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The Isolation and Characterization of Novel Mycobacteriophages

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Faculty Mentor: Dr. Small

PROJECT TITLE: Isolation and Characterization of Novel Mycobacteriophages

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I have reviewed this completed senior honors thesis with this student and certify that it is a project commensurate with honors level undergraduate research in this field.

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Comments (Optional):

Elizabeth did an outstanding job on her research project

The Isolation and Characterization of Novel Mycobacteriophages

Senior Honors Project

In The Department of Microbiology

Mentor: Dr. Pamela Small

Elizabeth Fleming

May, 2004

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Abstract

Mycobacterium ulcerans produces a cutaneous lesion, Buruli ulcer, which is refractory to antibiotic treatment. The localized nature of infection suggested that phage therapy might be useful for treatment, although until this time no mycobacteriophage lytic for *M. ulcerans* had been reported. Efforts were made to identify phages lytic for *M. ulcerans* by screening a set of known mycobacteriophages provided by Graham Hatfull, University of Pittsburgh, as well as by isolating new phages from the environment. Results of these studies showed that two of the fourteen known phages tested, D29 and Bxz2, were lytic for *M. ulcerans*.

During the course of these studies, four novel phages were isolated from environmental sources. Knox Zoo 1 and 2 were isolated from hay compost at the Knoxville Zoo and Island Home 1 and 2 from a compost pile in a local garden. These phages were tested to see if they could infect the mycobacteria: *M. smegmatis, M. phlei, M. fortuitum, M. marinum,* and *M. ulcerans.* All four newly isolated phages infected only *M. smegmatis.* Knox Zoo 1 formed large, haloed, cleared plaques and Island Home 1 formed turbid plaques. Knox Zoo 2 and Island Home 2 formed plaques that were somewhat cleared. DNA was prepared from Knox Zoo 1 and Island Home 1 and subjected to restriction fragment analysis using *Hind*III, *Xba*I, *Bam*HI, and *Eco*RI restriction enzymes. The genome size of Knox Zoo 1 was estimated to be approximately 80Kb while Island Home 1 was about 55Kb. DNA from Knox Zoo 1 and Island Home 1 was subjected to restriction fragment analysis using the *Sma*I restriction enzyme, ligated into the Puc19 vector, and subcloned. Four clones containing inserts from Island Home 1 were sequenced, and the sequences of the inserts were compared to the sequences of

known phages using a Blast search. Analysis of the sequenced segments of DNA from Island Home I indicates the phage is novel.

Mycobacterial host strains were isolated from the compost samples that yielded the phages. These strains were grown in a pure, broth culture, stained with an acid-fast stain, and subjected to PCR analysis using mycobacterial conserved sequences from the 16S rRNA gene to identify potential host species. The potential host species appear to be similar to *M. smegmatis*.

Introduction

Mycobacteria General Information

Mycobacteria are rod-shaped, gram-positive organisms which all share the

distinctive staining property of acid-fastness. Unique lipids called mycolic acids located

in the cell wall are responsible for the acid-fastness, and the complex that they form with

peptidoglycan gives the bacterial wall a hydrophobic, waxy consistency (Madigan *et al.*,

2003). Mycobacteria can be divided into two major groups, the slow-growers and the

fast-growers. Fast-growing mycobacteria form visible colonies within five days, but in

comparison to many other fast-growing bacteria such as *Escherichia coli* they grow much

slower (Colston & Cox, 1999). *Mycobacterium phlei*, *Mycobacterium smegmatis*, and

Mycobacterium fortuitum are all strains commonly used in the laboratory because they

are fast-growers and are less pathogenic than many other mycobacteria. The slow-

growing mycobacteria form visible colonies in more than five days, and some such as

Mycobacterium ulcerans can take up to a month (Colston & Cox, 1999). The pathogenic

mycobacteria: *M. tuberculosis, M. leprae*, and *M. ulcerans* are all slow-growers.

According to the World Health Organization, *M. Tuberculosis* causes the highest

number of mycobacterial infections in immunocompetent people followed by *M. leprae*

and *M. ulcerans* (Asiedu *et al.*, 2000). *M. ulcercans* is the causative agent of Buruli

ulcer, a necrotizing skin disease, which is currently endemic in thirty-two countries

(Asiedu *et al*, 2000). Many aspects of the disease including its mode of transmission are

unknown. To date *M. ulcerans* has not been isolated from the environment (Portaels *et*

al., 1996). The disease begins as a painless swelling under the skin, and then a nodule

develops. The mycolactone toxin that the bacteria produce destroys tissue and suppresses

the immune system, which can lead to massive skin ulceration *(Asiedu et al.,* 2000).

There are currently no good antimicrobial treatments for Buruli Ulcer, and so the

preferred treatment is surgical excision *(Asiedu et al.,* 2000).

Figure1: The ulcerative lesion caused by *M. ulcerans*

. Ulcerative lesion. (Photo: Kingsley Asiedu)

(photo from WHO website)

< http://www.who.int/gtb-buruli/publications/PDF/Buruli_ulcer_monograph.PDF>.

Mycobacteriophages General Information

Mycobacteriophages are DNA viruses that infect mycobacteria, and ones that lyse their host have been of interest for their potential as a therapy for mycobacterial infections. There are two major types of phages, lytic and temperate. Phages can be characterized by the way that the host bacteria respond to phage infection. Lytic phages cause the bacterial cell wall to lyse, which results in cell death and release of the progeny phage. Temperate phages in addition to having the ability to lyse the host bacteria can also possess the ability to produce stable lysogens in which a copy of the phage's genome is stably maintained in the host (Hatfull, 2000). Temperate phages and lytic phages can be distinguished by the appearance of the plaques, areas of clearing, on the bacterial host. Normally temperate phages form turbid plaques and lytic phages form cleared plaques, but it is not uncommon for phages to form intermediate plaques where it is not obvious if lysogens are present (Hatfull, 2000).

It has been proposed that mycobacteriophages could be used as treatment for mycobacterial infections such as tuberculosis. Past experiments on phage treatment in animals experimentally infected with *M. tuberculosis* have not produced desirable results (McNerney, 1999). Further studies into the treatment of tuberculosis with phage therapy have reported some success when there is some sort of supplement to help the phage reach the host bacteria (McNerney, 1999). In one study *M. smegmatis* was transiently infected with the lytic phage, TM4, and used to deliver the phage to *Mycobacterium avium* and *M. tuberculosis* residing in macrophages (Broxmeyer *et al.*, 2002). Although this experiment was successful *in vitro*, subsequent studies will have to show whether or not this type of phage therapy will be successful in an animal model. Although

Broxmeyer's study and several other studies have shown that mycobacteriophages alone are not effective against intracellular infections of *M. tuberculosis*, a study by Sulla *et al.* reported that phage treatment with the phage, DS-6A, was effective (1981).

In the past phages were useful in phage typing of mycobacteria, although now DNA fingerprinting has become the preferred method for studying the transmission of tuberculosis (McNerney, 1999). However, mycobacteriophages remain important tools for molecular studies of mycobacteria. Some temperate phages have the ability to integrate DNA into the host's chromosome (Hatfull, 2000) This property is of interest to researchers who study the virulence of the host bacteria, because it has been shown that many pathogenic bacteria have obtained toxin genes from segments of phage DNA integrated in the host's chromosome. Temperate phages are also useful for constructing vectors as well as in other genetic tool.

Bacteriophages are estimated to be the most populous group of organisms on the planet (Hendrix *et al.*, 1999). There have been hundreds of mycobacteriophages isolated since the first one was discovered in 1947 (McNerney, 1999). At the time researchers were interested in using the phages for typing the clinical isolates of mycobacteria from patients with *M. tuberculosis* infections. In addition to the phages which have a high specificity for their host and only infect a specific subgroup, there are also phages that infect a diverse host range including both fast and slow-growing mycobacteria (Hatfull, 1999).

Mycobacteriophages have been isolated from a variety of environments across the world, and they can be easily obtained from soil samples. The first mycobacteriophage isolated was collected from moist leaf composts from around Seattle (McNerney, 1999).

Although a large number of mycobacteriophages have been isolated, until 2003 only four phages L5, D29, TM4, and Bxb1 had been completely sequenced. In 2003 the genomes of ten more mycobacteriophages were published (Pedulla *et al.*, 2003). Although phages can be categorized by aspects of their biology such as plaque morphology, the only way to understand their true relationship to each other evolutionarily is to genetically characterize them (Hendrix *et al*, 1999).

One example of this characteristic is the case of the phages, L5 and D29. D29 is a lytic phage capable of infecting a broad range of mycobacterial hosts including slow and fast-growers (Russell *et al*, 1963). L5 is a temperate phage isolated from *M. smegmatis* (Doke, 1960), but under specific conditions it can also infect slow-growing mycobacteria (Fuller & Hatfull, 1997). The restriction maps of L5 and D29 are quite different from each other (Oyaski & Hatfull, 1992). Despite the difference in many biological aspects, recent genomic studies of the two phages have revealed that they are very similar. D29 appears to have been a derivative of L5, possibly created during propagation of the phage shortly after it was isolated (Ford *et al.*, 1998). It appears that a deletion of the repressor gene in L5 caused the phage to loose its ability to form stable lysogens in the host (Ford *et al.*, 1998).

The temperate mycobacteriophage Bxb1 was recently isolated and sequenced. Notable features of the phage are that it infects *M. smegmatis* and does not infect any slow-growing strains, and it forms plaques with large haloes (Mediavilla *et al.*, 2000). The formation of haloed plaques has not been described for any previous mycobacteriophages, however it is not uncommon in other bacteriophages (Mediavilla *et al.*, 2000). Even though many features of this phage are not similar to L5 or D29,

genomic analysis has shown that it is a member of the L5/D29 family (Hatfull, 2000). However, Bxb1 shares less similarity with L5 or D29 than L5 and D29 share with each other (Hatfull, 2000).

TM4 is another mycobacteriophage that has been sequenced, but it appears to be quite different than the L5/D29/Bxb1 family (Hatfull, 2000). The ten new phages that were sequenced suggest that mycobacteriophages may be more diverse than previously expected, and the bacteriophage population as a whole may represent the largest reservoir of currently unsequenced information in the biosphere (Pedulla *et al.*, 2003). In general mycobacteriophages significantly differ genetically, but most have similarly sized genomes and have similar arrangements of structure and assembly genes (Pedulla *et al.*, 2003). The new analysis of the fourteen known phage genomes has shown that they share a mosaic relationship with each mosaic piece a single gene (Pedulla *et al.* 2003). In addition many unexpected genes have been discovered within mycobacteriophage genomes such as genes implicated in mycobacterial latency, cellular and immune response to mycobacterial infection, and autoimmune disease (Pedulla *et al.*, 2003).

Objective

Because of the lack of good, non-surgical treatments for Buruli ulcer infections, it is desirable to examine alternative methods of treatment. The localized nature of an *M. ulcerans* infection suggested that phage therapy might be useful for treatment, although until this time no mycobacteriophage lytic for *M. ulcerans* had been reported. During the course of this study efforts were made to identify phages lytic for *M. ulcerans* by

screening a set of known mycobacteriophages provided by Graham Hatfull, University of

Pittsburgh, as well as by isolating new phages from the environment.

Materials and Methods

Phage Spot Tests

Stocks of fourteen phages: TM4, L5, D29, Barnyard, SS, PB11, Black Raspberry,

Bxz2, Rosebush, Omega, PG2, Wildcat, Che8, and SB1 were obtained from Graham

Hatfull, University of Pittsburgh, and were tested on *M. smegmatis*, *M. fortuitum*, *M.*

marinum, and *M. ulcerans*. Spot tests were performed for each phage as described by

Sarkis and Hatfull (1998). Aliquots of 500 µl of *M. smegmatis* and *M. fortuitum* were

placed in separate test tubes for each phage. Then 4.5 ml of Middlebrook Top Agar

supplemented with 1mM $CaCl_2$ and prewarmed to 55°C was pipetted and mixed with the

bacteria. The agar and bacteria were then poured onto a Middlebrook 7H10 plate, and the

mixture was allowed to solidify. Dilutions from 10^{-1} to 10^{-8} of the stock phage were

placed in 5µl spots around each plate. The plates were then allowed to incubate overnight

at 37°C.

The protocol for *M. marinum* was the same except 700 µl of bacteria was used,

the top agar contained an OADC supplement (oleic acid 0.6g/L, bovine albumin 50g/L,

dextrose 20g/L, catalase 0.03g/L), and the plates were incubated at 32°C. The protocol for

M. ulcerans differed significantly. No top agar was used, instead 200 µl of bacteria was

plated on deep Middlebrook 7H10 plates and the plates were divided into four quadrants

with one plate for 10^{-1} to 10^{-4} spot dilutions and another for 10^{-5} to 10^{-8} spot dilutions.

The plates with *M. ulcerans* were incubated at 32°C and monitored over several months

because the bacteria grow very slowly.

Phage Isolation- Screening

Mycobacteriophages, Island Home 1 and Island Home 2, were isolated from a soil

sample from a local garden compost. Knox Zoo 1 and Knox Zoo 2 were isolated from a

hay compost at the Knoxville Zoo. Stocks of each phage were prepared as described by

Sarkis and Hatfull (1998). About 10-20 ml of sample was collected and about 25 ml of

phage buffer (10 mM Tris-HCL (pH 7.5), 10 mM MgSO4, and 68.5 mM NaCl) was

added to the sample to allow for the diffusion of the phage from the solid sample into the

buffer. The mixture was vortexed and then centrifuged for 5 minutes at a medium speed

to pellet the debris. The supernatant was then filtered with a 0.22 μm filter to sterilize the

sample. *M. smegmatis* $mc^2 155$ grown in Middlebrook 7H9 broth with OADC was used to

screen the samples for mycobacteriophages. Aliquots of 500 μl of bacteria were added to

separate test tubes, and 50 μl of each phage sample was added to each tube of bacteria.

Melted top agar with $CaCl_2$ was added, and the mixture was plated as previously

described. The plates were incubated several days at 37°C, and the plates were monitored

for plaque formation.

Figure 2: Plaque Morphology of Spot Tests on *M. smegmatis*

Island Home 1 forms turbid plaques   Knox Zoo 1 forms large, haloed plaques
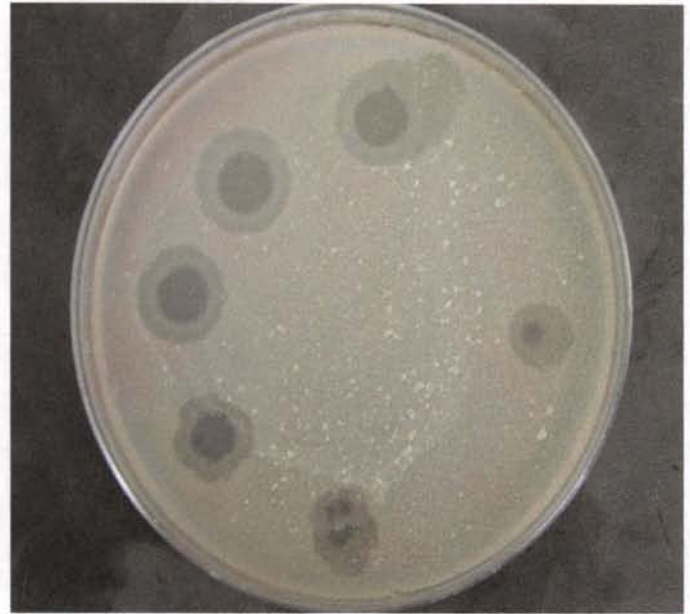
Phage Isolation- Plaque Purification

The tip of a pipette was gently touched in the center of a single plaque of each newly isolated phage, and the phage material on the tip was placed in 100 μl of phage buffer. The phages were then serially diluted at concentrations of 10^{-2}, 10^{-3}, and 10^{-4}. Each dilution of each phage was added to a test tube containing 500 μl of *M. smegmatis*, and the mixture was then plated and incubated as previously described. A pipette tip was again used to obtain a pure sample of each phage from one plate. The phage was placed in 100 μl of phage buffer and was serially diluted at 10^{-1}, 10^{-2}, and 10^{-3} concentrations. A 10 μl sample of the neat (undiluted) and 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} were each added to *M.*

smegmatis and again were plated with top agar and incubated as previously described.

Stocks of each phage were obtained by adding 4.5 ml of phage buffer to the plates and

allowing them to incubate overnight at 4°C. The next day the liquid lysate was siphoned

off the plates using a pipette, centrifuged to pellet the debris, and filtered with a 0.22 μm

filter. Spot test were then performed for each of the four newly discovered phages on *M.*

smegmatis, *M. fortuitum*, *M. phlei*, *M. ulcerans*, and *M. marinum* in the same manner as

used for the fourteen known phages.

High Titer Phage Stocks

In order to obtain a highly concentrated stock of the phages, a large amount of

lysate had to be harvested. Large agar plates with Agar Noble and Middlebrook 7H9

were used to propagate 1 ml of bacteria and phage, and the bacteria were plated with 10

ml of top agar and harvested with 10 ml of phage buffer. High titer CsCl phage stocks of

three of the newly discovered phages were made according to the protocol given by

Sarkis and Hatfull (1998). Lysates of each phage were combined until there was about

10^{12} total phage particles (about 50 ml of phage stock). The stock was then adjusted to

10% polyethelene glycol 8000 and 1M NaCl, and the phages were allowed to precipitate

overnight at 4°C. The phages were then pelleted by centrifugation and resuspended in 10

ml of phage buffer while gently rocking overnight at 4°C. The insoluble material was

then collected by centrifugation, and the supernatant was transferred to a 14 ml

polyallomer quick-seal centrifuge tube (Beckman). The phage buffer was then adjusted to

a density of 1.5 g/ml with CsCl. The tubes were then spun at 126,000g for 24 hours.

The phage was evident as a white band just above the center of the gradient. The phage was removed by first puncturing the top of the tube and then puncturing the tube just below the phage band with an 18G needle and slowly removing about 0.5 ml. Using the Slide-A-Lyzer dialysis kit (Pierce), 500 µl of each phage was dialyzed against 500 ml of phage buffer at room temperature for about four hours.

Isolation of Phage DNA

The DNA from Knox Zoo 1 and Island Home 1 was extracted from the high titer CsCl phage stocks as according to the protocol set forth by Sarkis and Hatfull (1998). After dialysis 250 µl of each phage was mixed with an equal amount of equilibrated phenol, centrifuged for 5 minutes, and the aqueous phase removed. In order to obtain clear phases the extraction procedure was repeated five times. The final extraction of the aqueous phase was performed with an equal volume of 24:1 chloroform/isoamyl alcohol and the aqueous phase was saved. The DNA was then precipitated with a 1/10 volume of 3M sodium acetate (pH 5.4) and 3 volumes of 95% ethanol. The DNA was pelleted by centrifugation and dried. The DNA was then resuspended in 200 µl of dH_2O overnight.

Restriction Analysis of DNA

Restriction fragment analysis using the enzymes *Hind*III, *Xba*I, *Bam*HI, and *Eco*RI was performed on DNA isolated from Knox Zoo 1 and Island Home 1. A mixture of 11µl of water, 5 µl of concentrated phage DNA, 2 µl of buffer, and 2 µl of enzyme was incubated in a 37°C water-bath for 2 hours. The cut phages, uncut phages, 1Kb ladder,

and lambda DNA cut with *Hind*III were visualized using a 0.4% (wt/vol) agarose stained

with ethidium bromide.

Figure 2: Gel Picture of Knox Zoo1 and Island Home 1

- Lane1& 14: 1 Kb ladder

- Lane 2 & 13: λ DNA w. *Hind*III

- Lane 3: KZ1 uncut

- Lane 4: KZ1 w. *Hind*III

- Lane 5: KZ1 w. *Xba*I

- Lane 6: KZ1 w. *Bam*HI

- Lane 7: KZI w. *Eco*RI

- Lane 8: IH1 uncut

- Lane 9: IH1 w. *Hind*III

- Lane 10: IH1 w. *Xba*I

- Lane 11: IH1 w. *Bam*HI

- Lane 12: IHI w. *Eco*RI
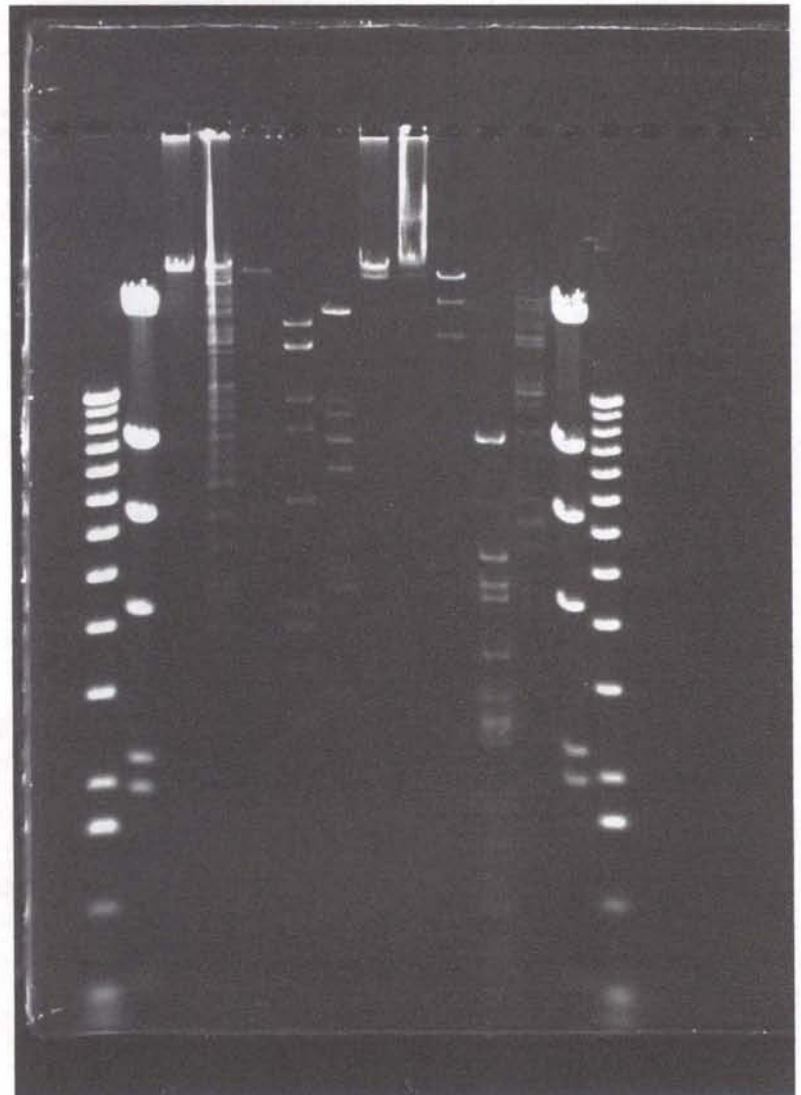
Genomic Analysis of the Phages

Separate reaction mixtures for each phage and for the vector were prepared. Each mixture containing 20 µl of concentrated phage DNA or the pUC19 vector and 20 µl of water, 5 µl of buffer, and 5 µl of the *Sma*I enzyme was incubated at 37°C for 4 hours. The products were visualized using a 1% (wt/vol) agarose gel stained with ethidium bromide. The DNA was purified according to the manufacturer's directions using the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences). The fragments of phage DNA were then ligated into the pUC19 vector using T4 DNA ligase. Ligation mixtures containing no phosphatase and several ratios of insert to vector were incubated at 4°C overnight. The following day the ligase was heat deactivated by incubating the mixture at 65°C for 5 minutes.

The products of the ligation mixtures were transformed using the TOPO TA Cloning kit (Invitrogen) using directions provided by the manufacturer. Clones containing the insert were screened using 5-bromo-4-chloro-3-indolyl-B-D-galactopyranoside (X-Gal)-mediated blue-white discrimination and ampicillin resistance. White, ampicillin-resistant transformants were selected and grown overnight at 37°C while shaking in Luria-Bertani (LB) broth containing ampicillin (50 µg/ml). Minipreps were prepared from 5 ml cultures using the Wizard Plus Minipreps DNA Purification System (Promega). The minipreps were digested with *Eco*RI and the products were visualized on a 1% (wt/vol) agarose gel stained with ethidium bromide. Clones containing the inset were grown in 25 ml volumes of LB broth containing ampicillin (50 µg/ml) overnight with shaking at 37°C. The Wizard Plus Minipreps DNA Purification System was again used to isolate plasmid DNA.

Bidirectional sequences from the cloned fragments from Island Home 1 were obtained from the University of Tennessee Molecular Biology Sequencing Facility (Knoxville, Tennessee) using the M13 forward and reverse primers (Invitrogen). The sequences obtained for the inserts from Island Home 1 were analyzed, and the sequence of a single continuous segment of DNA was constructed. This segment of DNA from Island Home 1 was compared to the sequences of the other known phages by performing a BLAST search.

Mycobacteriophage Host Isolation

In order to determine the mycobacterial host for the newly discovered mycobacteriophages, mycobacteria were isolated from the environmental samples which yielded the phages. Seventeen potential host bacteria were selected from the samples using two methods. Samples were decontaminated with the BBL MycoPrep (Becton Dickinson) according to the manufacturer's directions. The decontaminated samples were then tested for the presence of mycobacteria using the BBL MGIT kit (Becton Dickinson). The kit contained Middlebrook OADC enrichment, PANTA antibiotic mixture (containing: Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim, and Azlocillin), and Mycobacteria Growth Indicator Tubes which contained a fluorescent indicator. Decontaminated samples were also grown on Middlebrook 7H10 plates containing the PANTA supplement, which was prepared from the Bactec PANTA Plus Kit (Becton Dickinson). The PANTA supplement was reconstituted as stated by the manufacturer's instructions and 100 µl was added to each of the Middlebrook 7H10

plates. The plates and vials were incubated at 37°C and monitored for mycobacterial

growth, and potential host colonies were selected for further analysis.

Characterization of Potential Host

Potential mycobacterial host colonies were screened by preparing slides of the

bacteria and staining them with the Kinyoun stain procedure using the TB Stain Kit K

(Difco), as recommended by the manufacturer. Colonies that appeared to be mycobacteria

were grown in pure culture and stained again. The mycobacterial strains were grown in

7H9 broth with OADC at 37°C with shaking. Each of the four novel phages isolated

during the course of this experiment was tested on four of the potential host bacteria. Spot

tests were performed as previously described.

Figure 3: Morphology of potential host mycobacteria

(Note: Photos taken from before bacteria were grown in pure culture)
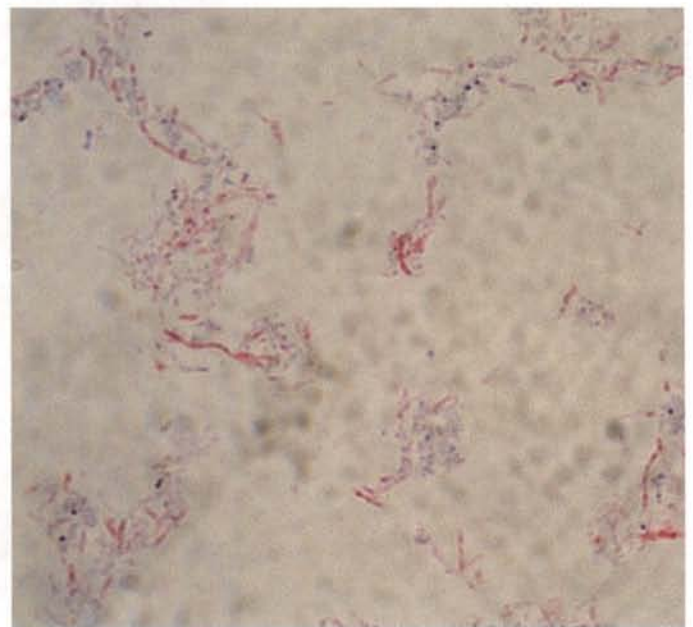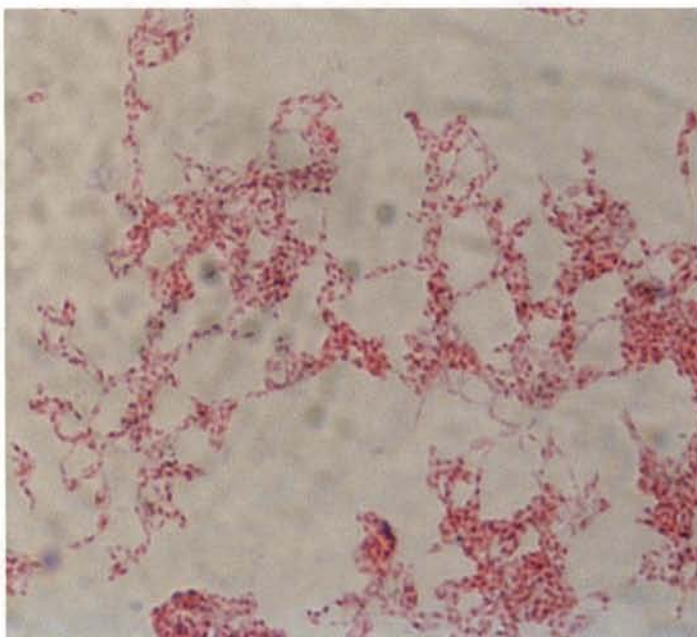
Genomic DNA Extraction

DNA was isolated from the four potential host bacteria tested using a rapid

boiling method. Briefly, 1 ml of each bacteria was collected and centrifuged in a

microcentrifuge for 10 minutes at 4°C. The supernatant was discarded and the pellet

resuspended in 200 μl of water. The tubes were placed in boiling water for about 10

minutes and then spun again in the microcentrifuge for 10 minutes at 4°C. The

supernatant was then collected and used for PCR.

Identification of Potential Host Mycobacteria

In order to characterize the mycobacteria isolated from the environmental

samples, PCR of the 16S rRNA gene was performed. A region of approximately 577-bp

of genomic DNA was used to confirm that the strains isolated were mycobacteria and to

identify the species of mycobacteria. This level of characterization is possible because in

mycobacteria there are conserved regions of DNA as well as two hypervariable domains

that are species-specific (El Amin *et al.*, 2000; Holberg-Peterson *et al.*, 1999). The

procedure is described by Daniel *et al.* (2004). The primers used for the amplification

were pA (5'-AGA GTT TGA TCC TGG CTC AD-3') and MSHE (5'-GCG ACA AAC

CAC CTA CGA G-3'). The PCR reactions contained 1 μl of genomic DNA and 49 μl of

PCR amplification mixture to give a total of 50 μl, which contained 1 U of Taq DNA

polymerase, 1mM primers, 1.5 mM $MgCl_2$, and 1 mM dNTPs (Promega). Amplification

took place in a thermocycler (Eppendorf), and the amplifications were performed with 4

minutes of denaturation at 94°C, 40 cycles of 94°C for 1 minute, 60°C for 2 minutes, and

72°C for 2 minutes, followed with a final extension period of 72°C for 7 minutes and then holding at 4°C.

The PCR products were visualized using a 1% (wt/vol) agarose gel stained with ethidium bromide. All potential host bacteria tested were determined to be mycobacteria, and the four strains of bacteria that the phages were tested on were chosen for further examination. The products of the ligation mixtures were transformed using the TOPO TA Cloning kit (Invitrogen) using directions provided by the manufacturer. Clones containing the inset were screened using 5-bromo-4-chloro-3-indolyl-B-D-galactopyranoside (X-Gal)-mediated blue-white discrimination and kanamycin resistance. White, kanamycin-resistant transformants were selected and grown overnight at 37°C while shaking in LB broth containing kanamycin (50 µg/ml). Minipreps were prepared from 5 ml cultures using the Wizard Plus Minipreps DNA Purification System (Promega). The miniprepes were digested with *Eco*RI and the products were visualized on a 1% (wt/vol) agarose gel stained with ethidium bromide. Clones containing the insert were grown in 25 ml volumes of LB broth containing kanamycin (50µg/ml) overnight with shaking at 37°C. The Wizard Plus Minipreps DNA Purification System was again used to isolate plasmid DNA.

Bidirectional sequences from the cloned fragments from Island Home 1 were obtained from the University of Tennessee Molecular Biology Sequencing Facility (Knoxville, Tennessee) using the M13 forward and reverse primers (Invitrogen). The sequences for three of the potential host mycobacteria were obtained and a BLAST search was performed to better identify them.
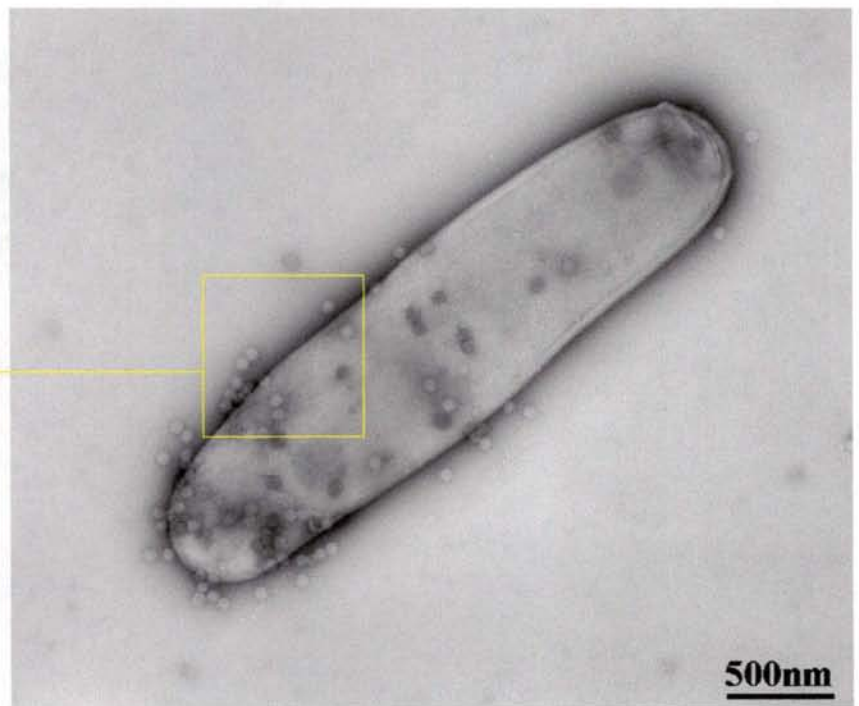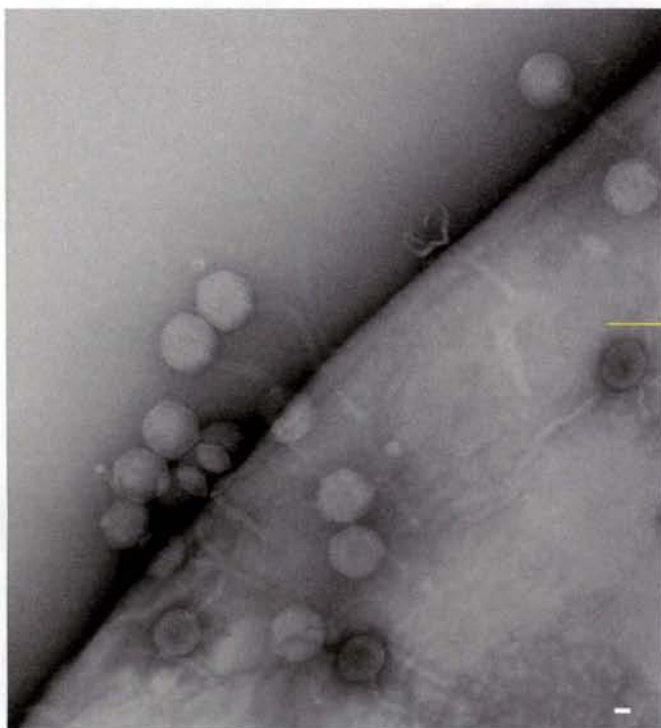
Results

The Known Phages

Previously no mycobacteriophage lytic for *M. ulcerans* had been reported. Of the fourteen phages provided by Gram Hatfull, University of Pittsburgh, two of them, D29 and Bxz2, were lytic for *M. ulcerans*.

Figure 4: Mycobacteriophage, D29, infecting a *M. ulcerans* bacterium

(Photo by Jan Rybniker)

The Novel Mycobacteriophages

None of the four newly isolated phages infected *M. ulcerans*. Of the bacteria with which the phages were tested, only *M. smegmatis* was infected. Knox Zoo 1 formed large, haloed, cleared plaques and Island Home 1 formed turbid plaques. Knox Zoo 2 and Island Home 2 formed plaques that were somewhat cleared. Of the four phages only two, Knox Zoo 1 and Island Home 1, were subjected to restriction fragment analysis. From this analysis the genome size of Knox Zoo 1 was estimated to be approximately 80Kb, and Island Home1 was about 55Kb.

In order to confirm that the phages were novel, one phage was selected for further genomic analysis. Analysis of sequenced segment of DNA from Island Home I indicates the phage is novel. Of the 1647bp sequenced there was a 377bp region with 82% homology to the mycobacteriophages, Che9c. This region includes the gp39 in Che9c genome. Although this phage has a region which is highly homologous to a gene in Che9, this region is flanked with large segments of DNA that are not homologous to any sequenced organism.

Host Bacteria

Four of the possible host bacteria that were isolated from the samples that yielded phage were tested to see if the phages could infect them. Island Home 1 infected three of the unknown bacteria, but unlike the plaques formed on *M. smegmatis* which were cleared the plaques on the unknown bacteria were turbid. Knox Zoo 1 formed a few turbid plaques on one of the strains. Island Home 2 formed some small, turbid plaques on

the other two strains that Island Home 1 infected, but it did not form plaques on the colony infected by Knox Zoo 1.

Analysis of sequence from the 16S rRNA gene from the three possible host mycobacterial strains indicated that they are *M. smegmatis*-like mycobacteria. Although it is impossible to define a host from these studies, it is reasonable to conclude that these phages infect a host similar to *M. smegmatis* in the environment. In the lab all four phages infected the laboratory strain of *M. smegmatis* mc^{2}155, and bacteria isolated from the same samples from which the phages were isolated contained bacteria that are genetically similar to *M. smegmatis*.

Conclusion

Although none of the new phages isolated were lytic for *M. ulcerans*, two known phages, D29 and Bxz2, were lytic. It is not surprising that D29 was one of the two phages able to infect *M. ulcerans* because the established host range for D29 is very large and includes both slow and fast-growing species. Jan Rybniker continued to study D29 by carrying out experiments, which tested its ability to be used as phage therapy in animal models infected with *M. ulcerans*. The four newly isolated phages only infected *M. smegmatis*, and appear to have a *M. smegmatis*-like host in the environment. It is not uncommon for phages to have a narrow host range such as these phages. Since no genomically defined mycobacteriophage has ever been isolated from the environment more than once (Pedulla *et al.*, 2003), it is reasonable to assume that the four phages isolated during this experiment are novel mycobacteriophages. Furthermore, DNA

segments isolated from one phage, Island Home 1, are significantly different from any other sequenced mycobacteriophage.

Further studies are needed to establish a complete host range of the novel phages. Hosts of interest include *M. tuberculosis, M. avium, M. scrofulacium*, as well as other environmental mycobacteria. Also, work could be done to complete more of the sequence of Island Home 1, and to begin sequencing the other three phages isolated. It would be interesting to determine the amount of homology among these geographically similar phages as well as to the other sequenced phages. The possible natural host bacteria could also be studied in much greater depth. Three of the bacteria examined have been identified as *M. smegmatis*-like bacteria, but more work is needed to determine whether they can be classified as *M. smegmatis*. Also, there are fourteen other possible host bacteria that were isolated and appear to be mycobacteria, but have not yet been genetically examined.

Works Cited

Asiedu, K., Scherpbier R., Raviglione, M. (eds) 2000. Buruli Ulcer: *Mycobacterium Ulcerans* infection. World Health Organization. < http://www.who.int/gtb-buruli/publications/PDF/Buruli_ulcer_monograph.PDF>.

Broxmeyer, L., D. Sonsnowska, E. Miltner, O. Chacon, D. Wagner, J. McGarvey, R.G. Barletta, and L.E. Bermudez. 2002. Killing of *Mycobacterium avium* and *Mycobacteria tuberculosis* by a mycobacteriophage delivered by a nonvirulent mycobacterium: A model for phage therapy of intracellular bacterial pathogens. *J. Infect. Dis.* **186**:1155-1160.

Colston, J. M. and Cox, R.A. 1999. Mycobacterial growth and dormancy, p. 198-219. In: C. Ratledge and J. Dale (eds), *Mycobacteria: Molecular Biology and Virulence*. Chapman and Hall, London, United Kingdom.

Daniel, A., R.E. Lee, F. Portaels, P.L.C. Small. 2004. Analysis of mycobacterium species for the presence of a macrolide toxin, mycolactone. *Infect. Immun.* 71:774-783.

Doke,S. 1960. Studies on mycobacteriophages and lysogenic mycobacteria. *J. Kunamoto Med. Soc..* **34**:1360-1371.

El Amin, N.M., H.-S. Hanson, B. Pettersson, B. Petrini, and L.V. von Stedingk. 2000. Identification of non-tuberculosis mycobacteria: 16S rRNA gene sequencing analysis vs. conventional methods. *Scand. J. Infect. Dis.* 32:47-50.

Ford, M.E., G.J. Sarkis, A.E. Belanger, R.W. Hendrix, and G.F. Hatfull. 1998. Genomic structure of mycobacteriophage D29: implications for phage evolution. *J. Mol. Biol.* **279**:143-164.

Hatfull, G.F. 1999. Mycobacteriophages, p. 38-58. In: C. Ratledge and J. Dale (eds),

Mycobacteria: Molecular Biology and Virulence. Chapman and Hall, London, United Kingdom.

Hatfull, G.F. 2000. Molecular genetics of mycobacteriophages, p 37-54. In: G.F. Hatfull and W.R. Jacobs, Jr (eds), *Molecular Genetics of Mycobacteria*. ASM Press, Washington, D.C.

Hendrix, R.W., M.C. Smith, R.N. Burns, M.E. Ford, and G. F. Hatfull. 1999. Evolutionary relationships among diverse baceriophages and prophages: all the world's phage. *Proc. Natl. Acad. Sci. USA* **96**:2192-2197.

Holberg-Peterson M., M. Steinbakk, K.J. Figenschau, E. Jantzen, J. Eng, K.K. Melby. 1999. Identification of clinical isolates of Mycobacterium spp. By sequence analysis of the 16S ribosomal RNA gene. *APMIS* **107**:231-239.

Madigan, M.T., J. M. Martinko, and J. Parker. 2003. *Brock Biology of Microorganisms*. Prentice Hall, Upper Saddle River, New Jersey.

McNerney, R. 1999. TB: The return of the phage. A review of fifty years of mycobacteriophage research. *Int. J. Tuberc. Lung Dis.* **3**:179-184.

Mediavilla, J., S. Jain, J. Kriakov, M. Ford, R.L. Duda, W.R. Jacobs Jr., R.W. Hendrix, and G.F. Hatfull. 2000. Genome organization and characterizaion of mycobacteriophage Bxb1. *Mol. Microbiol.* **38**:955-970.

Oyaski, M., and G..F. Hatfull. 1992. The cohesive end s of mycobacteriophage L5 DNA. *Nucleic Acid Res.* **20**:3251.

Portaels, F., Fonteyne, P.-A., de Beenhouwer, H., *et al*. 1996. Variability in 3'end of 16S rRNA sequence of *Mycobacterium ulcerans* is related to geographical origin of isolates. *J. Clin. Microbiol.* **34**:962-965.

Pedulla, et al. 2003. Origins of highly mosaic mycobacteriophage genomes. *Cell* **113**:171-182.

Russell, R.L., G.J. Jann, and S. Froman. 1963 . Lysogeny in the mycobacteria. II. Some phage-host relationships of lysogenic mycobacteria. *Am. Rev. Respir. Dis.* **88**:528-538.

Sarkis, G.J., and G.F. Hatfull. 1998. Mycobacteriophages. *Methods Mol. Biol.* **101**:145-173.

Sulla, L., J. Sulova, and M. Stolcpartova. 1981. Therapy of experimental tuberculosis in guinea pigs with mycobacterial phages DS-6A, GR-21 T, My-327. *Czech Med.* **4**:209-214.