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# Identifying Growth Deficient *Bacillus anthracis* Mutants via Transponson Mutagenesis

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**Degree Type**
Open Access Senior Honors Thesis

**Department**
Health Sciences

**First Advisor**
Dr. Lynne Shetron-Rama

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# IDENTIFYING GROWTH DEFICIENT *BACILLUS ANTHRACIS* MUTANTS VIA TRANSPOSON MUTAGENESIS

By

Michelle Hatto

A Senior Thesis Submitted to the

Eastern Michigan University

Honors College

in Partial Fulfillment of the Requirements for Graduation

with Honors in Clinical Laboratory Science

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Approved at Ypsilanti, Michigan, on this date June 5, 2012

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**ABSTRACT**

*Bacillus anthracis* is a spore-forming bacterium and etiological agent of the disease anthrax. How exactly *B. anthracis* spores germinate and grow into their vegetative form is a poorly understood concept, and the bacterium's metabolism and nutrient requirements within a host environment are largely unknown. Our aim was to start an investigation into the metabolic requirements of *B. anthracis* that help it to flourish so effectively in the host. The first step in this project was the creation of a *B. anthracis* mutant library. Creation of the library was facilitated by the use of transposon mutagenesis, where mobile transposons insert into the host genomic DNA causing a disruption of gene function. A library of nearly 400 mutants was generated and screened for growth impairment in minimal media using optical density measurements. Arbitrarily selected mutants with a defective growth phenotype were analyzed further by using a Southern Blot to confirm the presence of the transposon, indicating a disruption in a gene or genes necessary for effective growth. This library of mutants can be studied further in order to identify the metabolic pathways affected, thus revealing novel targets for therapy.

## INTRODUCTION

### Microbe background

*Bacillus anthracis*, the etiological agent of the disease anthrax, is a soil dwelling, spore forming bacterium. They are classically differentiated from other *Bacillus* species by their absence of motility and hemolytic activity on Blood agar, and susceptibility to penicillin and to the γ bacteriophage, though exceptions have occurred with all the aforementioned traits [10]. *B. anthracis* is recognized morphologically as a gram positive rod with a tendency to form long, snaking chains. Colony morphology is opaque to off-white, and often described as having irregular borders or protrusions with a "ground glass appearance" [10].

*B. anthracis* is the only obligate pathogen of the *Bacillus* genus. The bacterium is widespread geographically and is primarily a zoonotic disease. Anthrax is most frequently a disease of grazing ruminants, though any mammal is susceptible to infection [24]. Human infections are largely acquired as a result of occupation, such as exposure to infected livestock or contact with contaminated textiles. Due to a massive livestock vaccination effort, disease is infrequently encountered in developed countries [24].

The current research interest in this organism is due to its potential use as a bioterrorist agent. In 2001, spores were deliberately unleashed through the United States mail. Twenty-two individuals were exposed, and due to swift administration of antibiotics, all but five survived [20]. However it is evident that if aerosolized spores were dispersed in a large scale assault, medical supplies could be exhausted and the economical impact could be devastating [24].

In the environment under nutrient deprivation or other unfavorable conditions, the microbe assumes the endospore state, which is the state capable of initiating anthrax disease. Upon arrival in a mammalian host, these spores germinate into vegetative bacilli and can cause serious, uncontrollable infection. Entry of these spores into a human host can occur by three routes with drastically different clinical outcomes.

Cutaneous anthrax is a response of spore infiltration into open wounds or abrasions of the skin. Painless papules with accompanying edema present days after contact with spores, eventually transforming into a black eschar [20]. With early intervention, this form of the disease is rarely fatal with a mortality rate of only 1% [24]. Gastrointestinal anthrax occurs after consumption of spore-tainted meat, and is exceedingly rare outside of underdeveloped countries. Clinical presentation is that of intense abdominal pain, lower GI tract bleeding and hematemesis [23]. This form of anthrax frequent results in fatality, as the vague symptoms cloud the true diagnosis, causing a delay in proper treatment [20, 24].

Inhalational anthrax is the most severe form of disease, resulting from the entrance of spores into the respiratory tract. Spores small enough to evade clearance by the mucociliary system in the upper respiratory tract journey down to the alveoli of the lungs [20]. There they are consumed by alveolar macrophages as well as dendritic cells, which transport them to the mediastinal lymph nodes [22].Germination occurs as the spores assume the vegetative state and escape from the phagocytes. Toxins are synthesized and bacilli multiply until the lymph node environment is overwhelmed, causing a surge of toxins and bacteria to enter circulating blood. Host tissue is damaged by edema, hemorrhage, and necrosis, and eventually septic shock ensues [20].

The exceptional virulence of *B. anthracis* stems from the toxins and capsule synthesized by genes residing on the microbe's plasmids, pOX1 and pOX2. Both plasmids must be present in order for full virulence expression [1]. pOX2 carries the genetic information needed to synthesize a poly-γ-D-glutamic acid capsule. Three genes are required for the formation of the capsule, which likely functions to elude phagocytes of the innate immune response [24]. pOX1 encodes two protein toxins, edema toxin and lethal toxin, composed of lethal factor, edema factor and protective antigen. These subunits assemble together on host cell membranes where they exert their deleterious effects [1, 20, 24].

Edema factor is a calmodulin dependent adenyl cyclase that functions to convert ATP to cAMP. When cAMP levels begin to escalate, water homeostasis and cellular signaling is disrupted, leading to edema and a depressed immune response from phagocytes [20]. Lethal factor is a zinc metalloprotease that cleaves mitogen-activated protein kinase kinases, again causing a major disruption of cellular signaling. Lethal factor has also demonstrated the ability to lyse macrophages [1, 20]. Protective antigen is the central constituent of the toxin that works with the other factors in a binary fashion to damage host cells. The factors bind to the protective antigen to produce toxins that are imported across cell membranes into the cytosol where they will inflict their damage, chiefly by downregulating cellular signaling processes [1]. Though antibiotics may be effective in destroying the vegetative bacilli present in infection, it is the overabundance of these toxins that will overwhelm the host [1].

**Transposon mutagenesis**

One of the most popular and efficient tools used to study microbial genetics is the process of transposon mutagenesis. Transposons are mobile DNA fragments that possess the ability to randomly insert into different positions of genomic DNA. This insertion causes mutations that disrupt gene function and often alters the organism's phenotype. The simplest form of a transposon is comprised of a fragment of DNA bordered by sequences of inverted repeats [8]. These terminal repeats are discerned by a transposase enzyme which allows the transposon to reintegrate within the genome.

In forward genetic studies, the desired outcome is to produce an array of mutants each containing random insertions. These mutants can be grouped into a transposon library, with each cell in the library containing one fragment of an organism's genome. Thus, the entire genome is represented in a population [23]. This library of amassed isolates can then be screened for imperfections in a specific phenotype of interest. The subsequent recognition of genes that were disrupted by these transposon insertions is then suggestive of their role in that specific

phenotype [4].

Historically, *B. anthracis* libraries were created using transposon *Tn917*, though it was established this transposon had a propensity to insert into plasmid sequences rather than genomic DNA [25]. Transposon *Tn10* has also been used to create mutant libraries, and though the randomness of insertions was enhanced, the incidence of plasmid retention could be subject to improvement [26]. Ultimately, *mariner*-based transposons have proven to be the most proficient at generating random insertion libraries for *B. anthracis* and other microbes [2, 4, 12, 13, 16, 25, 26].

**Project rationale**

How exactly *B. anthracis* germinates and grows into its vegetative form is a poorly understood concept, and *in vivo* nutritional requirements are not clearly defined. Much of the research on this organism has been focused on its virulence factors and vaccine development. Metabolism is essential to the production of virulence factors which result in a disease, and is likely adjusted by the pathogen based on nutrient availability and host-pathogen interactions. The roles of carbon, sugar, and fatty acid metabolism have only recently begun to be appreciated for potential contributions to pathogenicity [6].

This project aims to begin an investigation into the metabolic requirements of *B. anthracis* that help it to flourish so effectively in the host. By identifying what the bacterium requires to thrive and what metabolic pathways are employed, perhaps we can better understand how to inhibit its growth. Thus, the goals for this project were to create a library of *B. anthracis* mutants using a *Himar1* transposon system, develop and conduct screening assays to detect growth deficiencies in minimal media, and identify specific mutants that can be further characterized based on the future mapping of insertion sites.

## MATERIALS AND METHODS

### Bacterial strains and growth conditions

The avirulent 34F2 Sterne strain of *Bacillus anthracis* ($pOX1^+$, $pOX2^-$) was used in this study. *B. anthracis* was routinely grown on Brain Heart Infusion (BHI) media or in BHI broth at 37°C. *Escherichia coli* strains DH5α and SCS110 were grown at 37°C on Luria-Bertani (LB) media supplemented with erythromycin (400 μg/ml) or ampicillin (100 μg/ml) where appropriate.

| *E. Coli* Host Strain | Plasmid | Antibiotic Resistance | Description |
|---|---|---|---|
| SCS110 | pRP1083 | Erythromycin | Transposon Vector |
| DH5α | pSS1827 | Ampicillin | Conjugation Helper |

Table 1: Characterization of *E. coli* strains employed in transposon mutagenesis

### Transposon delivery vector

The *mini-Himar1* transposon was delivered to *B. anthracis* via the plasmid delivery vector pRP1083 obtained from researchers at the Center for Biologics Evaluation and Research, Food and Drug Administration. pRP1083 uses shuttle vector pBKJ236 as its backbone, which harbors temperature sensitive replication machinery for contingent replication in *B. anthracis*, erythromycin resistance, and an origin of transfer for conjugation [7]. *Himar1* C9 transposase and a kanamycin resistance gene were components incorporated from pMarB previously used in the mutagenesis of *Bacillus subtilis* [12]. The transposable cassette consists of the kanamycin resistance gene flanked by *mariner* inverted repeat sequences that allows for recognition by the transposase enzyme, and an R6Kγ origin of replication situated between these inverted terminal repeats [16]. The map of the plasmid vector is shown in Figure 1.

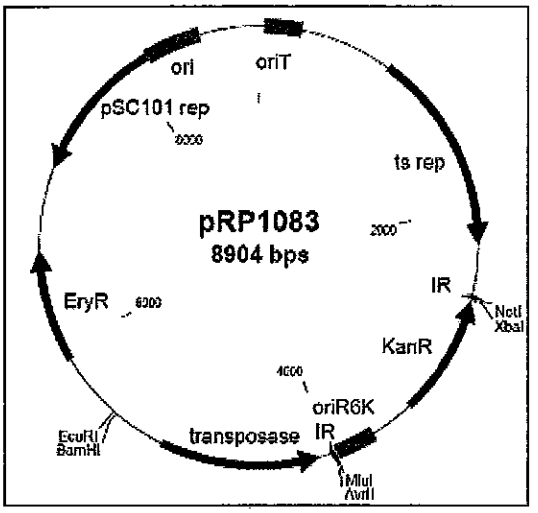



Fig. 1. Vector map of pRP1083. Vector contains temperature sensitive replication machinery derived from pBKJ236 allowing plasmid transfer at 24°C but not at 37°C, *Himar1* C9 transposase, erythromycin resistance, and an origin of transfer for conjugation. The transposable cassette encodes a kanamycin resistance gene used as a selectable marker, an R6K origin of replication and several restriction sites positioned within the inverted repeats that denote the ends of the transposon that can be utilized in future alterations [16].

## Conjugation

*B. anthracis*, *E. coli* SCS110 and DH5α were streaked concurrently in a "triparental cross" [1] on BHI and incubated for 24 hours at room temperature. Growth from this media was streaked onto BHI supplemented with erythromycin (5 μg/ml) and polymyxin B (60 units/ml) and incubated at room temperature for 48 hours. *B. anthracis* colonies were identified as being well separated from nonresistant background growth and used to inoculate 5 ml of BHI broth containing erythromycin (5 μg/ml). Broth culture was incubated at the non-permissible plasmid replication temperature of 37°C on a shaker overnight. Broth was then centrifuged for 10 minutes at 3000 rpm to pellet the cells. The supernatant was removed and the pellet was resuspended in fresh BHI. This wash was repeated and the resulting pellet was again resuspended in BHI. Serial dilutions were prepared beginning with a 1:10 dilution using 50 μl of overnight broth culture added to 450 μl BHI. 50 μl of this 1:10 dilution was then added to 450 μl BHI broth, and this process was carried out to a $10^{-8}$ dilution. 100 μl of each dilution was plated

to BHI supplemented with kanamycin (20 μg/ml) and incubated overnight at 37°C.

**Isolation of mutants**

Kanamycin resistance encoded on the transposon was used as a selectable marker for this study. Transposant candidates were initially selected on BHI supplemented with kanamycin (20 μg/ml). Colonies were propagated by picking and restreaking onto BHI supplemented with the same antibiotic concentration as previously described and grown overnight at 37°C.

**Growth assay development and minimal media preparation**

R and XO minimal media [19, 5] were used in this study to identify transposants that showed growth impairment relative to wild type *B. anthracis*. XO media was prepared as previously described with minor modifications. Our preparation of XO media contained glucose (2.5 g), ferric ammonium citrate (20 μg), glycine (100 μg), L-methionine (20 μg), L-proline (20 μg), L-serine (20 μg), L-threonine (20 μg), L-glutamic acid (1 mg), $(NH_4)_2SO_4$ (1 mg), sodium citrate (500 μg), $KH_2PO_4$ (6 mg), $K_2HPO_4$ ( 2 mg), $MgSO_4$ (.0025 mg), $MnSO_4$ ( .00125 mg). The final pH of the media was 5.7. R media was prepared following the recipe outlined by Ristroph and Ivins [19]. Initial pH of solution was 8.3, which was adjusted to 8.0 using dilute HCl. Both media were filter sterilized under positive pressure using a 0.2 μm pore size filter (Corning), aliquoted in 100 ml portions and stored at 4°C.

To assess the growth characteristics of wild type *B. anthracis* in these minimal media compared to rich media, optical density (OD) measurements were recorded using a BioRad SmartSpec 3000 spectrophotometer. 1 ml of overnight growth was added to 10 ml of BHI, R,

or XO media. Broths were placed on shaker and incubated at 37°C for 1 hour prior to the first OD measurement. Readings were taken every hour over a 5 hour time period, and after each reading the broths were returned to the incubated shaker. Before each reading, the spectrophotometer was blanked using uninoculated media. Three trials using each media were carried out, and an average of the trials was used to construct a graph in Microsoft Excel.

**Growth curve preparation and data analysis**

Wild type *B. anthracis* was grown overnight at 37°C in sterile Falcon tubes containing 2 ml of BHI broth. Arbitrarily selected mutants were grown overnight at 37°C in sterile Falcon tubes containing 2 ml of BHI supplemented with kanamycin (20 μg/ml). 1:5 dilutions were prepared containing 100 μl overnight growth and 400 μl of each media to be assayed. 150 μl of each preparation was added to 96-well microtiter plate wells (Corning). Three wells containing the same mutant in each media were used in each trial. Control blanks of uninoculated media were also run in triplicate. Microtiter plates were incubated at 37°C in a VersaMax tunable plate reader programmed to record absorbance readings at 600 nm. Readings were obtained every 30 minutes over a 24 hour time period with three seconds of shaking in between each reading. Data was acquired using SoftMax Pro® software and averages of the triplicate well sets were obtained using Microsoft Excel.

**Sporulation assay**

Overnight cultures of wild type and transposant mutant *B. anthracis* were grown in BHI and R media. A tenfold dilution of overnight growth for each strain was prepared, and serial

dilutions were subsequently made by transferring 50 µl of the previous dilution into fresh media to a final dilution of $10^{-5}$. 100 µl of each dilution was plated to BHI. The remaining solution was heat treated for 20 minutes at 65°C to eliminate vegetative cells. Again, 100 µl of each of these heat treated dilutions were plated to BHI. Plates were incubated at 37°C overnight and colony counts were obtained.

**General molecular biology techniques and Southern Blot analysis**

Three randomly selected mutants identified as having growth impairment were subjected to Southern Blot analysis. Wild type 34F2 was included as a negative control. Genomic DNA was isolated from select transformants and 34F2 using the Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions. 40 µg of genomic DNA was digested with EcoRI overnight and resolved on a 1% agarose gel. DNA was denatured for 30 minutes in solution of .5 M NaOH and 1.5 M NaCl and neutralized for 30 minutes with .5 M Tris-HCl and 1.5 M NaCl. DNA was then transferred to a positively charged .45 micron nylon membrane (Magna) via capillary transfer in 20X SSC buffer overnight. Fixation of the DNA to the blot was done by cross linking with UV light.

A probe targeting the kanamycin resistance gene on the transposon was synthesized by PCR amplification using the plasmid shuttle vector pABG5 as a template. This plasmid harbors genes that encode resistance to kanamycin. Primers used were 5'Kan-2SmaI TCC CCC GGG CTA TAG AAT GGG CAA AGC and 3'Kan-2-SmaI TCC CCC GGG GAC ATC TAA ATC TAG GTA C. The probe was then purified using the Genejet™ Gel Extraction Kit (Thermo

Scientific) following manufacturer's protocol. The probe was labeled using the DIG Gel Shift Kit (Roche) following manufacturer's instructions and denatured at 100°C for 5 minutes. Membrane bound DNA was hybridized with 25 µl probe in a heat-sealed bag at 55°C in a rotating water bath overnight. Chemiluminescent probe detection was performed using the DIG High Prime DNA Labeling and Detection Starter Kit (Roche) following the manufacturer's recommendations. Visualization was achieved using a BioRad ChemiDoc™ XRS with Image Lab™ software.

## RESULTS

### Creation of transposon library

The first goal in our investigation of *B. anthracis* metabolic requirements required the generation of mutant library. This was facilitated by the use of the forward genetic tool of transposon mutagenesis. We used a mini-*Himar1* based transposon system, as it has been shown to efficiently produce random insertions in a variety of microbes. Plasmid pBKJ236, originally used in *B. anthracis* allelic exchange studies by Janes and Stibitz [7], served as the vector backbone in the design of pRP1083 [16] used in our research. pBKJ236 was engineered to contain temperature dependent replication machinery, where plasmid replication is allowed at room temperature but hindered at 37°C. Modifications to this backbone came from elements of pMarB [12], which were a *Himar1 C9* transposase and a kanamycin resistance gene.

A key attribute of this transposon system is that the transposase gene is situated outside of the transposable element itself. The gene resides on the plasmid backbone, allowing for transposon insertions to remain settled without continuous selective pressure. This transposable element contains a kanamycin resistance gene as a selectable marker that could be followed throughout this study. Thus plating to BHI with kanamycin was the final positive selection step in our library creation, as growth on this media indicates retention of the transposon.

### Isolation of mutants

Kanamycin resistance encoded on the transposon serves as the positive selection marker in the identification of transposant candidates. Candidates were passaged to BHI media supplemented with kanamycin in two rounds at the non-permissible plasmid replication temperature of 37°C. Colonies growing in the presence of antibiotic after these successive passages were selected for inclusion in our mutant library.

A library of nearly 400 mutants was rapidly generated, with transposition occurring at a frequency of $1.4 \times 10^{-6}$ per cell, comparable to frequencies reported in other *Himar1* based studies

[13, 16]. Transposition frequency was calculated by dividing the colony count on BHI with kanamycin by the number of colonies on BHI without antibiotic. Mutants were placed in storage at -80°C in BHI broth with the addition of 20% glycerol prior to analysis of growth kinetics.

**Development of growth assay**

Before screening the mutant bank for growth impaired isolates, the growth kinetics of vegetative wild type 34F2 in minimal media were examined. Optical density measurements using a spectrophotometer were taken hourly at an absorbance of 600 nm, and this data was plotted to produce a growth curve (Figure 2). Growth in nutrient rich BHI produced the classic bacterial curve typified by a lag, log, and stationary phases, with entrance into the stationary phase after six hours. Bacilli maintained in R media had significantly less exponential growth, reaching absorbance levels that were 50% less than that observed in protein-rich BHI. Growth in XO media was almost nonexistent in these trials, with only a minute increase of absorbance levels noted between hour one and two and stationary phase entrance at hour three.

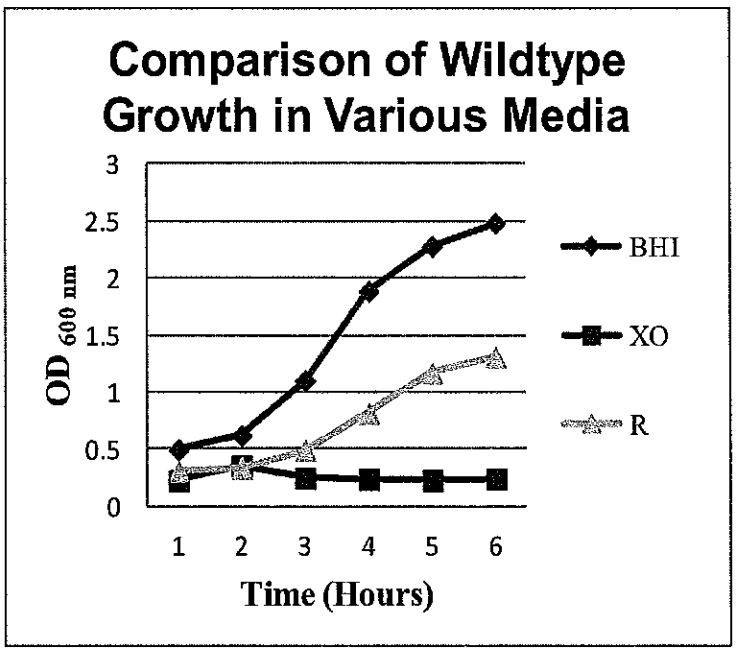



Fig. 2. Kinetic growth analysis of 34F2 in nutrient rich and minimal media. Optical density measurements were taken hourly at 600 nm.

**Phenotypic screening**

Mutants identified as kanamycin resistant were grown in BHI, minimal XO and R media in microtiter wells, with optical density measurements collected every thirty minutes over the course of 24 hours. An increase in optical density correlates with an increase in bacterial population. As bacterial cells amplify in number, light is scattered more and the spectrophotometer detects higher absorbance values [17]. Turbidity of the media is a visual indication of increased bacterial proliferation (Figure 3).

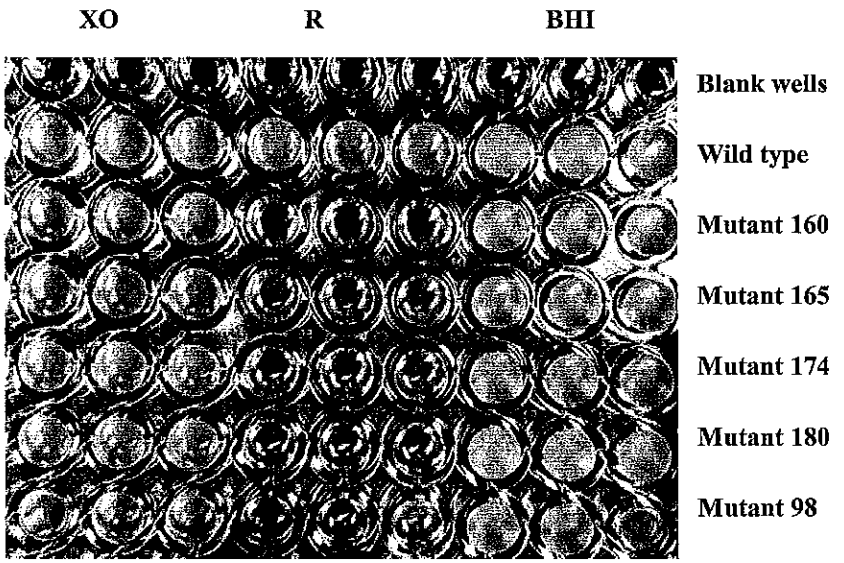



Fig. 3. Microtiter wells containing BHI, R, and XO media. Growth is indicated by turbidity of media.

In nutrient rich BHI, mutant growth did not approach the same levels as 34F2 achieved, though growth was still appreciable (Figure 4). Overall, mutants grew poorly in R media (Figures 5-7). Only two of the screened isolates showed any appreciable growth, which was still nearly 60% less than wild type. Nine isolates failed even to reach an absorbance level of 0.1. Those isolates that grew expressed similar growth kinetics of short log phase and extended stationary phase. Entrance into stationary phase was quite variable, with some isolates entering as early as five hours into the trial, and some reaching this phase after fifteen hours. Wild type 34F2 was strikingly similar in these trials, which was in contrast to initial observations in the assay development trials where growth was much more appreciable in R media.

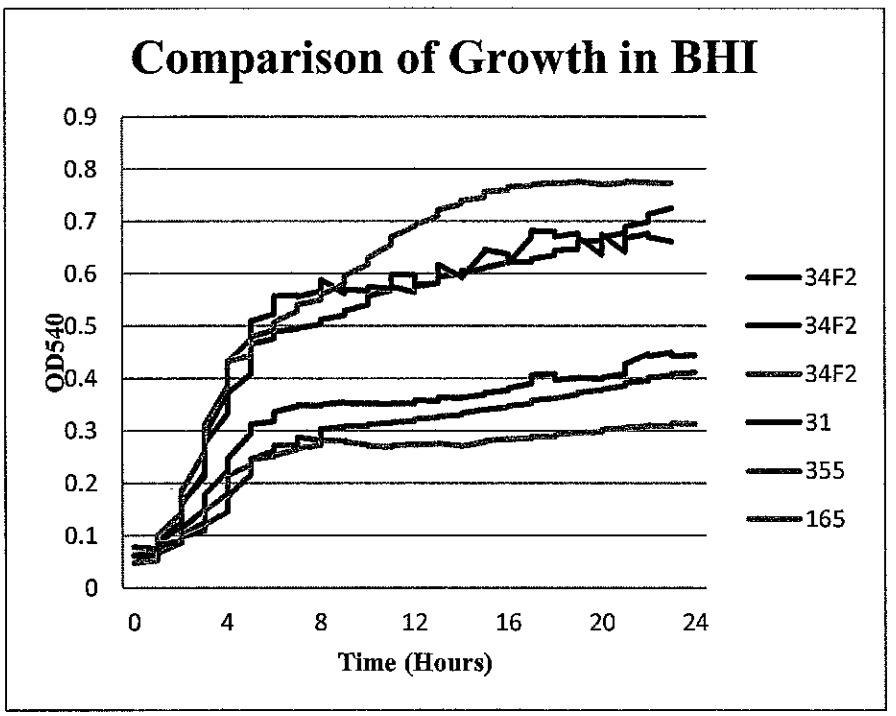
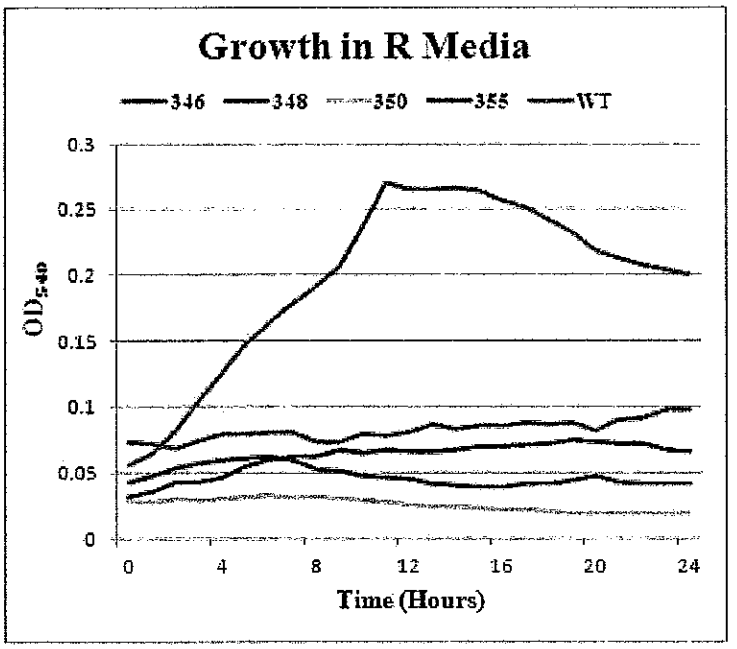
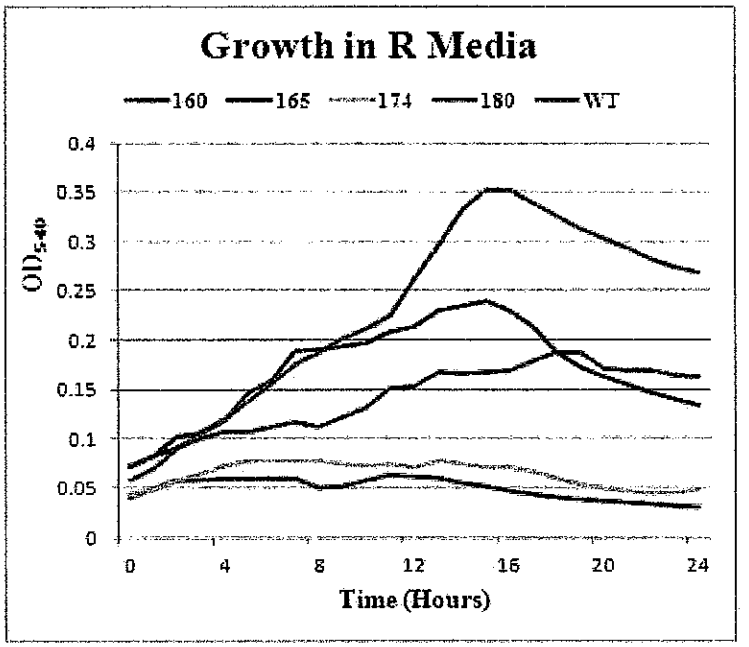
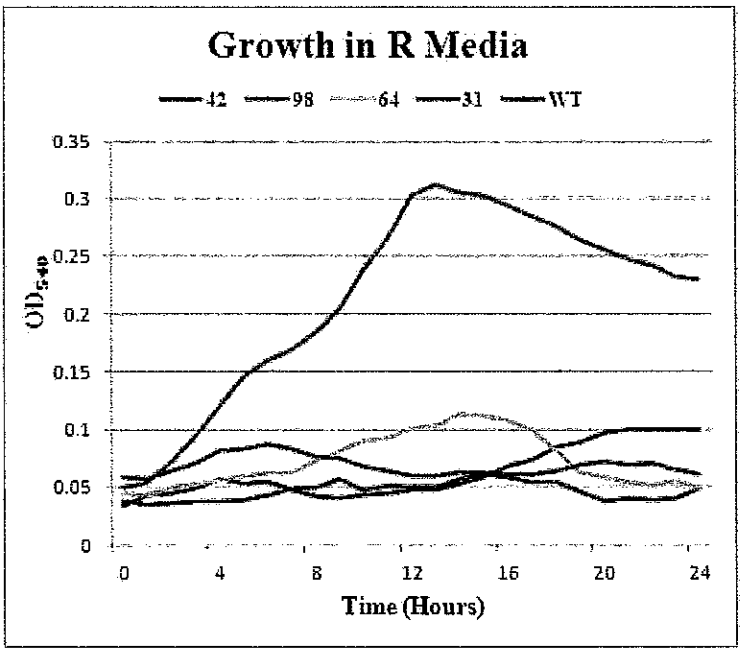


Fig. 4. Wild type 34F2 growth in BHI compared to mutant isolates 31, 165, and 355.




Figs. 5-7. Mutant growth in R media. Wild type 34F2 shown in light blue for comparison.

XO is a more minimally defined media; however growth levels of mutants were higher in this media than in R media on average (Figures 8-10). In contrast to mutant growth in R media, only two isolates failed to reach absorbance level readings of 0.1. Mutants also transitioned into stationary phase at a much earlier time point, averaging around hour twelve.

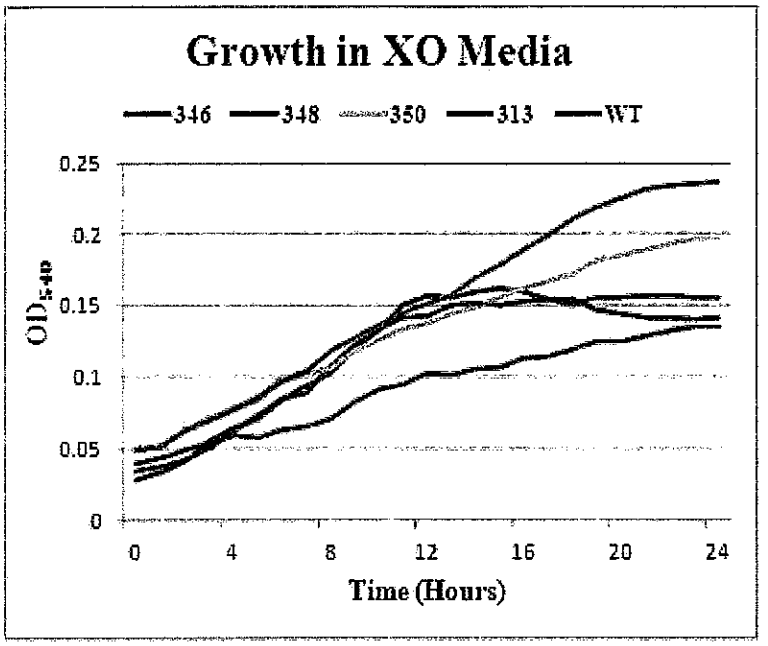
These growth assays were conducted with mutants kept in the presence of kanamycin, as well as trials using media free of antibiotics. Data was similar for both circumstances, indicating stability of the transposon insertions with or without prolonged selective pressure.

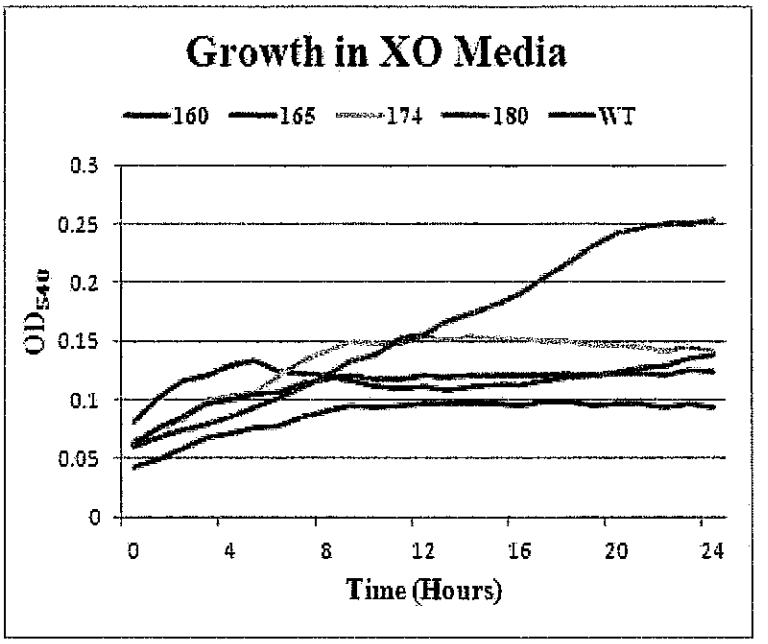
**Sporulation assessment**

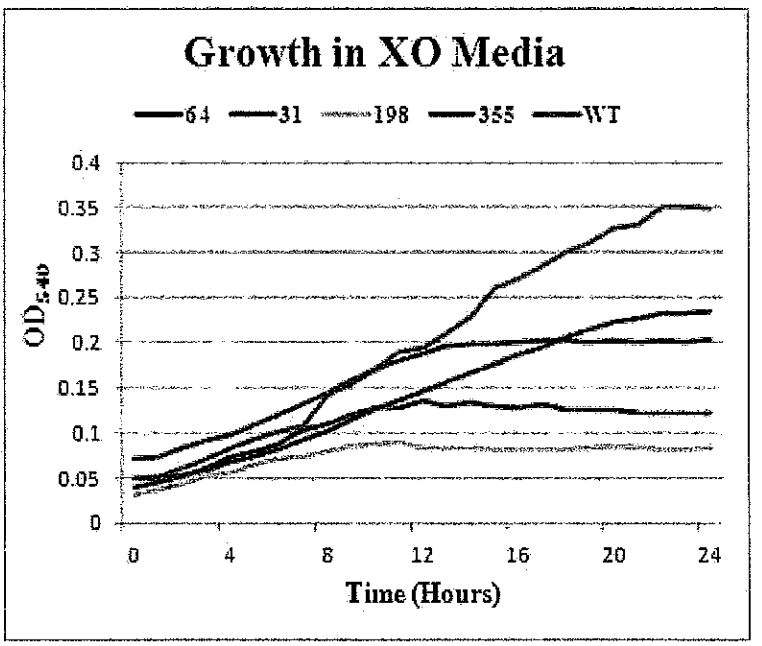
A sporulation assay was performed to compare mutant and wild type sporulation in rich and minimal media to discover if different growth responses were related to sporulation differences. This assay is used to establish the number of colony forming units (CFU) that were in either spore or vegetative state. To calculate CFUs/ml, the number of colonies visible on heat treated plates was divided by the number of colonies present on unadulterated BHI plates. Data from this assay is presented in Table 2. As expected, wild type and mutant *B. anthracis* both showed negligible sporulation in protein-rich BHI. Interestingly, the mutant isolate had a lower spore percentage than wild type in minimal media. Looking at its performance in the phenotypic screening growth assays, the mutant was also more growth deficient in R media than XO media.

| Strain and Media | % Spores |
|---|---|
| Wt 34F2 BHI | 2.4% |
| Wt 34F2 R | 62% |
| Mu 31 BHI | 1.8% |
| Mu 31 R | 45% |

Table 2. Spore percentage of wild type and mutant isolate in BHI and minimal R media.




Figs. 8-10. Mutant growth in XO media. Wild type 34F2 shown in light blue for comparison.

## Verification of transposition

Three randomly selected isolates demonstrating kanamycin resistance were subjected to Southern Blot analysis (Figure 11). Genomic DNA was extracted and cut with EcoRI, a restriction enzyme that does not cut within the transposon itself. Hybridization of digested DNA with a probe specific for the kanamycin resistance encoded on the transposon resulted in single bands of various sizes for two of the selected isolates, while one isolate showed the presence of two bands. Wild type 34F2 was used as a negative control and a hybridization signal was not distinguishable.

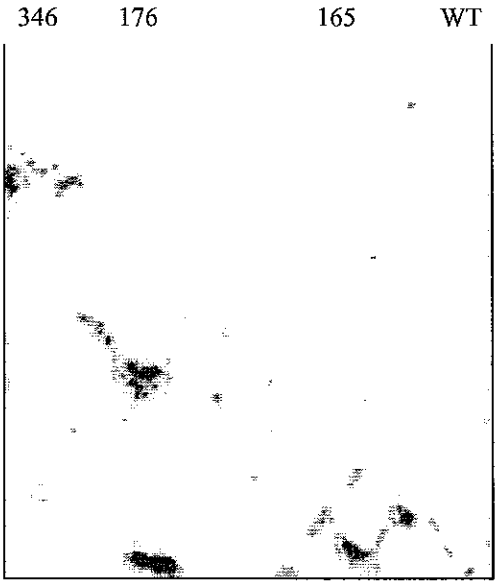



Fig. 11. Southern Blot analysis of isolates 346, 176, 165, and wild type (WT).

## Investigation of a novel phenotype

In the course of this study, one of the transposants demonstrated production of a unique pink pigment when growing on BHI supplemented with kanamycin. Colonies originated as off-white and small, with their appearance becoming altered after approximately 48 hours of incubation. The colony morphology apart from the pigmentation was typical of *B. anthracis* (Figure 12). After 48 hours, colonies appeared large with irregular edges and were off-white with pigment originating from the center of the colonies (Figure 13). The isolate grew well on Blood agar, and demonstrated some hemolytic activity not classically associated with *B. anthracis*. PCR was performed to detect the lethal factor coding *lef* gene found on pOX1, for which the isolate was positive.

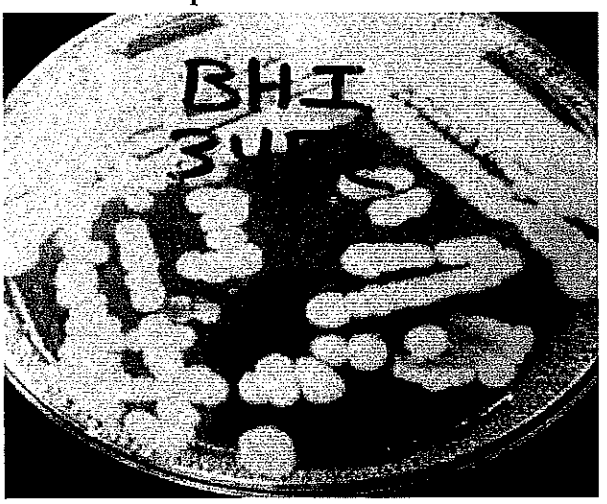



Fig. 12. Wild type strain 34F2 on BHI agar after 48 hours of growth.

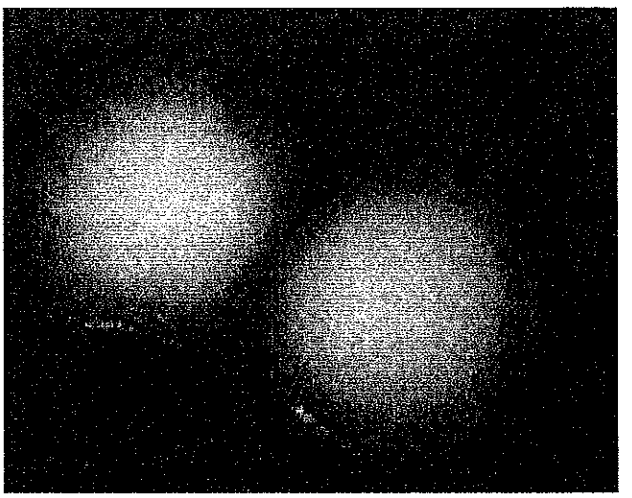


Fig. 14. Isolate 346 colonies on Blood agar after 48 hours of growth.

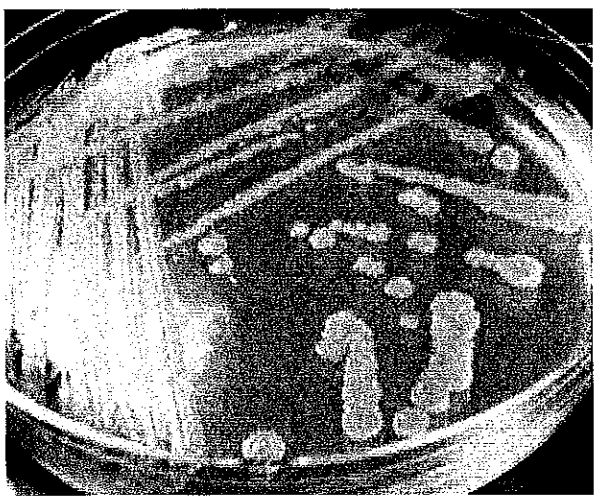


Fig. 13. Pigmented isolate 346 on BHI after 72 hours of growth.

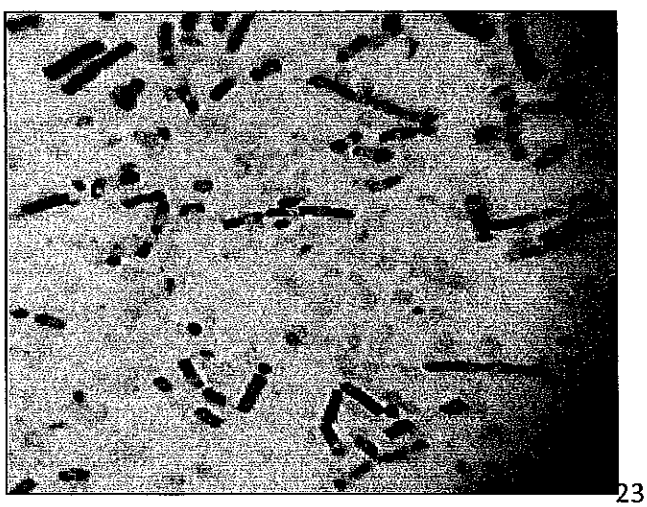


Fig. 15. Sporulating mutant 346 from BHI plate after 72 hours of growth.

## DISCUSSION

*Bacillus anthracis* is a highly virulent pathogen that causes rare but lethal infections in humans. Elucidating host-pathogen interactions is of great significance to researchers due to its potential use in acts of bioterrorism. Though many research endeavors have focused on the organism's virulence factors, there is still much to uncover regarding the metabolism and nutritional requirements of *B. anthracis*. Optimal management of nutrient acquirement and metabolism are likely paramount in host niche dominance of microbes associated with *in vivo* infections [6]. By better deciphering what the organism is using to survive, perhaps this knowledge could be used to inhibit its growth *in vivo*. The principal objective of this project was to create a library of *B. anthracis* mutants using transposon mutagenesis that could be phenotypically screened for growth deficiencies. In order to generate this bank of screenable mutants, a *Himar1* transposon was employed.

*Himar1* is a member of the *mariner* family of transposable elements, originally extracted from the horn fly *Haematobia irritans* [11]. They are exceptionally powerful mutagenesis tools due to their minimal requirements for transposition and demonstrated ability to insert randomly across a wide array of prokaryotic genomes [11, 15]. In vitro transposition is driven simply by the transposase enzyme, with transposition occurring via a cut and paste reaction when the transposase enzyme interacts with terminal repeat sequences [15]. The transposon is excised and relocated to a new genomic position, which is almost exclusively at a TA dinucleotide, making it well suited for use in bacteria with a low GC content such as *B. anthracis* [11, 15].

We utilized a transposon delivery system based upon temperature sensitive plasmid transfer where a *Himar1* transposon was delivered from donor *E. coli* to recipient *B. anthracis*. Therefore conjugation was carried out at room temperature where transfer is permissible. Subsequent plating of growth from the conjugation plate to media containing polymyxin B allows for negative selection, as *E. coli* is susceptible to this antibiotic and *B. anthracis* can

tolerate its presence in moderate quantities [1]. Colonies apparent at this stage were then transferred to broth with erythromycin and moved to a 37°C incubation, allowing for inhibition of plasmid replication.

Mutants that demonstrated resistance to kanamycin and were susceptible to erythromycin were considered positive for transposition. Arbitrarily selected mutants were then screened in minimal R and XO media to detect isolates that could not grow when faced with limited nutrients. Growth assays were performed in both the presence and absence of kanamycin to establish the stability of the selectable marker, and results for each experiment were similar. This indicates that the inclusion of kanamycin in initial screenings was not a variable that inhibited growth.

In the assay development trials, wild type 34F2 grew much more in R media, reaching absorbance levels four times greater than levels observed in XO media growth. However, in the phenotypic screening assays, wild type growth in these medias were strikingly similar in all of the conducted trials. The pronounced difference in initial development trials may be related to differences in the assays used for optical density measurements. For the assay development trials, the bacteria were allowed more oxygen exposure and more shaking of the broth media. The pronounced growth in R media in that assay may indicate that the supplied nutrients are better utilized under more aerobic conditions. R media contains seventeen amino acids, glucose as a carbon source, nucleobases adenine and uracil, and various inorganic salts [19]. XO media is the most minimal defined media for growth of *B. anthracis*, containing six amino acids, glucose as a carbon source, several inorganic salts, and ferric ammonium citrate as a source of iron [5].

Mutant growth in protein rich BHI was more pronounced than in minimal media as expected. Though growth was not identical to wild type strains, all isolates doubled, if not tripled in growth compared to minimal media absorbance levels. An ideal transposant would grow with some normalcy in BHI and struggle in minimal media. This would illustrate a transposon that

disrupted a gene in a metabolic pathway essential to growth in minimal environments but not nutrient rich environments.

Mutant growth in minimal media was overall less efficient than wild type 34F2, with a more pronounced deficiency in R media. Nine isolates failed to reach absorbance levels of 0.1 in this media in comparison to two isolates in XO media that did not reach this level of growth. Higher growth was initially expected in R media as it supplies a variety of amino acids which are hypothesized to play a major role in *B. anthracis* metabolism. The fact that mutants grew less efficiently in R media than XO media may indicate a disruption in an amino acid pathway that allows for *B. anthracis* to thrive. The addition of an iron source in XO media may also explain the heightened growth in this media as it has been proposed to be the most critical element in bacterial fitness.

Nutritional requirements for *B. anthracis* are ill-defined and likely vary between strains [10]. It is also important to recognize that the wild type strain 34F2 is attenuated in that it is void of plasmid pOX2. This plasmid contains 78 genes with no assigned function, which may end up contributing to *B. anthracis* metabolic processes [1].Amino acids have been suggested as being essential in *B. anthracis* metabolism based on the identification of multiple amino acid efflux systems, transporters, and utilization genes not seen in other *Bacillus* genomes [18]. Genes encoding an array of proteases and peptidases also suggest *B .anthracis* is suited for existence in a protein-rich environment [18].

Iron is widely accepted to be the most important nutrient in bacterial fitness [1, 3]. Iron deprivation has been shown to result in diminished expression of inorganic ion metabolism and an increase in amino acid metabolism in *B. anthracis* [3]. *B. anthracis* has a multitude of iron-acquisition genes, most notably genes focused on the production of siderophores [18]. These iron-scavenging compounds are a method of coping with the limited bioavailability of an element crucial for bacterial survival [1, 3].

Verification of transposon integration was conducted by means of a Southern Blot to detect the kanamycin resistance encoded on the transposon. Three isolates were assayed, and two revealed single bands indicating hybridization of the probe to genomic DNA. The other isolate showed what appeared to be two separate bands, suggesting a double insertion. The bands were of different sizes which typically corresponds to random insertion, though insertion site mapping is needed to definitively confirm random insertion.

Screening for altered colony morphology was also conducted during the course of the growth deficiency screens. One isolate producing a pink pigment after 48 hours of incubation was identified. This isolate was also grown on Blood agar (Figure 14) and media containing crystal violet to check for any other bacterial contamination. Growth readily occurred on blood agar with some degree of hemolysis and was absent on crystal violet media. The lack of growth on media with crystal violet further supports the identification of the bacterium as being gram positive, as these organisms will not thrive due to the inhibitory nature of crystal violet. The hemolysis observed on blood agar from our isolate is not normally associated with *B. anthracis* in conventional identification schemes. In fact, beta-hemolysis has classically been used to distinguish *B .anthracis* from *B. cereus* [10]. However, hemolytic activity has been seen in non-clinical isolates and hemolysins have been identified in the Ames strain sequencing project and other studies [18, 21].

Several other species of *Bacillus* have been identified as pigment producers [9, 14]. Outside of a host, pigment synthesis is believed to play a role in spore survival, as pigmentation offers further defense from UV damage [14]. Spores dwelling *in vivo* may also benefit from pigmentation, as this may confer resistance to oxidation from phagocytes [9]. A gram stain was made of pigmented colony growth and indeed revealed sporulating, gram positive bacilli (Figure 15). In depth characterization of this pigment cannot be concluded in the absence of specific biochemical tests, though based on other studies we assume it to be a carotenoid [9, 14].

Future work is still necessary to completely characterize the mutant isolates. DNA sequencing from the inserted transposon will pinpoint the location of the transposon using the completed *B. anthracis* genome sequence [18]. Sequencing will also provide insight on the randomness of insertions and allow for any consensus sequence present to be revealed, apart from the initial TA dinucleotide insertion site that is to be expected with *Himar1* transposons [11, 15].

Complementation studies involving reconstruction of the mutants back into the wild type strain can be completed in order to more efficiently characterize the phenotype. Ideally, the transposon has interested into a gene of interest that resulted in an altered phenotype. Once it is realized where this transposon has inserted using DNA sequencing, a mutant can be constructed that is lacking that disrupted gene by plasmid manipulation. However transposons can not only affect the gene they directly insert into, they may cause a downstream effect. Therefore the creation of a mutant that stays within frame is critical to ensure that the phenotype is not a result of a downstream effect. Further characterization implementing tissue culture techniques can also be done to demonstrate inhibited growth in a minimal cellular environment. This will better illustrate if growth deficiency would be discernible in an *in vivo* infection.

We believe our results at this stage highlight the usefulness of *Himar1* transposons to generate banks of screenable mutants to probe the genetic foundation of *B. anthracis* metabolism. Our investigation has revealed several growth impaired isolates, as well as one isolate with altered colony morphology. Future works for this project are extensive. Modifications can be made to the transposon system in order to add reporter genes or phage promoters to provide more insight on *B. anthracis* physiology. We hope that identifying metabolic pathways needed by the organism for successful infection will reveal novel bacterial enzyme targets that can be utilized for future therapies.

**SUMMARY**

*Bacillus anthracis* is a highly virulent pathogen that is proficient at evading host defenses and multiplying to lethal levels. The bacterium's metabolism and nutrient requirements are ill defined, as are the details regarding early stages of infection when spores germinate into their vegetative state intracellularly. Recognizing what the bacterium requires for survival within the host will allow for the development of novel countermeasures. This study focused on the creation of a mutant *B. anthracis* library by utilizing the forward genetic tool of transposon mutagenesis. A bank of nearly 400 mutants was generated, with transposition occurring at a frequency of $1.4x10^{-6}$ per cell. These mutants were screened for growth deficiencies in minimal media environments. Arbitrarily selected mutants that demonstrated defective growth were analyzed by a Southern Blot, which revealed random transposon insertions. A novel pigment producing phenotype was also encountered in the course of this study. Future directions stemming from the creation of this library are extensive. DNA sequencing of the insertions and implementing tissue culture techniques to simulate an *in vivo* infection need to be performed in order to fully characterize the growth impaired mutants.

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