The Cloning of Putative Genes From Within the *Mycobacterium ulcerans* AGY99 Genome That May be Involved in Mycolactone Efflux

Brandon Ferguson
Mentor: Dr. Gold

Winter 2011
Abstract

*Mycobacterium ulcerans* secretes mycolactone, a macrolide toxin that causes the severe ulcers seen in Buruli ulcer. The mechanism by which mycolactone is secreted from the cell is unknown. This study describes the cloning of four genes (MUL 1481, MUL 1658, MUL 3757 and MUL 4484) from within the *M. ulcerans* AGY99 genome that may be involved in mycolactone efflux.

Introduction

*Mycobacterium ulcerans* is the causative agent of the third most common mycobacteriosis in the world (Johnson et al. 2005) and is the only mycobacterium known to produce a toxin (George et al. 1999). Furthermore, *M. ulcerans* exerts its toxic effects extracellularly unlike most mycobacteria (Cosma et al. 2003). Human infection by *M. ulcerans* leads to Buruli ulcer, a severe necrotic skin disease that causes large, ulcerated lesions of the skin and subcutaneous tissues; in serious cases these lesions can even affect the bone (Johnson et al. 2005). Most cases of Buruli ulcer are found in humid tropical regions of Asia, Africa and Latin America. The incidence of Buruli ulcer has surpassed that of tuberculosis and leprosy in some areas of the world (Silva et al. 2009).

Early lesions may appear as a subcutaneous nodule, which can be easily treated by surgical excision and suture. If left untreated these lesions advance to large, painless, open ulcers that lead to physical disability, social stigma and loss of livelihood even after treatment (van der Werf et al. 2005). Unfortunately, the painlessness of the ulcers, a lack of understanding of the disease, poor economic circumstances and limited transportation options often result in delayed treatment allowing the disease to progress to the more severe state. These delays result in the need for more complex surgical procedures that often postpone healing and place greater financial burden on the patients and their families (Kibadi et al. 2006).
George et al. (1999) isolated the toxin produced by *M. ulcerans*, identified it as a macrolide and determined that mycolactone is required for *M. ulcerans* virulence. Mycolactone alone resulted in necrotic ulcers in guinea pigs. The mycolactone toxin has also been directly linked to the painlessness observed in Buruli ulcer (En et al. 2008).

Though efforts by the World Health Organization have increased interest in Buruli ulcer within the scientific community, much about the pathogenesis of *M. ulcerans* is unknown. There is currently no effective Buruli ulcer vaccine (Johnson et al. 2005) and though recent studies point to aquatic insects as likely vectors of the disease, the exact mode of transmission is still not known (Marsollier et al. 2007, Mosi et al. 2008). Drummond and Butler (2004) note the need for the development of cost-effective therapies for Buruli ulcer patients.

While mycolactone is a key virulence factor in the pathogenesis of *M. ulcerans*, the mechanism for mycolactone secretion from the bacterium itself has yet to be determined. This study describes the cloning of four genes (MUL 1481, MUL 1658, MUL 3757 and MUL 4484) from within the *Mycobacterium ulcerans* AGY99 genome (Stinear et al. 2007), which may be involved in the secretion of mycolactone.

Methods

*Determination of Optimal PCR Conditions*

All PCR reactions in this study were carried out with a five minute initial denaturing step at 95° C. Each reaction then cycled 30 times through three steps: first, a 30 second denaturing step at 95° C, second, an annealing step at a temperature consistent with the melting temperatures of each respective primer set and third, an extension step at 72° C for two min/kb of
amplicon. All reactions were terminated with a final seven minute step at 72°C and then stored at -20°C until analysis (Table 1).

To determine the optimal concentration of *M. ulcerans* AGY99 template DNA, nine separate 20 μL PCR reactions were set up in nine individual 0.5 mL thin-walled microcentrifuge tubes. All tubes contained a 1X PCR Master Mix (Promega Corporation). The primers MUL 1481-F1 and MUL 1481-R1 (Table 1) were added to the first seven tubes at a concentration of 0.1 μM along with *M. ulcerans* AGY99 genomic DNA at 5 ng/μL, 2.5 ng/μL, 1 ng/μL, 0.5 ng/μL, 0.3 ng/μL, 0.2 ng/μL, and 0.1 ng/μL. The primers BSF349 and BSR1114 (Nucl. Acids Res. (2004) 32 (suppl 1): D101-D103.), designed to replicate the 16s rRNA gene, were used as controls along with 5 ng/μL or 1ng/μL *M. ulcerans* AGY99 genomic DNA. Each tube was placed into a Biometra T3000 thermocycler and cycled using an annealing temperature of 62°C for the MUL 1481-F1/MUL 1481-R1 primer set and 55°C for the BSF349/BSR1114 primer set. Each PCR reaction was analyzed using agarose gel electrophoresis with Hyperladder I (Bioline Inc.) as a sizing standard.

**Cloning of Putative Macrolide Efflux Genes**

PCR was used to amplify genes MUL 1481, MUL 1658, MUL 3757 and MUL 4484 from *M. ulcerans* AGY99 genomic DNA. An initial PCR reaction was set up by adding AGY99 genomic DNA at 1 ng/μL, 1X PCR mastermix (Promega) and sterile deionized water to a final volume of 80 μL. This reaction was divided into four 0.5 mL thin-walled microcentrifuge tubes in 20 μL aliquots. Each tube received a forward (F1) and reverse (R1) primer specific to the gene to be amplified at a concentration of 0.1 μM each (Table 1). PCR results were confirmed via agarose gel electrophoresis. Each amplicon was individually cloned into a separate pCR2.1
TOPO vector (Invitrogen Corporation) according to the protocol provided by the manufacturer. pCR2.1 TOPO constructs were transformed into *Escherichia coli* NEB 5alpha (New England Biolabs, Incorporated) following the manufacturer’s protocol. Each transformation was plated onto LB Agar plates containing 50 µg/ml Kanamycin Sulfate.

PCR was used to screen 9 clones from each construct transformation using the T7 promoter primer and the respective reverse primer for each gene insert (Table 1). Agarose gel electrophoresis confirmed the presence of the desired insert in each of the individual constructs. Verified clones were used to inoculate 5 mL of LB broth containing 50 µg/ml Kanamycin Sulfate and incubated at 37° C overnight. Plasmid isolations were performed using the QIAprep Spin Miniprep (QIAGEN) kit according to the protocol provided by the manufacturer. Each construct was sequenced by SeqWright DNA Technology Services using the M13 forward and reverse universal primers.
Table 1: The forward (F1) primers, reverse (R1) primers and annealing temperatures (Tm) used to amplify and verify insertion of MUL 1481, MUL 1658, MUL 3757 and MUL 4484 using PCR. The underlined bases represent a restriction site to be used in downstream cloning and expression experiments.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Tm °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUL 1481F1</td>
<td>5'-GAATTCCAGTAACACCAACACGCAGGAGAGAG-3'</td>
<td>62</td>
</tr>
<tr>
<td>MUL 1481R1</td>
<td>5'-GAAAGCTTCTGAGCAGCAGAAGACTAAACTC-3'</td>
<td></td>
</tr>
<tr>
<td>MUL 1658F1</td>
<td>5'-GAATTCTAAGCGGCGAGGGATTTCAGGAAGTC-3'</td>
<td>70</td>
</tr>
<tr>
<td>MUL 1658R1</td>
<td>5'-GAAAGCTTCTGACGAACGGAACTCTGGGATGACTC-3'</td>
<td></td>
</tr>
<tr>
<td>MUL 3757F1</td>
<td>5'-GAATTCTCGACGCGGTAAGTCAATCTCCAAAGAC-3'</td>
<td>56</td>
</tr>
<tr>
<td>MUL 3757R1</td>
<td>5'-AGGGCCGGCAAGTAGACCAACACAGACGAC-3'</td>
<td></td>
</tr>
<tr>
<td>MUL 4484F1</td>
<td>5'-GGATTCATTGCCATGCGAGCGTTGTCTTACC-3'</td>
<td>65.2</td>
</tr>
<tr>
<td>MUL 4484R1</td>
<td>5'-GAATTCTGCAGCGGCGAGGTGTTGTCTTACC-3'</td>
<td></td>
</tr>
<tr>
<td>BSF349</td>
<td>5'-GGGTTGCACGCTGCCTCTCCTCCT-3'</td>
<td></td>
</tr>
<tr>
<td>BSR1114</td>
<td>5'-GGGTTGCACGCTGCCTCTCCTCCTCCTCCT-3'</td>
<td></td>
</tr>
<tr>
<td>T7 Promoter Primer</td>
<td>5'-TCCCGCGAAAATAATACGACTCAC-3'</td>
<td>55</td>
</tr>
</tbody>
</table>

Results

**Determination of Optimal PCR Conditions**

PCR of *M. ulcerans* genomic DNA with the MUL1481F1/MUL1481R1 primer set should yield a single band of approximately 3.9 kb. A band corresponding to this size was observed only when 1 ng/μL of AGY99 genomic DNA was used in the PCR reaction (Figure 1). This same concentration of genomic DNA was successful in amplifying the 16S rRNA region of the genome, yielding a band of approximately 0.9 kb (Figure 1).
Cloning of Putative Macrolide Efflux Genes

Agarose gel electrophoresis confirmed amplification of each putative macrolide efflux gene. The MUL 1481-F1/MUL 1481-R1 primer set yielded a single band at an approximate length of 3.9 kb. The MUL 1658-F1/MUL 1658-R1 primer set yielded a single band at an approximate length of 3.5 kb. The MUL 3757-F1/MUL 3757-R1 primer set yielded a single band at an approximate length of 4.1 kb. The MUL 4484-F1/MUL 4484-R1 primer set yielded a single band at an approximate length of 2.3 kb (Figure 2). Of the 36 colonies screened for a recombinant insert using PCR with the T7 forward primer and the reverse cloning primer for each respective gene, colonies possessing cloned inserts were identified for each of the four \textit{M. ulcerans} genes (Figure 3). Sequencing data provided by SeqWright DNA Technology Services further confirmed the presence of each amplicon within the selected clones.

![Image of agarose gel](image)

Figure 1: A 1% agarose gel showing 1ng/μL to be the optimal concentration of AGY99 genomic DNA for amplifying MUL 1481 which shows as a band in lane 4 and the 16S rRNA region of the genome in lane 10.
Figure 2: A 1% agarose gel showing the amplification of MUL 1481 (lane 2) at 3.9kb, MUL 1658 (lane 3) at 3.5kb, MUL 3757 (lane 4) at 4.1kb and MUL 4484 (lane 5) at 2.3kb. Each gene was amplified using a 1 ng/µL concentration of AGY99 genomic DNA.

Figure 3: A 1% agarose gel showing those NEB 5alpha colonies that were successfully transformed with their respective TOPO constructs. MUL 1481 lane 2 shows the only successful MUL 1481/TOPO construct. MUL 1658 lane 8 shows the only successful MUL 1658/TOPO construct. Six successful MUL 3757/TOPO constructs are seen in the MUL 3757 lanes 1, 3, 5, 6, 7 and 9. The only two MUL 4484/TOPO constructs are seen in MUL 4484 lanes 5 and 6.
Discussion

Determination of Optimal PCR Conditions

This study determined 1 ng/μL of AGY99 genomic DNA to be the optimal template concentration for the amplification of MUL 1481, MUL 1658, MUL 3757 and MUL 4484 using the primers and annealing temperatures in Table 1. Typically template concentration is not the most important factor when doing PCR but it becomes increasingly significant when amplifying long (>3 kb) sections of DNA. In such cases it is recommended that no more than 1 ng/μL of template DNA be used (Dieffenbach and Dveksler 2003). This study confirms these recommendations but also found that anything less than 1 ng/μL was not sufficient for the desired amplification.

Cloning of Putative Macrolide Efflux Genes

According to primer design the length of the projected amplicons were as follows: MUL 1481 at 3.1 kb, MUL 1658 at 2.7 kb, MUL 3757 at 3.1 kb and MUL 4484 at 2.1 kb. Gel electrophoresis confirmed these projections for MUL 3757 and MUL 4484 however the remaining two inserts appeared to be approximately 0.8 kb larger than was expected (Figure 8). This could be due to nonspecific binding of one or more of the respective primers though sequencing data confirmed that at least the first 1 kb of the open reading frame for each gene was present suggesting that the forward primer annealed correctly. To be certain that the entire gene is present in each construct, further sequencing is necessary.

Stinear et al. (2007) noted that genes MUL 1481, MUL 1658, MUL 3757 and MUL 4484 may be involved in macrolide efflux. A protein blast search using the amino acid sequences of these four genes confirmed an up to 99% alignment of MUL 1481, MUL 1658 and MUL 3757
with known ABC transporters and a 97% alignment of MUL 4484 with a known multidrug efflux pump. This alignment is promising as one of the functions of ABC transporters in bacteria is the secretion of toxins and multidrug efflux pumps are promiscuous with respect to substrate specificity (Schaechter 2004).

Identification of the mycolactone efflux mechanism used by *M. ulcerans* could provide new opportunities for treating Buruli ulcer. Assuming that the efflux pump is a membrane protein, it may also serve as a strong immunogen therein providing a possible avenue for the development of a Buruli ulcer vaccine. The cloning described in this study is a significant step toward determining the function of the products of the cloned genes and sets the stage for future studies that may identify the mechanism for mycolactone efflux.

**Acknowledgments**

I would like to thank the BYU-Hawaii Biology faculty for their guidance, support and all the effort that goes into the Biology Research and Thesis class. I would like to thank the FAST program for funding my research. I would also like to thank Dr. Jean Engohang-Ndong for introducing me to *Mycobacterium ulcerans* and for providing me with *M. ulcerans* AGY99 genomic DNA for this study. Most of all, I would like to express my deepest gratitude to my mentor Dr. Roger Shane Gold for his tireless support and patience, for making himself ever available and for the confidence that he demonstrated in my wild ideas and tiny abilities. I will be forever grateful for this experience.
References


