IDENTIFICATION OF KNOCK-OUT GENES TO CREATE A TEMERATURE SENSITIVE MUTATION IN pMR10 PLASMIDS

A Senior Scholars Thesis

by

SARAH KATHERINE MURPHY

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

April 2011

Major: Biomedical Science

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Approved by:

Research Advisor: Thomas Ficht
Director for Undergraduate Research: Sumana Datta

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ABSTRACT

Identification of Knock-Out Genes to Create a Temperature Sensitive Mutation in pMR10 Plasmids. (April 2011)

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Brucella spp. is a gram-negative, cocobacillus, non-motile bacteria that causes a zoonotic disease called brucellosis. Cases of brucellosis remain prevalent in Middle Eastern countries, and it is the most common laboratory-acquired infection in the United States. It typically causes a mild infection but if left untreated it can lead to chronic problems in both humans and animals. There is currently no vaccine against brucellosis approved for human use; because of its highly communicable nature and global prevalence, one is needed. Finding the knock-out genes for temperature sensitivity in a broad-host-range plasmid would be an asset in developing a *Brucella* mutant for use in a vaccine. In this study random mutation with hydroxylamine was preformed in vitro on the broad host range plasmid pMR10. Mutated plasmids were then transformed into DH5-α competent cells and individual colonies were isolated and grown in nutrient broth. The broth was stamped onto agar plates and grown at selective (42°C) and non-selective (37°C and 30°C) temperatures. The plates were then screened for growth at the lower or non-selective temperatures and no growth at higher or selective temperatures.

Of the 23,000 colonies screened in the project none were found to be temperature sensitive.

DEDICATION

This thesis is dedicated to my family. To my mother who taught me no dream is too big. To my father who made me realize being a nerd is actually cool. And, to my brother who was always there to swap lab stories with me. Without their love, support and guidance I would not have been able to accomplish this, thank you.

ACKNOWLEDGMENTS

This project could never have been completed without support from the amazing people around me; I would like to thank them all for their help.

First, I would like to thank Dr. Ficht for being a truly great mentor. He not only encouraged me to apply for the program, but guided me the entire process. The experiences and knowledge I have gained while working under him has been one of the highlights of my undergraduate experience.

I would also like to thank the amazing staff in the lab, without them none of this would have been possible. To Annie To, to list everything she has done for me would take up several pages; I cannot thank her enough for her support. Thank you to Dr. Melisa Kahl-McDonagh, for her guidance on the project and proof reading my writing and Penny Prochaska-Berg, for her support. Finally, thanks to the consultants at the Texas A&M statistics department for their help on the probability analysis in this project. I cannot thank all of you enough for what you have done.

NOMENCLATURE

CDC	Center for Disease Control
pMR10	Gram-Negative Broad Host Range RK-2 Based Plasmid
TLR	Toll-Like Receptors
Ts	Temperature Sensitive

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CHAPTER I

INTRODUCTION

Brucellosis: an overview

The bacterium in genus *Brucella* was first discovered in 1887 by British army Captain David Bruce. He successfully isolated the first sample of what is now known as *Brucella melintensis* from the spleen of a British solider who died from a febrile disease common at his post in Malta. *Brucella* was isolated again in 1897 when Bernhard Bang found *B.abortus* to be the causative agent in cases of spontaneous abortion in cattle. In 1917 these two pathogens were found to be identical and the bacterium was given the name *Brucella* after Captain Bruce[1].

Brucella is a gram-negative, facultative intercellular, non-motility, coccobacillus bacteria[2]. The condition caused by an infection of *Brucella spp.* is termed brucellosis; it is also commonly referred to as Mediterranean fever or Malta fever because of its prevalence in the Mediterranean and Middle East. The infection is typically found in animals but is communicable to humans making it a zoonotic disease. There are six known species of *brucella,* there classification is based mainly on host preference and

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This thesis follows the style of *Nature*.

pathology*: B. melitennsis* (sheep and goats)*, B. abortus* (cattle)*, B. canis* (dogs)*, B. suis* (swine)*, B. neotomae* (rodents) and *B. ovis* (sheep)[3,4].

Transmission of *Brucella* to humans is typically through direct contact with an infected animal or ingestion of unpasteurized dairy products, but aerosol transmission is also possible if the bacterium becomes airborne. Due to it's highly transmittable nature and low dosage needed to establish infection (10 to 100 CFU) *Brucella* has become the most common cause of laboratory-acquired infections and was used in early bioterrorism development in the United States. The weapons developed were never used but this led the CDC to classify the organism as a class B select agent[5].

Pathology

Clinical symptoms do not occur until 4 to 6 weeks after initial contact with *Brucella.* This extended asymptomatic incubation period is due the organism's unique interaction with the immune system. Once the pathogen enters the body it colonizes local tissue without a typical inflammatory response; this occurs because the organism is able to evade recognition by the innate immune cells that's initially respond. Once in the tissue, the infection spreads to organs rich in reticuloepithelial tissue (such as spleen, kidney and lymph nodes) and joints where it begins to invade and replicate in the host's phagocytic cells. The organism remains evasive from opsinization by the innate immune cell because of its altered pathogen-associated molecular patterns (PAMP); which are the immune systems method of recognizing foreign cells in the body. These alterations

include, an extended lipid a carbon chain so that TLR 4 cannot recognize it, altered flagella to evade recognition by TLR5, and O antigens lacking the hydroxyl group needed for c3 to bind and start the alternative complement pathway[5-8]. This continues for 4 to 6 weeks when the patient begins to show clinical symptoms of an infection. The common symptoms of brucellosis in humans are non-specific flu like symptoms that include: head ace, anorexia, nausea, back pain, and undulant fever. Infected animals experience a more adverse reaction; common symptoms include: abortion, orchitis, undulant fever and sterility[2]. It is considered chronic if the infection persists for six months and symptoms evolve to weight loss, nausea, depression and onset arthritis[5,9].

Diagnosis and treatment

Diagnostic techniques to identify brucellosis are commonly serology tests, including enzyme linked immunosorbent assay (ELISA) and agglutination tests. These tests are useful in clinical setting but can sometime result in false-positives. Cultures made from the patient's blood are the most reliable test but it is a more labor-intensive technique[5,10].

Treatment for an infection is a regiment of multiple antibiotics that are capable of working intercellularly and in highly acidic environments. The World Health Organization has recommended a 6-week regiment of doxycycline and either rifampicin or streptomycin[5].

Vaccines: present and future

Eradication attempts to stop the spread of *Brucella* in both animals and humans have been ongoing since it's identification, the disease has been nearly eradicated in the United States but continues to be a problem in areas in Europe and the Middle East. There are still as many as 500,000 emerging cases of brucellosis in humans reported each year and an even larger number of animal cases[11]. The numerous reported cases coupled with *Brucella's* ability for use in bioterrorism make the need for a viable human vaccine a pressing issue. Work on finding one has been in progress for years and promising advances have been made.

Vaccines have been USDA approved for animal use these include: Strain 19 (*B. abortus)*, strain RB15, and strain Rev1 (*B. melitensis)*. When first developed strain 19-BA and 104M vaccines were used on human patients in the USSR and China, but because of harsh side effects on the patient it was restricted to only animal use[3]. The creation of a live attenuated vaccine that would be strong enough to elicit protective immunity against an infection but not cause adverse effects post vaccination would be an ideal vaccine for human use.

Making a temperature sensitive mutant

Temperature sensitive mutants (Ts) are widely used in laboratories for studying the genetics behind cell functions, such as DNA replication and protein production. There are several published articles detailing the creation of temperature sensitive plasmids and

the knock-out genes involved, but none of these publications discuss findings in a broad host range plasmid type required for use in *Brucella*. Finding the knock-out genes to induce Ts in a plasmid like pMR10 would be an asset in developing a vaccine for brucellosis.

Types of mutagenesis

There are several procedures that can be used to randomly mutate a plasmid in hopes of finding knock-out genes that will induce temperature sensitivity, some are more effective then others. The options I explored included: PCR, UV mutagenesis, chemical mutagenesis and cell lines with deficient DNA repair mechanisms.

PCR, UV mutagenesis and repair enzyme deficient cells are all viable options for mutagenesis, but based on previous success in other laboratories with hydroxylamine I decided it would be the most promising method to use[12]. The protocol I chose to use was in vitro mutagenesis with hydroxylamine (NH_2OH). Hydroxylamine causes point mutations in DNA by deaminating cytosine to uracil, changing CG base pairs to AT[13]. The type of mutation caused by this mutagen is ideal for random mutation that could lead to temperature sensitive knock-outs.

Candidate knock-out genes

A knock-out that will induce instability at higher temperatures will need to be a mutation in part of the genome that code for essential cell function, such as cell division, protein production or DNA/RNA synthesis. The mutation will have to allow the cell to perform normal function at low or permissive temperature but cease to, or reduce, function at higher temperatures[14]. Previous work with Ts mutants have found that these mutations are generally in the origin or replication section of the genome, they are generally missense mutations that alter some aspect of replication[12]. Based off of the finding from other laboratories I propose that a point mutation in the ori region of pMR10, in the 6004 to 6715 bp region seen in Figure 1 below, will lead to temperature sensitivity in the plasmid.

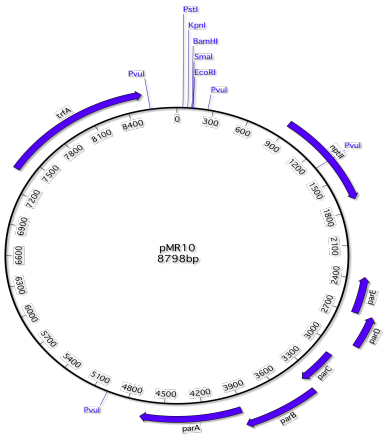

Figure 1 Map of the pMR10 plasmid genome

CHAPTER II

PLASMID PURIFICATION AND MUTAGENESIS

Introduction

The method chosen for this study was in vitro mutagenesis with hydroxylamine and isopropyl dialysis. This method was chosen due to its previous success in studies over genetic engineering and finding temperature sensitive mutants.[12,14] Hydroxylamine works by causing missense mutations, changing cytosine to uracile, in the DNA. In addition, the DNA damage leads to error-prone repair mechanisms in the cell which can cause further point mutations in the DNA.[13] This type of point mutations is ideal for finding a knock-out to cause temperature instability.

Once exposed to hydroxylamine DNA will continue to mutate at a steady rate until it is removed from the solution. The removal must be done at a precise time ensure the greatest degree of mutation without rendering the plasmid non-functional. Removal of the DNA was done with isopropyl precipitation and dialysis because of its ability to completely remove the hydroxylamine from the DNA to stop mutation.

Transformation was done into a strain of competent *E. coli*, used because of its ability to harbor a broad host range plasmid used needed for use in *Brucella.* There are several strains of *E. coli* cells I explored including XL Blue XL-1 Red, DH5-α, HB101 and C600. DH5-α was chosen because of its high efficiency rate when used with the Z-

Competent cell transformations kit; it has the potential to transform 10^9 colonies per 1ng of DNA added to it.[15] Because the goal of this study is to screen several thousand colonies a high yield competent cell is ideal.

Materials and methods

DNA purification

Cells containing pMR10-CM^R plasmid were inoculated into 50ml of Luria-Bertani broth containing 50µl of chloramphenicol. The culture was incubated overnight at 37°C with moderate shaking (200-300rpm). The liquid culture was then used to extract and purify pMR10 plasmids following the IsoPure™ Maxi II DNA Purification kit manufacturers protocol[16]. The purified DNA product was then scanned to determine nucleic acid concentration and stored in a 2ml microfuge tube at -20°C.

In-vitro mutagenesis

The plasmid DNA was mutated following the in-vitro mutagenesis with hydroxylamine and isopropyl dialysis protocol. The hydroxylamine solution was prepared by mixing .35g hydroxylamine hydrochloride with 4.55ml of cold dH_2O; then 450µl of 5M sodium hydroxide was added drop wise until the pH was 6.7. Then 25ng of purified plasmid was added to the solution and it was left to mutate for 18 hours at 37°C. The DNA was then precipitated out with ethanol. This was done by dividing DNA solution evenly into two 2ml microfuge tubes; then adding 2µg of pellet paint, 2.7µl of sodium acetate, 1.4µl of

$MgCl_2$ and 1000µl of ethanol to each. The solution was left at -20°C for one hour to allow DNA to participate out. It was then centrifuged at 15000 x g for 10 minutes. Supernatant was removed and disposed of in hydroxylamine waste and both pellets were then washed with 500µl of 70% ethanol and the two tubes were combined. The solution was centrifuged a second time at 15000 x g for 10 minutes, the supernatant was disposed of the pellet was left to air dry for 15 minutes. The mutated DNA was re-suspended in 20µl of dH_2O and stored in the -20°C freezer until needed.

Cell transformation and Ts screening

Competent DH5-α cells were formed into Z-Competent™ cells and then transformed with mutated pMR10-CM^R plasmids using the Z-Competent *E. coli* Transformation Kit and Buffer Set™ manufacturers protocol[15]. 50µl of transformed cells were platted onto pre-warmed LB + CM agar plates and incubated for 12 to 24 hours at 30°C. The colonies formed were then individually picked using sterile toothpicks and inoculated into a 300µl well containing LB+CM broth, in a 96 well plate. The well plates were incubated for 12 hours at 30°C. After incubating the well plates were stamped onto 6 LB+CM agar plates, the first 48 (side I) wells on three plate and the second 48 (side II) on three plates. The three plates from side I and II were incubated at 30°C, 37°C and 42°C, respectively. After a 12 to 24 hour incubation period there plates were removed and screened for discrepancies in growth between the temperatures.

CHAPTER III

RESULTS

23,000 colonies were screened in the experiment; all were found to be negative for the desired phenotype. My goal of 88,700 colonies screened was unfortunately not reached in the given time period of the experiment due to several unforeseen issues. A major delay in the experiment occurred when switching to a new transformation kit. I experienced difficulty getting correct readings from a photospectrometer in the laboratory; I had to follow the procedure 3 times before obtaining cells. Also after transforming the isolated cells with my mutated plasmid they had extremely low yield. The DH5-α one shot preformed competent cell kit I had been using previously was yielding 10^8 colonies per 2µl of mutated DNA whereas the new kit had a yield of 5 to 10 colonies using the same amount of DNA. After several attempts with the new transformations kit I switched back to the one shot kit. Several other minor infractions set my project back; this coupled with the time constraint lead me to fall short of my goal.

CHAPTER IV

SUMMARY AND CONCLUSIONS

Making a *Brucella* mutant using counter-selection is currently in use, but an improvement on the system has been proposed. The new method utilizes a temperature sensitive plasmid encoding a flippase. In the first step, the gene(s) to be deleted is replaced using a construct containing upstream and downstream flanking DNA sequences to promote exchange with the genome and an antibiotic resistance marker flanked by Frt sites. The plasmid encoding the flippase is introduced into this background and flippase expression results in removal of the resistance gene by excision at the Frt sites. The secondary plasmid is then removed from the organism by simply incubating it at a higher temperature. This would be a stark improvement to the laborious work involved in counter-selection. Not only would it save time and materials it would also leave the organism unmarked, enhancing its use in a vaccine.

Extensive work on finding a temperature sensitive plasmid has been done by other lab members prior to the beginning of this project. The focus has mostly been on XL1-Red cells, which are *E. coli* cells lacking the self-repair genes *mutS, mutD, and mutT* increasing the mutation rate 5,000 fold.[17] This work found four temperature sensitive colonies; but upon further screening it was discovered that the mutation was in the antibiotic resistance area of the genome not the desired plasmid maintenance region, this growth pattern is illustrated in part A of Figure 2.

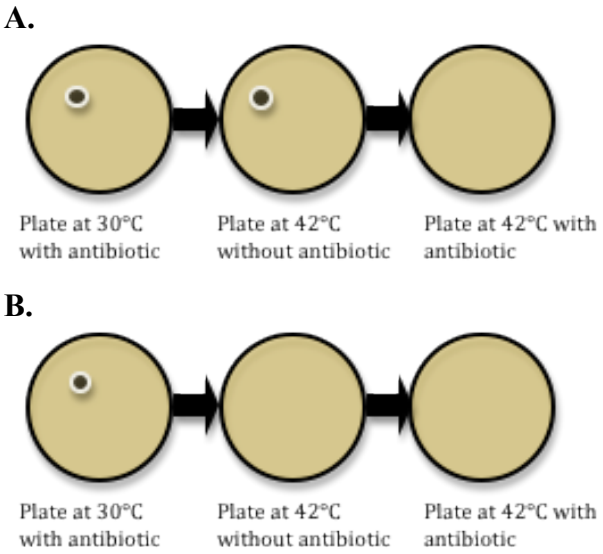

Figure 2 Growth Diagram. Part A. shows the undesired results of a mutation in region of the genome coding for antibiotic resistance. Part B. shows the desired result of a mutation in the part of the genome coding for plasmid

Work with hydroxylamine had also previously been done. I chose to continue this work because it causes the desired mutation type for finding temperature sensitivity, similar to XL1-Red cells, illustrated in Figure 3. The previous work with hydroxylamine did not screen a high enough volume of colonies to ensure finding a plasmid that is temperature sensitive. Therefore, I believed further colony screening was beneficial.

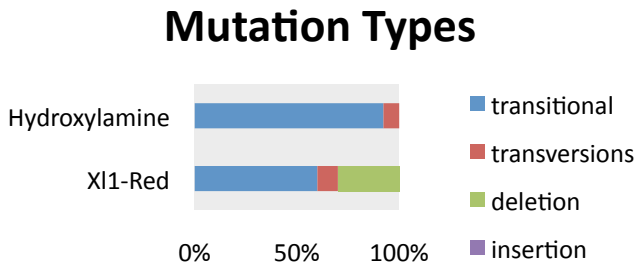

Figure 3 Mutation Type. Depicts the type of DNA mutation caused by hydroxylamine and XL1-Red cells.

Using the formula provided below it was determined with 99% confidence that each base pair would be mutated at least one time would require the selection and screening of at least 4,555 colonies. This figure makes several assumptions that may not be consistent with the mutation process. First, it assumed that each of the base pairs in the genome had the same probability (p) of being mutated; second, it assumed that the plasmids were mutating at the appropriate rate (averaging 26.394 bp per plasmid). Therefore, while the math predicts that in 23,000 colonies the identification of the wanted mutant was probable, it was not guaranteed.

If Y_i= the number in n plasmid that base pair I mutates then the probability is

$$P[Y_1 \geq 1, Y_2 \geq 1, \ldots Y_{8798} \geq 1] = .99$$
$$[1 - (1-P)^n]^{8798} = .99$$
$$n = 4555$$

Hydroxylamine was used throughout the experiment but its validity as a mutagen was never confirmed. DNA incubated in hydroxylamine for 18 hours should cause 3bp mutations per 1000bp, this number is based on the data illustrated in Figure 4. Therefore, plasmid pMR10 mutated with this protocol should cause an average of 26.394bp mutations per plasmid. This however was not tested in this experiment and it may be a source of error. Future experiments will evaluate the mutation rate directly by sequencing selected regions of the plasmid to determine whether mutation was occurring at the predicted rate.

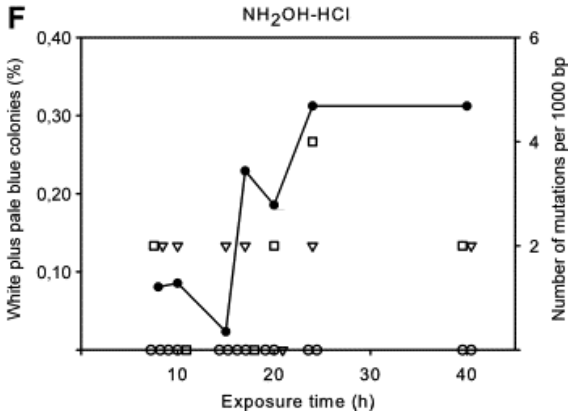

Figure 4 Mutation Rate. Shows the number of base pair mutations per 1000 base pairs when mutated for different lengths of time.

Even though the Ts plasmid was unfortunately not found in this study it laid the groundwork for future experiments. I believe the model used will eventually produce a temperature sensitive plasmid; a few changes could be made to reduce the time need to screen colonies. For example, replica plating of whole plates instead of picking individual colonies or increasing mutations in the plasmid by incubating in hydroxylamine for longer periods of time could expedite finding the desired mutation. A temperature sensitive plasmid would be a valuable asset to the development of a *Brucella* mutant and work should continue to search for one.

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