately 6.51 kb in a *Pvu*II digestion and a fragment of approximately 15.39 kb in an *Eco*RI digestion.

In an effort to determine what gene had been interrupted by the insertion of the transposon in the mutant 1615:tnp6w, several attempts at sequencing directly from the genomic DNA were made. These attempts were unsuccessful. Next, many attempts to clone the transposon with its flanking regions of genomic DNA to be sequenced from the newly created plasmid were made by both the shotgun method and by attempting to isolate and clone only the 6.51 kb *Pvu*II fragment of genomic DNA. These also were

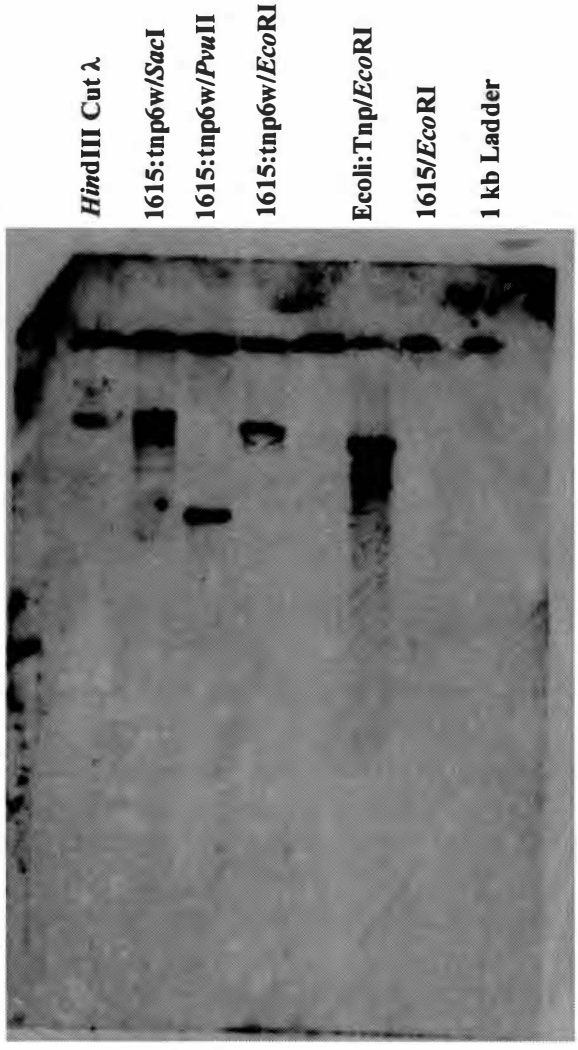

Figure 17. Film exposed by the Southern blot of mutant 1615:tnp6w genomic DNA probed with a probe complementary to a 1.1 kb region of the transposon. Digestion of 1615:tnp6w genomic DNA with *Sac*I was incomplete. A single band of *Pvu*II digested genomic 1615:tnp6w DNA of approximately 6.5 kb was hybridized by the probe; while, a single band of *Eco*RI digested genomic 1615:tnp6w DNA of approximately 15.39 kb was hybridized by the probe. *Eco*RI digested genomic DNA from *E. coli*:tnp, a confirmed transposon insertion mutant, and from wild-type *M. ulcerans* 1615 were used as a positive and negative control, respectively.

unsuccessful.

To obtain sequence data from the regions of genomic DNA flanking the transposon, a two part arbitrarily primed PCR technique was employed. The first round of PCR utilized a primer 150 bp within the transposon and a random primer containing sequences common in mycobacteria. This PCR reaction produced DNA fragments of varying lengths, all flanking the transposon (and containing over 100 bp of it). The second round of PCR in this arbitrarily primed PCR protocol used the first round products as template and an arbitrary (rather than random) primer which was nearly identical to the random primer with out the random bases. The second primer used in this reaction was a primer complementary to the transposon in a region 50-100 bp within the transposon. This amplified many fragments of DNA of the same length flanking the tranposon. This concentrated amplification of DNA adjacent to the transposon was sequenced using the sequencing primers included in the transposon kit.

The sequence obtained from the reactions which amplified DNA downstream of the transposon was poor; and, when a Blast search was performed, no strong homologies were identified. The sequence obtained for the DNA upstream of the transposon, however, was 97% identical to the *M. marinum* ferric uptake regulator homolog (FurA) gene and 89% identical to the same gene (*fur*A) of *M. tuberculosis.* The arbitrarily primed PCR protocol was repeated and the final products sequenced. The sequences obtained were identical to the ones previously obtained. At this time, it was uncertain where the transposon was inserted in relation to the *fur*A homologue of *M. ulcerans*.

Several months after this mutant was found to have an insertion in the *fur*A homologue of *M. ulcerans*, Dr. Armand Mve-Obiang independently identified a second

mycolactone-negative mutant (mutant 97) which also has an insertion in the same gene. This reinforced the suspicion that the *fur*A homologue of *M. ulcerans* could be a regulator of mycolactone. For complementation studies, the sequence of the *M. marinum* *fur*A homologue was obtained and used to search the Burulist database (http://genopole.pasteur.fr/Mulc/BuruList.html), a database organized by the Pasteur Institute to allow access to the unannotated genomic DNA sequences of *M. ulcerans*, for the *M. ulcerans* homologue of *furA*. This database identified the *fur*A homologue of *M. ulcerans* to be on a 7.6242 kb contig designated mu0181G02F PhrapUL 7.6242. The entire sequence of this contig was obtained from Dr. Tim Stinear (Pasteur Institute, Paris, France) and within it the *fur*A homologue was identified. At this time, the sequence data obtained from the forward and reverse products of the arbitrarily primed PCR protocol were pieced together and compared to the sequence of this region available in the contig. The transposon insertion of mutant 1615:tnp6w appeared to be located, not in the actual structural gene of the *fur*A homologue, but approximately 270 bp upstream of this gene. This region of DNA is not homologous to any know prokaryotic genes and could be a noncoding region of the *M. ulcerans* genome.

The sequence obtained from the mu0181G02F PhrapUL 7.6242 contig was used to create primers to amplify the *fur*A homologue of *M. ulcerans* and approximately 500 bp upstream and downstream of the gene. These primers were used to amplify the corresponding region of DNA of wild-type *M. ulcerans* 1615. This region of DNA was cloned into the shuttle vector pPR27HI and the construct used to complement the mutant 1615:tnp6w and mutant 97. Several transformants were obtained when the mutant 1615:tnp6w was electroporated with this construct. (No mutant 97 transformants were

obtained.) However, after several months incubation, none of the transformants had turned yellow. Cytotoxicity assays on L929 murine fibroblasts treated with sterile filtrate of the transformants revealed that the transformants did not produce any cytotoxic compounds present in the media.

In search of an explanation for the lack of mycolactone production upon complementation of the mutant:tnp6w with the *fur*A homologue of *M. ulcerans*, this mutant was analyzed via PCR for the presence of two genes, one encoding an enoyl reductase and the other a thioesterase (II), recently identified to be essential for the production of mycolactone. These genes are in areas of genetic instability and are thought to be frequently lost due to recombination. While mutant 97 contained both of these genes as evidenced by the PCR reaction, the thioesterase was not present in the mutant 1615:tnp6w. The mutant 1615:tnp6w had a mycolactone-negative phenotype due to the deletion of the thioesterase (II) gene required for mycolactone production, rather than the insertion in the *fur*A homologue. However, the possibility of the FurA homologue of *M. ulcerans* playing a role in the regulation of mycolactone production can not be eliminated.

Discussion

Through many attempts over many months to create mycolactone-negative mutants of *M. ulcerans* through transposon mutagenesis, only a total of five PCR-confirmed insertion mutations arose. This can be contributed to the extremely low transformation efficiency of *M. ulcerans* combined with the statistical improbability of transposition. While it might seem highly fortuitous to some that a mutant of the

phenotype desired--a mycolactone-negative mutant—would be present in a pool of only five insertion mutants; constructing a mycolactone-negative mutant of *M. ulcerans* was not seen as an endeavor that would require the production of large libraries of mutants. At the time of this study, based on the knowledge of other polyketides, it was hypothesized that the genetic elements required for the synthesis of mycolactone would be comprised of more than 100 kb of DNA and would include two polyketide synthase (PKS) genes, an acyl transferase, and a p450 hydroxylase. For this reason, it was not surprising that in even a small pool of insertion mutants there appeared to be a mutant with an insertion in such a large genetic target.

The putative mycolactone-negative mutant was recognized as such by its lack of pigmentation. Wild-type *M. ulcerans* has a very obvious yellow color which was thought to be due to the presence of mycolactone, a compound with the conjugated double bonds that cause molecules to be pigmented. Strains of *M. ulcerans* that had spontaneously lost pigmentation after repeated culturing in the lab had been found to be mycolactone-negative. The putative mycolactone-negative insertion mutant remained white after many months of growth. All other insertion mutants obtained were yellow and have remained so. To rule out the remote possibility that the putative mycolactone-negative mutant was not *M. ulcerans* but some contaminant, the mutant was verified by PCR to have the IS2404 insertion element, a genetic element unique to *M. ulcerans*.

The predominant species of mycolactone produced by *M. ulcerans* 1615, the strain from which the insertion mutants originated, is know to have an R_f value of approximately .23 when analyzed via TLC using the solvent system used in these experiments. To analyze the composition of the ASLs of mutant 1615:tnp6w and to

determine if they contained mycolactone or any molecules of similar size and polarity, the ASLs of a log-phase culture were isolated and analyzed via TLC using the ASLs of phenotypically normal transposon insertion mutant 1615:tnp3 and wild-type *M. ulcerans* as controls. While the ASLs of the mutant 1615:tnp3 contain a band at approximately R_f .23 as do the ASLs of *M. ulcerans* 1615 this band or one comparable to it is not present in the ASLs of the mutant 1615:tnp6w.

Though by TLC analysis of ASLs, mutant 1615:tnp6w appeared to be mycolactone-negative, cell cytotoxicity assays were performed on the sterile filtrate of this mutant using L929 murine fibroblasts. The sterile filtrates of *M. ulcerans* 1615 and mutant 1615:tnp3 were used as positive controls; while, sterile M7H9 + OADC was used as a negative control. Mycolactone, which is present in the sterile filtrate of strains that produce it, is known to cause cell cycle arrest in fibroblasts by rearrangement of the cytoskeleton. In cell culture, this is made apparent by rounding of the cells followed by their detachment from the monolayer. This affect, as expected, was seen in cells treated with the sterile filtrate of both *M. ulcerans* 1615 and the phenotypically normal mutant 1615:tnp3; however, the sterile filtrate of mutant 1615:tnp6w produced no such effect, and after 48 hours cells treated with it looked identical to cells treated with sterile M7H9 + OADC. Similar assays (not shown) on the ASLs of this mutant 1615:tnp6w produced identical results. It is clear that mutant 1615:tnp6w is not cytotoxic.

To definitively prove that this mutant does not produce mycolactone, its ASLs were analyzed via mass spectroscopy. The mass spectrum of mutant 1615:tnp6w did not have peaks at either *m/z* 765.5 or *m/z* 445.3, which represent mycolactone and its core lactone, respectively. It should also be noted that the mass spectrum of the ASLs of this

mutant did not have any peaks which represent any of the less common congeners of mycolactone. Mutant 1615:tnp6w is clearly mycolactone-negative as evidenced by this.

Though the EZ::TN™ <KAN-2>Tnp transposon is designed so that sequence data can be obtained by sequencing out from either end of the transposon directly from genomic DNA, this was not feasible with *M. ulcerans*. *M. ulcerans* has an extremely GC-rich genome, and sequencing directly from the genome is further complicated by the inability to isolate high concentrations of clean genomic DNA from this organism. After several attempts at obtaining sequence data directly from the genome, new approaches were considered. Many attempts at shotgun cloning using various enzymes and vectors were unsuccessful. The Southern blot of *Pvu*II and *Eco*RI digested mutant 1615:tnp6w genomic DNA revealed that when digested with these enzymes, the fragment containing the transposon was approximately 6.5 kb and 15.4 kb, respectively. While the 15.4 *Eco*RI fragment might be difficult to clone, the smaller 6.5 kb *Pvu*II was thought to be readily clonable. Many attempts at digesting mutant 1615:tnp6w genomic DNA with *Pvu*II, running the DNA on a gel, excising the bands of DNA corresponding to approximately 6.5 kb, extracting the DNA from a gel and ligating the DNA to a *Pvu*II cut vector were unsuccessful. It is very difficult to isolate from *M. ulcerans* the large quantities of DNA needed for cloning and even more difficult to isolate unsheared, pure DNA from this organism. This lack of DNA was exacerbated by the need to purify DNA from a gel. Several methods for doing this were tried and compared. All result in the loss of a significant percentage of the DNA to be isolated. The inability to to obtain sequence data from direct sequencing or from cloning the transposon containing fragment

necessitated alternative methods for determining which gene had been interrupted by the insertion of the transposon.

The arbitrarily primed PCR protocol proved to be a very useful tool for identifying the gene which had been interrupted by the transposon. With this method, sequence data was obtained that suggested that the transposon had inserted into the *fur*A homologue of *M. ulcerans*. Not long after it was determined that the transposon had possibly interrupted the *fur*A homologue of *M. ulcerans*, an independently created mycolactone-negative mutant (mutant 97 from Dr. Armand Mve-Obiang) was also found to have a single interruption in the same gene. This was the impetus to continue characterization of this mutant after discovering that the gene which was interrupted—the *fur*A gene—was most likely not directly involved in the synthesis of mycolactone.

The FurA protein of *M. marinum* is an orthologue of the *E. coli* transcription factor Fur (ferric uptake regulation). The *fur* genes of *M. tuberculosis* are thought to be transcriptional regulators of genes involved in functions other than acquisition of iron (14). In mycobacteria, *fur*A is ubiquitously linked to the catalase-peroxidase gene *kat*G and has been shown to be a negative regulator of *kat*G (60, 86). It is thought that *fur*A may regulate virulence factors other than *kat*G.

Complementing the mutant 1615:tnp6w with the *fur*A homologue of *M. ulcerans* did not result in the production of mycolactone; however, mutant 97 was not able to be transformed. Via PCR analysis, the lack of restored mycolactone production in mutant 1615:6w has been explained by a deletion in the thioesterase (II) encoding gene which has recently been identified as essential for mycolactone production (73). This deletion is most likely the cause of the mycolactone-negative phenotype, rather than the insertion

of the transposon into the *fur*A homologue of *M. ulcerans*. Electroporation is associated with increased mutation rates and may have caused this deletion in the thioesterase (II). It should also be noted that *fur*A, possibly functioning as an iron uptake regulator in *M. ulcerans*, could protect the cell from damage due to excess iron. It is possible that in a FurA-minus mutant, increased levels of iron within the cell would lead to an increased mutation rate. The possibility that FurA plays some role in the regulation of mycolactone can not be ruled out at this time.

IV. Optimization of the Electroporation Efficiency of *Mycobacterium ulcerans* and *Mycobacterium fortuitum*

Introduction

The development of the electroporation technique--in which electrocompetent bacterial cells are subjected to a brief, strong electrical impulse which permeabilizes their membranes, allowing them to take-up DNA--was a great advance in the field of genetic engineering. The electroporation protocol developed for use with mycobacteria was based on the traditional protocols which are generally highly effective when used on other genera of bacteria (61). These protocols require that cells are washed and remain at 4°C at all times to achieve optimal transformation efficiencies (30). Such technique has been found comparably effective for the transformation of *Mycobacterium smegmatis* and possibly other fast-growing mycobacteria (40, 61, 83). However, for other strains of mycobacteria, transformation efficiencies achieved with these techniques are significantly lower. With *M. ulcerans*, these techniques are not effective at all. There are no published reports of successful electroporation into *M. ulcerans*; nevertheless, electroporation is considered the best method of transformation for any mycobacteria (40). An effective electroporation protocol is greatly needed for *M. ulcerans.* Without this tool, prolific complementation studies and studies implementing transposition mutagenesis are impossible.

In 1996, Wards and Collins published a study demonstrating that *M. bovis* cells prepared for electroporation at room temperature have a significantly higher

transformation efficiency than those washed at the traditional 4℃ (83). In 2000, Talaat and Trucksis, found that the same is true for *M. marinum* (74). In addition, Wards and Collins also demonstrated that for *M. bovis*, *M. tuberculosis*, and *M. intracellulare* the highest electroporation efficiencies are achieved when the cells are electroporated at 37℃; while, for *M. smegmatis*, the highest electroporation efficiencies are achieved when the cells are electroporated at the traditional 4℃ (83).

The following experiments were performed to determine the effect of the temperature of the cell at the time of electroporation on transformation efficiency in *M. ulcerans*. The findings of these studies were then tested on the faster growing *M. fortuitum*, another atypical mycobacteria for which high transformation efficiency is not readily achieved. Studies are also described here which are aimed at determining the effect of mycolactone on the electroporation efficiency of *M. ulcerans*.

Results

There have been no published accounts of successful transformation into *M. ulcerans*. Transformation of *M. ulcerans* at the time this study was begun had been achieved, though with minimal success, only by washing cultures of *M. ulcerans* grown in Middlebrook 7H9 with 10% oleic acid-albumin-dextrose-complex (M7H9 + OADC) with ice-cold 10% glycerol and electroporating the resulting cells at 4℃ with parameters set at 2.5 kV, 800 Ω, and 25 μF, parameters similar to those used with other mycobacteria. Because electroporation at higher resistances has been shown to result in higher efficiencies in other species, the number of transformants obtained by electroporating three identical aliquots of 10% ice-cold glycerol washed *M. ulcerans* at

each of 800 Ω, 900 Ω, and 1000 Ω resistance was compared. For each pulse, capacitance and voltage were held constant at 25 μF and 2.5 kV, respectively. All steps were carried out at 4℃. Immediately after electroporation, cells were transferred to 2 mL of M7H9 + OADC and placed in a rollerdrum at 32℃. After 72 hours, the cells were plated on selective media. None of the nine aliquots resulted in a single transformant. As a result, it can not be determined with certainty at which resistance the electroporation efficiency of *M. ulcerans* would be highest. However, it is likely that the highest electroporation efficiency would occur with electroporations at 800 Ω resistance. Arcing seemed to increase with an increase in resistance. All samples pulsed at 1000 Ω arced; while, only one arced when pulsed at 900 Ω. None of the samples arced when pulsed at 800 Ω. Arcing is known to greatly decrease electroporation efficiency in other species of mycobacteria, because it results in massive cell death. This seems to be true for *M. ulcerans* as well. *M. ulcerans* cells plated on non-selective media after an arcing pulse took six weeks to grow up (compared to four weeks for cells subjected to a non-arcing pulse) and failed to form a lawn on the plate as was seen on the non-selective media plates of a non-arcing pulse. These observations of greatly decreased survival of *M. ulcerans* subjected to arcing compared to *M. ulcerans* subjected to a non-arcing pulses suggests that the resistance least likely to result in arcing, 800 Ω, should be used. All subsequent electroporation experiments with *M. ulcerans* were carried out with pulses of the parameters 2.5 kV, 800 Ω, and 25 μF. It was determined that it was imperative to examine factors other than resistance which may improve electroporation efficiency in *M. ulcerans.*

The Effect of Temperature on Electroporation Efficiency

M. ulcerans cells had previously been washed at 4℃ as prescribed by protocols for the preparation of electrocompetent cells of nearly all other bacteria; however, two studies had been published demonstrating that electroporation efficiencies of *M. bovis* and *M. marinum* increase when the cultures are washed at room temperature (74, 83). In a preliminary electroporation study to determine if an increase in temperature of the *M. ulcerans* cells at the time of electroporation would result in a higher transformation efficiency, a method for preparing electrocompetent *M. ulcerans* at room temperature was tested. The cells of a 400 mL culture of *M. ulcerans* were harvested and washed at room temperature with successively smaller volumes of room temperature 10% glycerol. The final pellet was resuspended in 2 mL of 10% glycerol and the cells were apportioned into 60 µL aliquots. Three aliquots were held at each of three temperatures, 1) 4℃, 2) room temperature, and 3) 37℃, for 10 minutes prior to their electroporation with 1 µL (of a stock at a concentration of .3 µg/µL) of the hygromycin B resistance conveying plasmid pPR27HI. No arcing occurred with incubation at any temperature with these room temperature washed cells. Immediately after electroporation the cells were transferred to 2 mL pre-warmed M7H9 + OADC and incubated in a rollerdrum at 32℃ for 72 hours. After this time, the cells were plated on selective media. Though transformants appeared by 6 weeks of incubation, colony counts were made at 8 weeks of incubation (Table 1). No transformants resulted from cells electroporated after incubation at either 4℃ or room temperature; however, both trials after incubation at 37℃ produced transformants.

Table 1. The number of transformants resulting from the electroporation of *M. ulcerans* grown in M7H9 + OADC after incubation at 4°C, room temperature, and 37°C after preparation at room temperature.

		Temperature		
		4°C	Room Temperature	37°C
Total Number of Transformants	No DNA Control	0	0	0
	DNA Trial 1	0	0	76
	DNA Trial 2	0	0	58

In a subsequent experiment to further investigate the effect of temperature on electroporation efficiency, the cells of a 250 mL culture of *M. ulcerans* 1615 grown in M7H9 + OADC were harvested, washed at room temperature with 10% glycerol, and aliquoted as described previously. Five aliquots of approximately 5×10^5 cells each (one control and four experimental) were held at room temperature (25℃), 37℃, 40℃, and 45˚C for ten minutes prior to electroporation with 1 μg (concentration of 1 μg/μL) of the hygromycin B conveying plasmid pPR27HI. After electroporation, the cells were incubated at 32℃ in a rollerdrum for 72 hours before being plated on selective media as described previously. Controls plated on non-selective media grew up within four weeks; while, many transformants appeared by six weeks. After eight weeks, the transformants were counted (Figure 18). Cells electroporated after incubation at 25˚C had any average of 6 transformants/μg of DNA; while, those electroporated after incubation at 37℃ had an average of 23 transformants/μg of DNA—a small but significant increase in electroporation efficiency. Cells electroporated after incubation at 40℃ had an average transformation efficiency of approximately 45 transformants/μg of DNA; while, those electroporated after incubation at 45℃ had an average transformation efficiency of approximately 39 transformants/μg of DNA. However, due to great variability in the number of resulting transformants among aliquots electroporated after incubation at 40℃ and 45℃, there was no significant increase in transformation efficiency after incubation at either of these temperatures when compared to those electroporated after incubation at 37℃. However, with cells electroporated after incubation at 55℃, which had an average transformation efficiency of 159 transformants/μg of DNA, there was a significant increase in transformation efficiency. There were no spontaneous hygromycin resistant

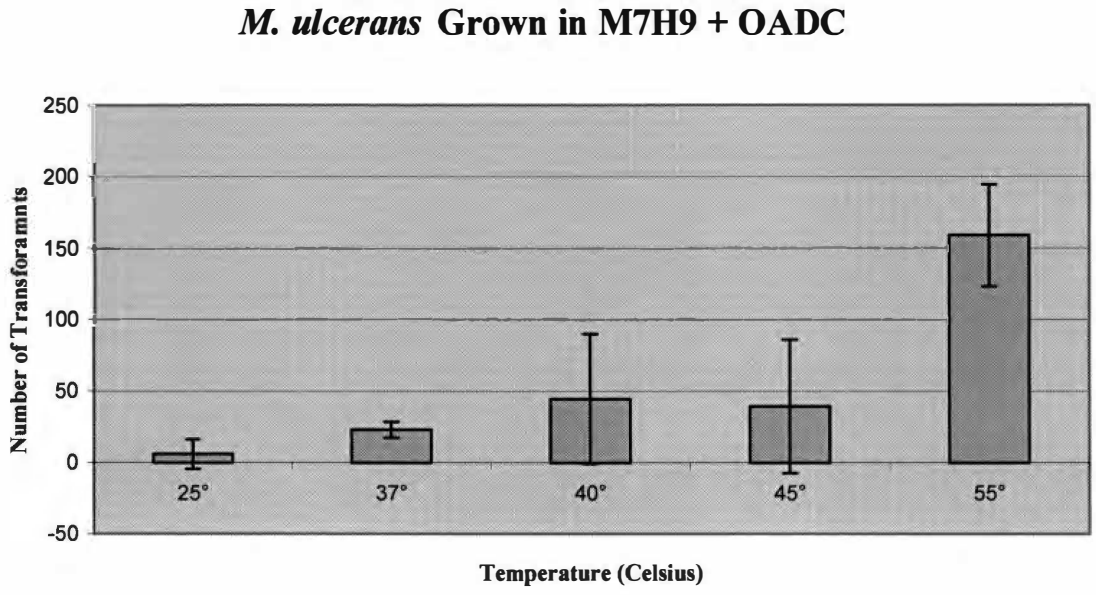

Figure 18. Average number of transformants resulting from electroporations of *M. ulcerans* grown in M7H9 + OADC at each temperature.
Average was computed from sample size n = 3. Error bars represent ± 1 standard deviation.

mutants arising from any of the cells electroporated after incubation at each temperature without pPR27HI; however, the transformants were confirmed via PCR amplification of the *hph* hygromycin resistance gene contained on the plasmid.

To determine if the trend of increased electroporation efficiency after incubation at higher temperatures could be applied to improve the electroporation efficiencies of other atypical mycobacteria, this experiment was repeated using *M. fortuitum* grown in M7H9 + OADC. *M. fortuitum* is another atypical mycobacterium for which there is no published protocol for transformation. *M. fortuitum*, like *M. ulcerans*, has a lipid-rich cell wall; however, unlike *M. ulcerans*, it is considered a fast grower, reaching log phase in three to four days. This is a particularly attractive characteristic to those who would like to express mycolactone (and perhaps other products of slow growing mycobacteria) in *M. fortuitum* to decrease the time required for its production; however, *M. fortuitum* can not be useful for such purposes until it can be efficiently transformed. *M. fortuitum* cells were harvested, washed, and aliquoted as described for *M. ulcerans* (approximately 5×10^5 cells). The cells were electroporated with 1 µL of DNA (at a concentration of 1 µg/µL) after incubation for 10 minutes at room temperature (25℃), 37℃, 40℃, and 45˚C at 2.5 kV, 25 µF, and 800 Ω. After electroporation, cells were transferred to 2 mL M7H9 + OADC and shaken for 5 hours at 37℃. Because *M. fortuitum* was found to be resistant to hygromycin B, the aparamycin resistance conveying plasmid pYUB185 had to be used rather than pPR27HI. Therefore, transformants were selected on M7H9 + OADC with 30 µg/mL aparamycin and appeared after 5 days of incubation at 37℃ (Figure 19). The average number of transformants for cells electroporated after incubation at 4℃ was

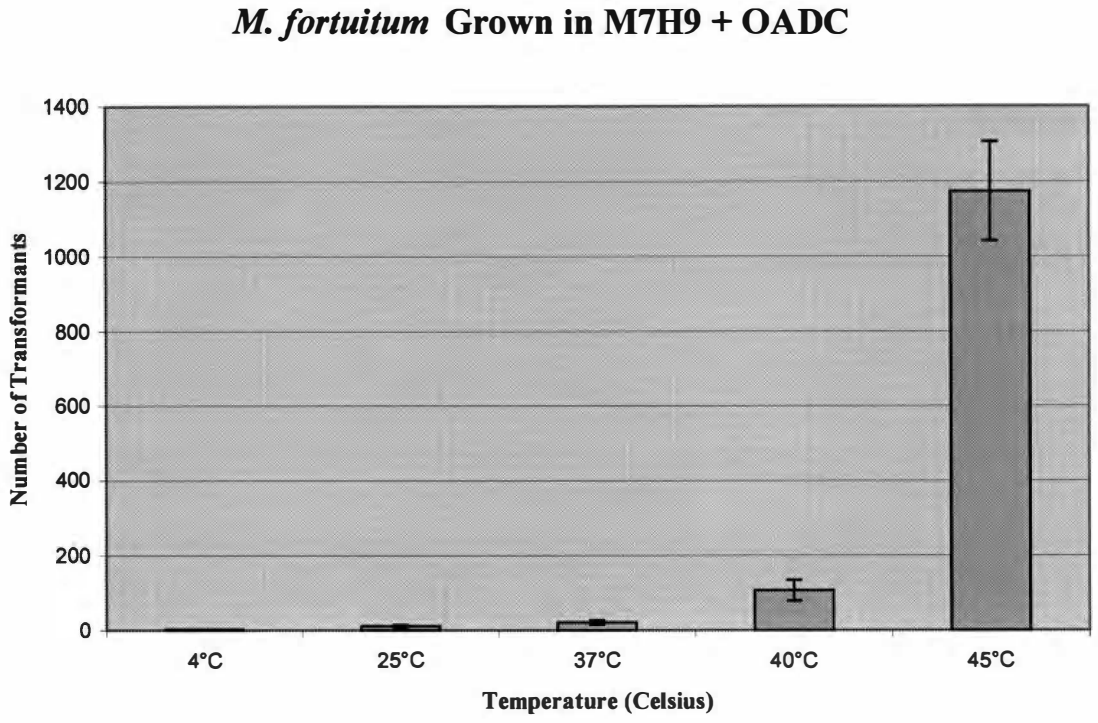

Figure 19. Average number of transformants resulting from electroporations of *M. fortuitum* grown in M7H9 + OADC at each temperature.
Average was computed from sample size n = 3. Error bars represent ± 1 standard deviation.

2 transformants/μg of DNA; while, that for cells electroporated at 25℃ was approximately 11 transformants/μg of DNA. A further increase in electroporation efficiency was seen with cells electroporated at 37℃, which had an average number of transformants of approximately 21 transformants/μg of DNA. The electroportation efficiency of cells electroporated after incubation at 45℃, approximately 1,173 transformants/μg of DNA, was greater than ten-fold higher than that of cells electroporated after incubation at 40℃, 106 transformants/μg of DNA.

The Effect of Mycolactone on Electroporation Efficiency

Though the increase in electroporation efficiency of *M. ulcerans* achieved by increasing the temperature of the cells at the time of electroporation are a marked improvement, efficiencies of 10^2 transformants/μg of DNA pale in comparison to the 10^6 and higher transformants/μg of DNA transformation efficiencies of other bacteria. Other methods of improving the electroporation efficiency of *M. ulcerans* need to be explored. It is thought that a great hindrance to transformation of *M. ulcerans* is its thick, polyketide-enriched cell wall. Because mycolactone is thought to be a major constituent of this cell wall, experiments were undertaken to determine the effect of mycolactone on electroporation efficiency. Mycolactone has been found to constitute approximately 10-20% of the total ASLs produced by a given *M. ulcerans* culture. Previous studies in the lab suggested that *M. ulcerans* grown in either Sauton's synthetic medium or Reid's synthetic medium produces significantly less ASLs (and therefore less mycolactone) than *M. ulcerans* grown in M7H9 + OADC;while, *M. ulcerans* grown in M7H9 with 20% OADC supplement (M7H9 + 2[OADC]) produces significantly more ASLs (and therefore mycolactone). To determine the effect of mycolactone on electroporation

efficiency, 500 mL cultures of *M. ulcerans* grown in Sauton's synthetic media, Reid's synthetic media, M7H9 + OADC, M7H9 + 2[OADC], and of a mycolactone-negative *M. ulcerans* PKS knock-out mutant 115 grown in M7H9 + OADC were divided into two 250 mL portions: one portion to be subjected to the electroporation experiment described above aimed at determining the effect of temperature on the electroporation efficiency of *M. ulcerans* and acetone soluble lipids (ASLs) to be extracted from the other portion. For the electroporation experiment, cells were harvested, washed, and aliquoted as described above. Five aliquots (one control and four experimental) of approximately 5×10^5 cells each were held at room temperature (25℃), 37℃, 40℃, and 45℃ for 10 minutes prior to electroporation with 1 μL (concentration 1 μg/μL) of the hygromycin B resistance conveying plasmid pPR27HI with a single pulse of constant parameters (2.5 kV, 25 μF, and 800 Ω). (*M. ulcerans* grown in M7H9 + OADC was also electroporated after incubation at 55℃.) Because a previous study (data not shown) demonstrated that cells from *M. ulcerans* cultures grown in Sauton's or Reid's synthetic medium were more likely to recover if transferred to M7H9 + OADC (than the medium in which they were grown) and then plated on M7H10 + OADC, cells were transferred to 2 mL M7H9 + OADC immediately after electroporation. After 72 hours of incubation in a rollerdrum at 32℃, the cells were plated on selective media.

In order to isolate ASLs, total lipids were extracted with chloroform:methanol (2:1, vol/vol). After drying off the chloroform:methanol under nitrogen, ice-cold acetone was added to the desiccated lipids. ASLs were resuspended in the acetone and transferred to another vial along with the acetone. The total ASLs extracted were massed

(Table 2), and approximately 60 μg of each was analyzed via thin-layer chromatography (TLC) (Figure 20).

Though all non-selective plates contained growth within eight weeks, no transformants resulted from the electroporations of cells grown in either Sauton's or Reid's synthetic medium. The number of transformants obtained for *M. ulcerans* grown in M7H9 + OADC is shown in Figure 21 and was discussed previously; while, the number of transformants obtained for *M. ulcerans* grown in M7H9 + 2[OADC] is shown in Figure 21. While *M. ulcerans* cultures grown in Reid's and Sauton's synthetic media do produce less mycolactone, they do not produce transformants when electroporated after incubation at any temperature. These media are not appropriate for growing cultures for transformation. The same was true for the mycolactone-negative PKS mutant 115 of *M. ulcerans*. While *M. ulcerans* grown in M7H9 + 2[OADC] does seem to produce more mycolactone than *M. ulcerans* grown in M7H9 + [OADC], the number of transformants resulting from cells grown in M7H9 + 2[OADC] electroporated after incubation at 25℃, 37℃, and 40℃ is not significantly different from the number of transformants resulting from cells grown in M7H9 + [OADC] at those same temperatures, respectively. However, the average transformation efficiency of cells grown in M7H9 + 2[OADC] and electroporated after incubation at 45℃ (approximately 119 transformants/μg of DNA) is nearly three-fold that of cells grown in M7H9 + OADC (approximately 39 transformants/μg of DNA).

Table 2. The mass of total ASLs isolated from 250 mL of each culture.

Culture	Mass of Total ASLs Isolated From 250 mL of Culture (mg)
M. ulcerans mutant #115, a PKS knock-out mutant	.7847
M. ulcerans Grown in Reid's Synthetic Medium	2.7456
M. ulcerans Grown in Sauton's Synthetic Medium	2.5867
M. ulcerans Grown in M7H9 + OADC	3.5133
M. ulcerans Grown in M7H9 + 2[OADC]	5.3447

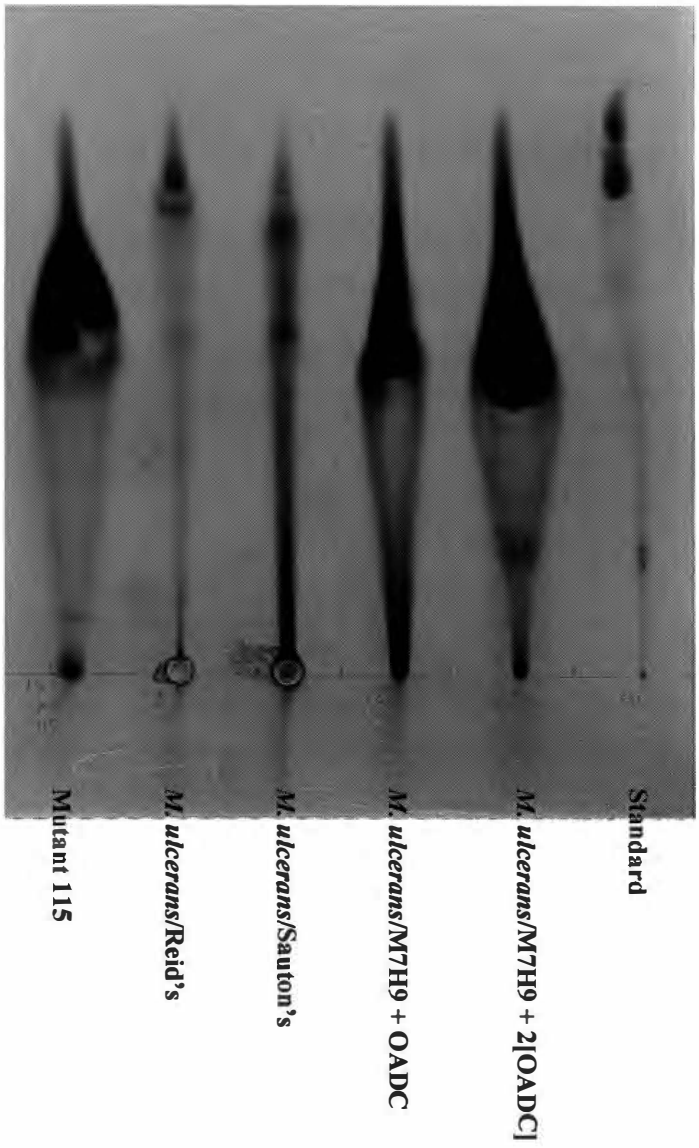

Figure 20. TLC analysis of ASLs isolated from a mycolactone-negative *M. ulcerans* PKS knock-out mutant 115 grown in M7H9 + OADC, *M. ulcerans* grown in Reid's synthetic media, Sauton's synthetic media, M7H9 + OADC, and M7H9 + 2[OADC]. ASLs were resuspended in acetone, and approximately 60 µg of each was loaded. The standard is 30 µg of previously isolated ASLs. The solvent system used was 90:10:1 chloroform:methanol:water (vol:vol:vol).

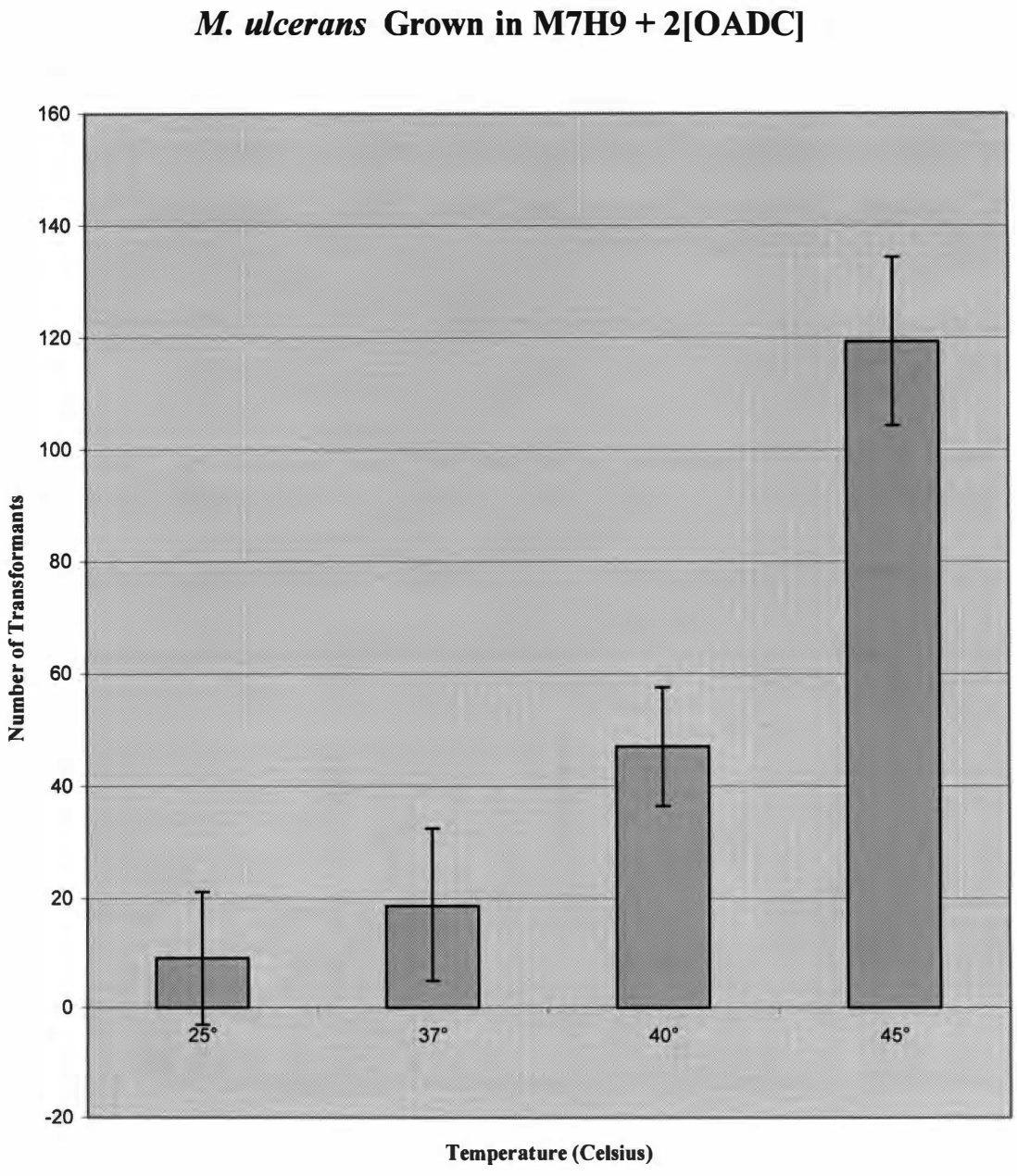

Figure 21. Average number of transformants resulting from electroporations of *M. ulcerans* grown in M7H9 + 2[OADC] at each temperature.
Average was computed from sample size n = 3. Error bars represent ± 1 standard deviation.

Discussion

The attempts to construct a mycolactone-negative mutant of *M. ulcerans* via transpon mutagenesis discussed in the previous chapter were greatly hindered by the poor transformation efficiency of *M. ulcerans*. For transposon mutagenesis to be a valuable tool with regard to this organism, it was necessary to determine the parameters under which transformation could consistently be carried out. In addition, it was certain that a reliable method for transforming *M. ulcerans* would be highly useful, if not necessary, in future genetic analysis of this organism. While electroporation is considered the best method of transformation for any mycobacteria, including *M. ulcerans*; there are no published reports of successful electroporation into it (40). At the time these studies were begun, the electroporation efficiency of this organism was effectively 0 transformants/μg of DNA. A round of electroporation utilizing all aliquots of *M. ulcerans* obtained from the washing of a 500 mL culture would yield a total of less than ten transformants but often none at all.

Initially, it was necessary to determine what parameters of the pulse used in electroporation would yield the highest transformation efficiencies. Previously, several transformants of *M. ulcerans* had been obtained at Rocky Mountain Laboratories through electroporation at 2.5 kV, 25 μF, and 800 Ω, parameters used for the electroporation of *M. tuberculosis* and other related species. Because it is known that for other species higher resistances generally yield higher electroporation efficiencies as long as they do not result in arcing, the task of determining if a higher resistance used on *M. ulcerans* would yield a higher electroporation efficiency was undertaken. Because even small amounts of impurities in DNA stocks can lead to arcing due to increased conductivity, it

is important to use the smallest volume of DNA possible during each pulse, usually 1 μL. Three sets of three identical aliquots each of 10% glycerol washed *M. ulcerans* were electroporated with 1 μL of EZ::TN™ <KAN-2>Tnp Transposome™ at constant capacitance and voltage, but a resistance of 800 Ω, 900 Ω, or 1000 Ω. This experiment yielded no transformants; therefore, it could not be determined with certainty which resistance would be best to use in future experiments. Every aliquot pulsed at 1000 Ω arced; while, one aliquot pulsed at 900 Ω arced. The sparse and greatly delayed growth on the non-selective control plates of the aliquots subjected to arcing indicated that massive cell death occurs among *M. ulcerans* cells exposed to arcing during electroporation as it does for other species. As in other species, massive cell death is likely to greatly decrease electroporation efficiencies. It was decided to use 800 Ω resistance in all subsequent experiments; because, there was no arcing when pulsing the aliquots at this resistance, and non-selective control plates developed lawns within four weeks, demonstrating that the cells could recover when pulsed at this resistance.

In the preliminary experiment to determine if the temperature of the *M. ulcerans* cell at the time of electroporation might influence electroporation efficiency, aliquots of electrocompetent *M. ulcerans* were held at three temperatures, 1) 4℃, 2) room temperature, and 3) 37℃, for 10 minutes prior to electroporation with .3 μg of the hygromycin B resistance conveying plasmid pPR27HI. No transformants resulted from aliquots electroporated after incubation at either 4℃ or room temperature. Though for statistical purposes more trials were needed, the results of this experiment suggested that an increase in temperature results in an increase in electroporation efficiency. The electrocompetent cells used in this experiment were prepared at room temperature.

Because no arcing occurred during this experiment, it appeared that washing cells at room temperature is at least as effective at producing electrocompetent cells as washing on ice at 4℃. Washing cells at room temperature is a more convenient method, because it can be done without ice. It should also be noted that *M. ulcerans* cells are less likely to clump together at a warmer temperature, making them easier to manipulate at room temperature than at 4℃. Salts, which cause arcing, are trapped in clumps; therefore, the salts within non-clumped cells can be removed more efficiently than those within clumped cells. Electrocompetent *M. ulcerans* cells were prepared at room temperature for all subsequent experiments and were never again electroporated at 4℃.

In the following experiment, a higher range of temperatures was used and more aliquots were electroporated at each temperature. The data obtained from this experiment suggests that to obtain the optimal electroporation efficiency, *M. ulcerans* should be electroporated after incubation at 55℃. Cells electroporated after incubation at 37℃ showed a slight but significant increase in electroporation efficiency when compared to those electroporated after incubation at 25℃. While cells electroporated after incubation at 40˚C and 45˚C both had higher average number of transformants/μg of DNA than those electroporated after incubation at 37℃, this increase was not statistically significant due to the great variability among the number of transformants which arose from each aliquot. The average number of transformants which arose from electroporations after incubation at 55℃ was five-fold higher than that achieved after incubation at 37℃. Further tests might be valuable to determine if even higher temperatures may increase electroporation efficiency more; however, in a separate study (data not shown), *M. ulcerans* cells held at 65℃ for ten minutes prior to electroporation were completely killed. (I.e., They failed to

grow-up at all, even on non-selective M7H9 + OADC.) Therefore, it is likely that a temperature which after incubation at induces a higher electroporation efficiency, if not 55℃, is between 55℃ and 65℃.

Electroporation efficiencies of over 100 transformants/μg of DNA achieved when cells are electroporated after incubation at 55℃ represent an enormous improvement over the very poor and unreliable electroporation efficiencies (always less than 10 transformants/μg of DNA and often none at all) achieved with the protocols used at the time this study was begun. It is likely that increasing temperature of the *M. ulcerans* cell increases the fluidity of lipids in the cell wall. Though usually impervious to the effects of electroporation, the *M. ulcerans* cell wall of increased fluidity would be more likely upon electroporation to form the pores necessary for transformation. This phenomenon of increased electroporation efficiency with increased temperature of the cell was tested for another atypical mycobacteria with a lipid-rich cell wall—*M. fortuitum.* A similar trend was seen for *M. fortuitum*, also a bacterium for which a reliable electroporation protocol has not been developed. For this bacterium, the electroporation efficiency after incubation at 45℃, approximately 1,173 transformants/μg of DNA, was greater than ten-fold higher than that after incubation at 40℃, 106 transformants/μg of DNA.

It is thought that a great hindrance to transformation of *M. ulcerans* is its thick, polyketide-enriched cell wall. In order to determine if electroporation efficiencies could be increased by decreasing the amount of lipids and particularly the amount of mycolactone produced by the organisms, a series of experiments were performed to compare the mass of ASLs produced by and the electroporation efficiencies of the following cultures: 1) *M. ulcerans* grown in M7H9 + OADC, 2) *M. ulcerans* grown in

M7H9 + 2[OADC], 3) *M. ulcerans* grown in Reid's synthetic media, 4) *M. ulcerans* grown in Sauton's synthetic media, and 5) a mycolactone-negative PKS mutant 115 of *M. ulcerans*. Equal portions of each culture were used to 1) repeat the electroporation experiment previously described and 2) extract ASLs. As expected, the cultures grown in Reid's and Sauton's synthetic media produced a significantly smaller mass of ASLs than did the cultures grown in M7H9 + OADC; while, the culture of mutant 115 produced an even smaller mass of ASLs. Also as expected, *M. ulcerans* grown in M7H9 + 2[OADC] produced significantly more ASLs than did *M. ulcerans* grown in M7H9 + [OADC]. Despite the decrease in ASL production of cultures grown in Reid's and Sauton's synthetic media and the culture of mutant 115 grown in M7H9 + OADC, there was no increase in electroporation efficiency seen. In fact, no transformants resulted from these three electroporation studies. However, it can not be said with certainty that a decrease in mycolactone (and total ASL) production by some other means would not improve electroporation efficiencies. Sauton's synthetic and Reid's media both lack oleic acid, a major component of OADC. Including oleic acid in the growth medium of mycobacteria is known to increase the total percentage of lipids composing the cell (66). This is thought to account for the increase in ASLs seen for cells grown in M7H9 + OADC and the even greater increase in ASLs for cells grown in M7H9 + 2[OADC]. It can only be said with certainty that PKS mutant 115 and wild-type *M. ulcerans* grown in either Reid's or Sauton's synthetic media all have electroporation efficiencies far lower than electroporation efficiencies of wild-type *M. ulcerans* grown in M7H9 + OADC. It should be noted that mutant 115 appears to have a much slower growth rate than wild-type *M. ulcerans* 1615, requiring nearly twice as long to reach log phase. It is interesting to note

that while the M7H9 + OADC and M7H9 + 2[OADC] cultures have comparable electroporation efficiencies after incubation at 25℃, 37℃, and 40℃, the electroporation efficiency of the M7H9 + 2[OADC] culture after incubation at 45℃ is three times that of the electroporation efficiency of M7H9 + [OADC] culture. An additional experiment has been performed to determine if there is a significant difference between the electroporation efficiencies of the two cultures after incubation at higher temperatures (particularly after incubation at 55℃).

V. The Investigation of a Link between *Mycobacterium ulcerans* and Schistosome-Infected Snails

Introduction

The natural reservoir of *Mycobacterium ulcerans* is unknown. Though many attempts had been made; at the time this study was begun, *M. ulcerans* had never been isolated from the environment, not even from Buruli ulcer-endemic foci (19, 69). Despite this deficiency in evidence needed to construct a complete model of *M. ulcerans* infection, most researchers believe that *M. ulcerans* infections are in fact acquired from the natural environment, particularly through small injuries which puncture the skin allowing the bacteria access to the subcutaneous layer of fat (19, 53, 81). Such injuries may be so small that the patient may not remember having them by the time the infection is obvious.

There is significant data to suggest that infection with *M. ulcerans* is acquired through water. *M. ulcerans* has been detected via PCR from water samples taken in Buruli ulcer-endemic regions; while, no other environmental samples unassociated with water have been consistently PCR-positive for *M. ulcerans* (68, 71). However, the theory that *M. ulcerans* is acquired through contact with contaminated water is more supported by epidemiological data. One study reported on the high incidence of *M. ulcerans* infection among Rawandan refugees who had recently settled in the refugee settlement of Kanyara, Uganda located near marshy areas near the Nile river (47). Buruli ulcer was not prevalent in Rawanda; so, it was unlikely that the refugees brought the disease with

them. It was also unlikely that the refugees acquired Buruli ulcer from the other inhabitants of Kanyara, because there was little contact between the refugees and the other inhabitants. When the refugees were relocated to Kyangwali, a new area much further from the marshy land of the Nile, the incidence of Buruli ulcer greatly decreased within months, then virtually disappeared (2).

Similarly, in 1997 Ross, et al., reported on an outbreak of Buruli ulcer in a small town on Phillip Island of Australia that seemed to be centered around the irrigation system of a golf course (69). The irrigation system relied on water from a storage dam, which contained natural ground water and treated wastewater. The outbreak was preceded by the construction of a road which prevented nearby land from draining naturally, consequently creating a swamp. Before this outbreak, the town had had no previous cases of Buruli ulcer. It is believed that this manmade swamp may have provided a suitable environment for the growth of *M. ulcerans* and contaminated the irrigation system (69). Both the swamp and irrigation tank were PCR-positive for *M. ulcerans*; while, other environmental samples were not (69). The incidence of Buruli ulcer in this town greatly decreased after the source of water for the irrigation of the golf course was switched to an alternate source (76). These and other epidemiological studies have led to almost universal acceptance that infection with *M. ulcerans* is associated with rivers, streams, swamps, lakes, flooding, and possibly other natural and manmade bodies of water (7, 34, 58).

It is not feasible for people living in Buruli ulcer-endemic regions to prevent the disease by avoiding contact with bodies of water suspected of harboring *M. ulcerans*, because they may be dependent on them for their livelihood. If a practical plan for the

prevention of Buruli ulcer disease is to be created and implemented, more must be learned about its transmission in relation to water. This objective may be accomplished through the examination of correlations between Buruli ulcer disease and other factors related to water. One such factor was noticed in regions of West Africa where the incidence of Buruli ulcer has increased drastically, especially in areas adjacent to newly constructed river dams. In these same regions, there has also been a concurrent increase in schistosomiasis (87).

Schistosomiasis, or Bilharziasis, is an infection caused by digenetic trematodes of the genus *Schistosoma.* There are primarily three species which cause disease in humans: *S. haematobium*, the most common cause of schistosomiasis in Africa and the Middle East; *S. mansoni*, which is prevalent in Africa and Central and South America; and, *S. japonicum*, which is prevalent in the Far East (57). Two other less prevalent species of schistosomiasis are *S. intercalatum* and *S. mekongi*, which are limited to West Africa and South East Asia, respectively (10, 37, 75). Schistosomes become associated with water when their eggs are released into it through contamination with waste of an infected human (Figure 22). The eggs hatch in response to fresh water to release miracidia—the life stage of the schistosome infective to snails, the intermediate hosts of the schistosome. Only when the permissive species of snail is present can the miracidia of a specific species of schistosome establish an infection in the snail and continue its life cycle. At this stage in the life of a schistosome, asexual reproduction is carried out. A miracidium forms a primary sporocyst in the snail from which forms a secondary sporocyst. Each secondary sporocyst gives rise to thousands of cercariae--the larval stage infective to humans.

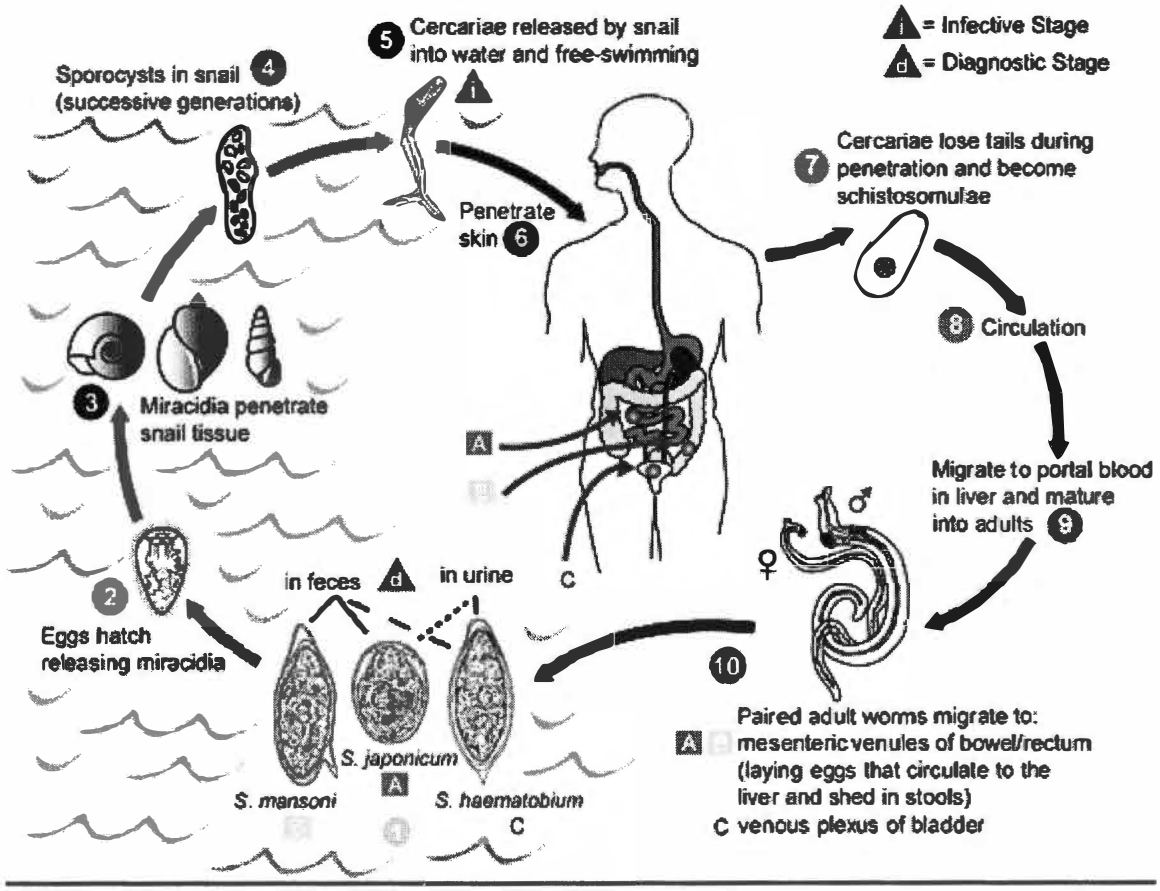

Figure 22. Life cycle of the human schistosome.
Schistosome eggs are deposited in water when the water is contaminated with the waste of an infected host (1). These eggs hatch, releasing miracidiae (2). Miracidiae infect snails (3). Each miracidia gives rise to a primary sporocyst, which gives rise to a secondary sporocyst (4). Cercariae are released from daughter sporocysts (5) and infect humans by penetrating the skin (6). After penetration, the cercariae become schistosomulae (7) and enter the circulation of the host (8). Schistosomulae mature into adult schistosomes in the liver of the host (9). They then migrate as sexually reproducing pairs to veins lining the intestines or bladder, depending on the species (10). (Adapted from CDC DPDx, 2004.)

Cercariae infect their definitive hosts by burrowing into the skin. Upon penetration, the cercariae loose their tails to become schistosomulae which enter the host circulatory system. The schistosomulae develop into adult worms up to 20 mm long in approximately one month. A male and female worm pair together and reside in the vessels lining the intestine or bladder, depending on the species of schistosome. At this stage, the schistosomes reproduce sexually. Each pair can excrete from several hundred to one thousand eggs everyday for an average of five years. Many of these eggs get excreted in the urine or feces of the host to complete the cycle of infection. Many eggs are not excreted and remain in the host causing an inflammatory response which over time damages vital tissues of the host.

It is this very inflammatory response that some researchers have blamed for the concurrent increases in Buruli ulcer disease and schistosomiasis. It is known that infection with schistosomiasis drives the host immune system toward a Th2 immune response while dampening the Th1 response (77). It has been demonstrated that the development of Buruli ulcer disease is likely to be fostered by anergy of Th1 lymphocytes (26, 27). A change from a Th1 immune response to a Th2 response following the development of Buruli ulcer disease has also been documented in a patient (28). For these reasons, it has been postulated that being infected by schistosomes predisposes patients to the development of Buruli ulcer disease (70, 87).

The following experiments were undertaken in an effort to test a different but related hypothesis. We wanted to determine if snails, the intermediate hosts of schistosomes, could harbor and transmit *M. ulcerans*. Because no specific element of the ecological niche of this bacterium had been identified at the time of these studies, it was

thought that even negative data on this subject would be valuable. In addition, we wanted to determine if there was an association between cercariae and *M. ulcerans*. Because the cercariae that infect humans must burrow into the skin, they may simply be responsible for the penetrating injury thought to be required for *M. ulcerans* to cause an infection. It is plausible that *M. ulcerans* is actually associated with the cercariae as is the bacterium, *Neorickettsia (Ehrlichia) risticii*—the causative agent of Potomac horse fever (62).

Results

Infection of Snails with *M. ulcerans*

In order to determine whether snails could be infected with *M. ulcerans*, six snails of the species *Biomphalaria glabrata* were placed in 25 mL of sterile, conditioned water to which 2.5 mL of *M. ulcerans* 1615 culture was added. As a control, six snails of the same species were placed in 25 mL of sterile, conditioned water to which 2.5 mL of M7H9 + OADC was added. After 24 hours, all snails in both the infection and control groups had died. Upon examination with the naked eye and low power dissection scope, the six snails in the infection group (I1-I6) had no apparent tissue damage that might be attributed to contact with mycolactone (or any compound within the medium). Likewise, the six snails of the control group (U1-U6) had no obvious change in appearance. In preparation for the examination of the snails for an association with *M. ulcerans*, all snails were rinsed repeatedly with sterile water and treated with amikacin for 36 hours, which is effective at killing extracellular *M. ulcerans*. Each snail was ground in a sterile tissue grinder, and the homogenate was divided into two portions: one for total

genomic DNA isolation and the other for treatment with a decontamination protocol aimed at eliminating all bacteria other than mycobacteria. The total genomic isolations produced a very small amount of DNA which was too dilute to be visualized via electrophoresis. However, this DNA proved to be suitable for PCR. Total genomic DNA isolations from all six infected snails (I1-I6) were PCR-positive for the IS2404 insertion element specific to *M. ulcerans*; while, the total genomic DNA isolations from all six uninfected snails (U1-U6) were PCR-negative for IS2404 (Figure 23). The plating of decontaminated snail tissue resulted in a multititude of bacterial colonies diverse in morphology (Table 3). Plates of snails 3 and 4 of the infectious group (I3 and I4) and snails 1 and 2 of the control group (U1 and U2) resulted in many colonies after two weeks that contained acid-fast bacteria. Though *M. ulcerans* will not grow up on a plate in two weeks, these acid-fast colonies were analyzed via PCR for the presence of IS2404. None were found to be PCR-positive for this insertion element; therefore, the possibility of them being *M. ulcerans* was eliminated.

Infection of Schistosome-Infected Snails with *M. ulcerans* through 1) Environmental Inoculation and 2) Foodborne Inoculation

In order to determine if schistosome-infected snails could be infected with *M. ulcerans*, two related infection studies were performed: 1) environmental infection and 2) foodborne infection studies. For both studies, two groups of ten snails of the species *B. glabrata* infected with *S. mansoni* were used. To allow for the schistosome infection to develop, these snails were maintained for four weeks before infection. The inoculum used for each of the two infections was washed with sterile water to remove any compounds within the medium which would result in immediate death of the snails.

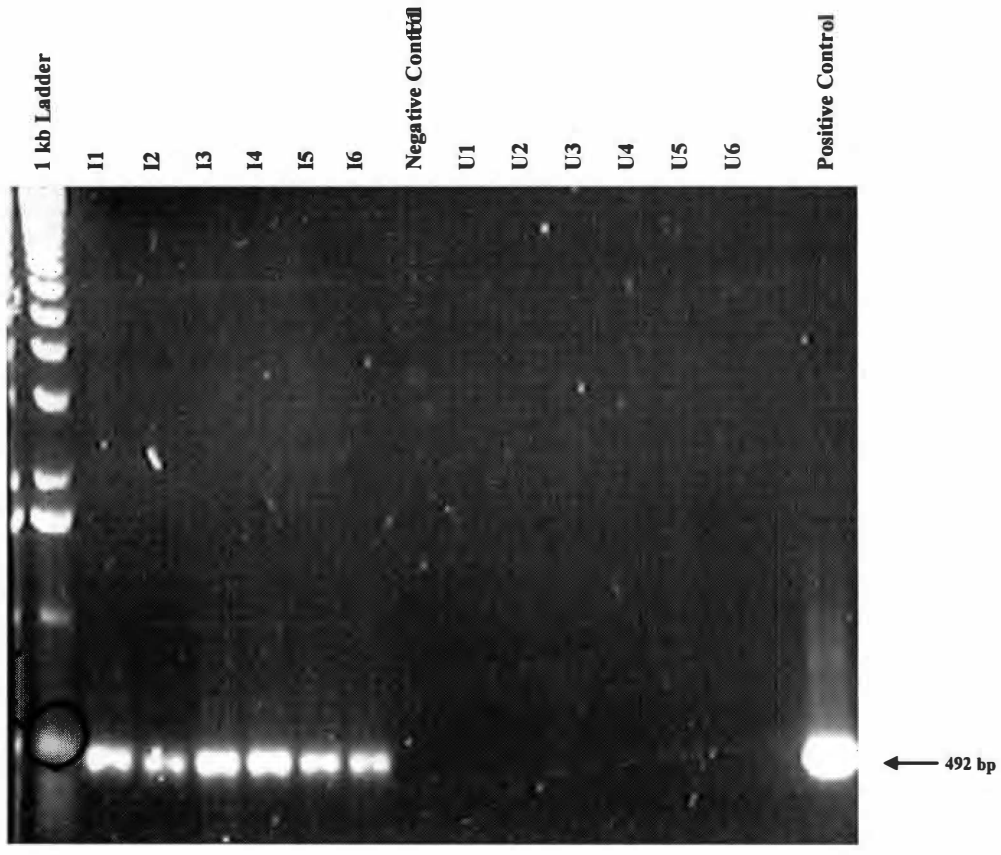

Figure 23. Detection of IS2404 via PCR in total genomic DNA isolations of *M. ulcerans*-infected snails (I1-I6) and uninfected snails (U1-U6).
All total genomic DNA isolations of infected snails are PCR-positive for IS2404, indicative of *M. ulcerans*; while, all total genomic DNA isolations of uninfected snails are PCR-negative for this element.

Table 3. Characterization of growth resulting from the plating of decontaminated tissue from the preliminary infection of snails of the species *Biomphalaria glabrata.*

Snail Plated		Description of Growth and Time of First Appearance	Acid-Fast
Infected Group	I1	None	N/A
	I2	One large, cream-colored colony appeared after two weeks.	-
	I3	One large, cream-colored colony appeared after two weeks.	+
	I4	One large, cream-colored colony appeared after two weeks.	+
	I5	Plate was overgrown with a lawn of 1) cream and 2) rose-colored microorganisms that extend into the agar after one week.	-
	I6	Two small areas with rose-colored growth similar to that of I5 were observed after one week. Four smooth, white colonies were observed after four weeks. These colonies were not morphologically similar to *M. ulcerans* and appeared to be yeast.	-/-
Control Group	U1	Three large, shiny, white colonies appeared after one week.	+
	U2	Twenty large, shiny, white colonies which were morphologically similar to those of U1 appeared after one week.	+
	U3	One smooth, shiny, yellow colony appeared after four weeks.	-
	U4	One large, shiny, white colony and a lawn of rose-colored growth appeared after one week.	-
	U5	One large, shiny, white colony appeared after one week.	-
	U6	None	N/A

For the environmental infection, the 10 snails of the infection group were placed in 250 mL conditioned water to which 2 mL of washed *M. ulcerans* culture was added; while, the 10 snails of the control group were held in 252 mL conditioned water. For the foodborne infection, the 10 snails of the infection group were held in 250 mL conditioned water where they were fed 1 g of food supplement which served as a matrix for the bacteria from 2 mL of washed *M. ulcerans* culture; while, the 10 snails of the control group were held in 250 mL conditioned water where they were fed 1 g of sterile food supplement. After 24 hours, all four experimental groups were returned to their respective containers and maintained as usual for eight weeks. Both groups tolerated infection well, and there were few premature deaths. Snails were divided relatively equally into two groups: 1) those to be preserved for the preparation of histological slides and 2) those to be homogenized, decontaminated, and plated.

The prepared histological sections proved to be an unreliable source of data. It was very difficult to discern the anatomy of the snail when viewing the sections which had been acid-fast stained. No difference between either infection group or their respective controls could be substantiated. Many regions on every snail for both infection studies and their respective controls had the appearance of acid-fast bacilli.

The plating of snail tissue proved to be a more valuable source of data in these experiments. A moderate array of microorganisms survived the decontamination procedure and grew up on the plates from snails from both infection groups as well as their respective controls (Table 4 and Table 5). At least one representative colony from every descriptive group of microorganisms on each plate was acid-fast stained. As noted in Tables 4 and 5, many of these colonies were acid-fast. Colonies that were acid-fast

Table 4. Characterization of growth resulting from the plating of decontaminated tissue from the infection of schistosome-infected snails of the species *B. glabrata* with *M. ulcerans* through environmental inoculation.

Snail Plated		Description of Growth and Time of First Appearance	Acid-Fast
Environmentally Infected Group	EMu1	Many small, bright yellow colonies appeared after two weeks.	+
	EMu2	Many small yellow colonies morphologically identical to those of EMu1 (acid-fast); three large, shiny white colonies (non-acid- fast); and 2 bright orange colonies (non-acid-fast) all appeared after two weeks.	+/-/-
	EMu3	Many yellow colonies identical to those of Emu1 (acid-fast); several bright orange identical to those of EMu2 (non-acid-fast); and many small white colonies (acid-fast) appeared after two weeks.	+/-/+
	Emu4	Many yellow colonies identical to those of Emu1 (acid-fast); several bright orange identical to those of EMu2 (non-acid-fast); and several white colonies (non-acid-fast) all appeared after two weeks.	+/-/-
Environmental Control Group	EC2	Several bright yellow colonies identical to those of EMu1, etc. (acid-fast); several white colonies (non-acid-fast); and several bright orange colonies identical to those of Emu2, etc. (non-acid-fast) all appeared after 2 weeks.	+/-/-
	EC3	Several bright yellow colonies identical to those of EMu1, etc. (acid-fast); several white colonies (non-acid-fast); and several bright orange colonies identical to those of Emu2, etc. (non-acid-fast) all appeared after 2 weeks.	+/-/-
	EC4	Several bright yellow colonies identical to those of EMu1, etc. (acid-fast); several white colonies (non-acid-fast); and several bright orange colonies identical to those of Emu2, etc. (non-acid-fast) all appeared after 2 weeks.	+/-/-

Table 5. Characterization of growth resulting from the plating of decontaminated tissue from the infection of schistosome-infected snails of the species *B. glabrata* with *M. ulcerans* through foodborne infection.

Snail Plated		Description of Growth and Time of First Appearance	Acid-Fast
Food Infected Group	FMu1	White and reddish slimy overgrowth of plate after one week.	-
	FMu2	Many small, bright yellow colonies and several large, white, dry colonies appeared after two weeks.	+/+
	FMu3	Many white colonies identical to those of FMu2 (acid-fast) and a slimy lawn very similar to that of FMu1 (non-acid-fast) apparent after two weeks.	+/-
	FMu4	Many small, yellow colonies identical to those of FMu2, etc. (acid-fast) and eight large, shiny white colonies (non-acid-fast) apparent after two weeks.	+/-
	FMu5	Similarly to those of FMu2, etc., many small, bright yellow colonies and several large, white, dry colonies appeared after two weeks.	+/+
	FMu6	Many small, bright yellow colonies; several large, white dry colonies; and one bright orange colony (non-acid-fast) appeared after two weeks.	+/+/-
Food Control Group	FC1	Several yellow colonies like those of FMu2, etc. (acid-fast) and one large purple colony (non-acid-fast) appeared after two weeks.	+/-
	FC2	Several yellow colonies like those of FMu2, etc. (acid-fast) and several white colonies (non-acid-fast) appeared after two weeks.	+/-
	FC3	Similar to FMu6, many small, bright yellow colonies; several large, white dry colonies; and several bright orange colonies (non-acid-fast) appeared after two weeks.	+/+/-

were analyzed via PCR for the presence of the IS2404 insertion element used to identify *M. ulcerans*. None were PCR-positive for the presence of this insertion element. Therefore, no *M. ulcerans* was recovered from the schistosome-infected snail in either infection study.

Discussion

In the preliminary snail infection, all snails in both the infection and control groups died with in 24 hours. Though mycolactone is likely to be harmful to snails, this was not the sole cause of death; because, snails in the control group did not come in contact with mycolactone. However, all snails did come in contact with M7H9 + OADC medium. It was clear that for any future studies designed to determine the effect of *M. ulcerans* on snails, the *M. ulcerans* used must be washed clean of the media before coming in contact with the snails.

In an effort to obtain PCR data with regard to the ability of *M. ulcerans* to infect snails as a part of the preliminary infection study, it was found that PCR reactions were inhibited by the presence of even very small quantities of the ground snail tissue used as template even after the sample was boiled. Dr. Fred Lewis of BRI reported (personal communication) that those is his lab had been successful at isolating total DNA from snail specimens for the purpose of PCR using the Wizard® Genomic DNA Purification Kit. The DNA obtained from this kit was too dilute to be visualized by electrophoresis. However, it did prove to be suitable for PCR. Because snails had been submersed in a solution of amikacin for 36 hours, this is suggestive that the *M. ulcerans* may be somehow internalized. It was clear that further studies aimed at histological examination

of the snail after a period of time sufficient enough for *M. ulcerans* to establish an infection would be valuable in confirming this.

It was thought that several problems with the decontamination protocol used for plating snail tissue during this preliminary study may have resulted in the killing of *M. ulcerans* indicated by PCR to be present in the snails of the infection group (I1-I6). The sample volumes were too small to neutralize quickly, and the pH paper proved to be an inefficient and cumbersome way of determining when the solution was neutralized. It is likely that the pH remained too high for too long to allow the survival of many mycobacteria that may have been present. Despite the difficulties in decontamination, plates of snails 3 and 4 of the infectious group (I3 and I4) and snails 1 and 2 of the control group (U1 and U2) resulted in many colonies after two weeks that were acid-fast; however, none were confirmed to have the IS2404 insertion element indicative of *M. ulcerans*. Plates were incubated for three additional months to allow for slow growing mycobacteria to appear; however, no new colonies appeared after the first two weeks of incubation. These colonies which had appeared had similar morphology and color and probably represent a mycobacterium species (or other relatively slow growing acid-fast bacterium) that is part of the normal flora of the snail. The existence of such organisms as a part of the normal flora of *B. glabrata* has been reported (55).

Because snails in the natural environment in areas where Buruli ulcer is endemic are intermediate hosts of schistosomes and play an integral role in the transmission of schistosomiasis, it would be more valuable to examine schistosome infected snails. The true aim of these studies is to examine any interaction between schistosome infected snails and *M. ulcerans*. For this reason, in the 1) environmental infection and 2) food-

borne infection studies *S. mansoni* infected snails of the species *B. glabrata* were used. To allow for the schistosome infection to develop, these snails were maintained for four weeks before infection. Washing the inoculum of *M. ulcerans* with sterile water prevented the immediate snail death observed in the preliminary infection study. Because the isolation of genomic DNA significantly decreases the amount of snail homogenate available for decontamination and plating, it was determined that for these studies no total genomic isolations would be undertaken; and thus, no snails from this study would be evaluated via PCR. Snails were either 1) fixed and prepared in sections or 2) ground, decontaminated, and plated. Unfortunately, the prepared sections proved to be an unreliable source of data. It was very difficult to discern the anatomy of the snail after acid fast staining. Many regions on every stained snail section from both infection studies and their respective controls had the appearance of acid-fast bacilli. No difference between either infection group or their respective controls could be substantiated by evaluation of the histological slides made. The plating of snail tissue proved to be a more valuable source of data in these experiments. The use of the pH indicator solution (rather than using pH paper) allowed a more accurate and quicker neutralization; however, no *M. ulcerans* was recovered. It is not clear if this was due to a still too harsh decontamination protocol or to the overgrowth of other organisms on the plates.

During the time that this work was being done, it was determined in several other labs that snails could not be persistently infected with *M. ulcerans*. Further epidemiological studies have shown no connection between schistosomiasis and Buruli ulcer. As a result, these studies have been discontinued.

VI. Conclusion

Efforts to create a mycolactone-negative mutant of *Mycobacterium ulcerans* via transposon mutagenesis were undertaken to elucidate the genes involved in the production of this toxin. Though only five PCR-confirmed mutants were produced, one of these, mutant 1615:tnp6w, was confirmed to be deficient in mycolactone production via thin-layer chromatography of acetone soluble lipids, cytopathicity assay of culture filtrate on L929 murine fibroblasts, and mass spectroscopy of ASLs. This mutant was found to contain an insertion near the upstream end of a gene 97% identical to the *M. marinum furA* gene but within a region of DNA not homologous to any other known prokaryotic gene. It was thought that this insertion may be interrupting the transcription of the *furA* homologue, because an additional mycolactone-negative mutant of *M. ulcerans* (mutant 97) independently created was found to have an insertion with in the structural gene of the *furA* homologue. However, the production of mycolactone was not restored when the mutant 1615:tnp6w was transformed with a construct containing the *furA* homologue of *M. ulcerans*. PCR analysis for the presence of genes recently found to be essential for the production of mycolactone revealed a deletion of such an essential gene—one encoding a thioesterase (II). The absence of this gene is the most probable and immediate cause of the mycolactone-negative phenotype. To further investigate the role of FurA in the regulation of mycolactone production, further attempts to complement mutant 97 should be made.

In an effort to determine the effect of temperature on the transformation efficiency of *M. ulcerans* via electroporation, electroporation experiments were

performed on *M. ulcerans* 1615 grown in Middlebrook 7H9 with 10% oleic acid-albumin-dextrose-complex supplement (M7H9 + OADC). Log phase cultures were harvested, washed with 10% glycerol, and apportioned into 60μL aliquots. Aliquots were briefly held at room temperature, 37℃, 40℃, 45℃, or 55℃ before electroporation in the presence of 1μg of pPR27H1, a hygromycin B resistance conveying plasmid. After 6 weeks, colonies were counted to calculate transformation efficiencies. It was found that *M. ulcerans* cells incubated at 55℃ prior to electroporation had a significantly higher transformation efficiency than those incubated at any other temperature prior to electroporation.

To determine if the presence of mycolactone affects electroporation efficiency, a similar experiment was repeated with *M. ulcerans* grown in Modified Reid's synthetic medium and *M. ulcerans* grown in Sauton's synthetic medium, both media which are thought to promote a decreased production of mycolactone; *M. ulcerans* grown in M7H9 with 20% OADC supplement (M7H9 + 2[OADC]), which is believed to foster an increased production of mycolactone; and, a polyketide synthase (PKS) knock-out mutant of *M. ulcerans* (mutant 115), which does not produce mycolactone, grown in M7H9 + OADC . *M. ulcerans* grown in Reid's or Sauton's synthetic media produced no transformants. *M. ulcerans* grown in M7H9 + 2[OADC] had a significantly higher transformation efficiency when incubated at 45℃ prior to electroporation than that grown in M7H9 + OADC and incubated at 45℃ prior to electroporation. Experiments to determine if *M. ulcerans* grown in M7H9 + 2[OADC] and incubated at higher temperatures (55℃ and 65℃) prior toelectroporation will result in even higher electroporation efficiencies have been performed; however, the results are not available.

Finally, though the natural reservoir of *M. ulcerans* is unknown, Buruli ulcer disease seems to be associated with water. For this reason, experiments aimed at investigating aquatic snails, also associated with water in Buruli ulcer endemic regions, as a component of the ecological niche of *M. ulcerans* were undertaken. Snails of the species *Biomphalaria glabrata* are intermediate hosts of the schistosome species *Schistosoma mansoni*; and, it is feasible that *M. ulcerans* could associate with cercariae, the schistosomal life stage infectious to humans, infecting humans through the wound caused by the cercaria entering its human host. Through various experiments, both healthy *Biomphalaria glabrata* and *Schistosoma mansoni* infected *Biomphalaria glabrata* were exposed to *M. ulcerans* and examined for *M. ulcerans* infection via PCR and plating of ground tissue. Though PCR analysis did indicate that the *M. ulcerans* was taken up by the snail in some way, plating of the snail tissue as well as histological sectioning was inconclusive. Despite decontamination protocols, there were great numbers of other species as bacteria present on the plates which may have outcompeated the *M. ulcerans*, which did not grow on the plates. Slides of snails infected with *M. ulcerans* and of uninfected snails were indistinguishable when stained by either hematoxylin-and-eosin or acid-fast staining. To definitively determine if there is an association between *M. ulcerans* and schistosome-infected snails, experiments involving fluorescently labeled antibodies against *M. ulcerans* or *M. ulcerans* expressing green-fluorescent protein would be useful.

Mycobacterium ulcerans is an organism of relatively new and fervent interests. With Buruli ulcer having been recognized by the World Health Organism as an emerging threat, many groups have been working to answer many of the questions addressed in the

experiments described in this work. Significant advancements both in the identification of the primary genes involved in the production of mycolactone and in the identification of the ecological niche of *M. ulcerans* have been published in the recent past.

Most recently, Stinear, et al., reported that at least many, if not all, of the essential genes required for the production of mycolactone are located on a 174 kb plasmid that has been named pMUM001 (73). They describe two large polyketide synthases (PKS), MLSA1 and MLSA2, which are responsible for the production of the 12-carbon core lactone; while, a third PKS, MLSB, is reportedly responsible for the side chain of mycolactone (73). The functions of these genes were assigned largely by data obtained from transposon mutants constructed by Dr. Armand Mve-Obiang using a phage delivery system which was found to be much more efficient at producing insertion mutants than the EZ::TN™ <KAN-2>Tnp Transposome™ Kit used previously to construct the mycolactone-negative mutant characterized in this work. While, still no definitive data is available on the role of FurA in the production of mycolactone; the possibility of it serving a regulatory role can not be ruled out.

Another important advancement in the knowledge of this organism was reported by Marsollier, et. al and contributes to the knowledge of the ecological niche of *M. ulcerans* (49). This group has for the first time ever reportedly isolated *M. ulcerans* from the environment. The organism was found in insects collected from Buruli ulcer-endemic regions of West Africa. These insects of the genus *Naucoris* were found to have salivary glands colonized by *M. ulcerans* (49). While the biting of insects carrying *M. ulcerans* is likely to contribute to transmission of Buruli ulcer; other environmental sources of this organism need to be identified. Knowing a more complete picture of the

epidemiology of this organism will definitely prove beneficial for decreasing the incident of Buruli ulcer and preventing outbreaks.

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Vita

Terri Alford, originally from Memphis, Tennessee, earned a Bachelor of Science degree in Microbiology from the University of Tennessee, Knoxville in May 2000. She began her graduate studies in microbiology at the University of Tennessee in August, 2000, graduating with a Master of Science degree in the Summer of 2004.