DEVELOPING A TEMPERATURE-SENSITIVE PLASMID TO CREATE *BRUCELLA* KNOCKOUT MUTANTS

A Senior Honors Thesis

by

MIDHAT SALEEM FAROOQI

Submitted to the Office of Honors Programs
& Academic Scholarships
Texas A&M University
in partial fulfillment of the requirements of the

UNIVERSITY UNDERGRADUATE
RESEARCH FELLOWS

April 2004

Major: Genetics

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ABSTRACT

Developing a temperature-sensitive plasmid to create

Brucella knockout mutants. (April 2004)

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Gene knockouts in an organism give valuable information about that gene's function. The current method for producing *Brucella* knockout mutants – sacB insertion followed by sucrose counterselection – is inefficient. A novel procedure for generating unmarked *Brucella* knockouts is proposed. First, the target gene is replaced by an antibiotic resistance marker via homologous recombination. Then, this marker is excised by flippase, an enzyme encoded by a temperature-sensitive plasmid. The plasmid is subsequently removed from *Brucella* by raising the temperature.

To obtain such a temperature-sensitive mutant, the broad-host-range plasmid pBBR1 was mutated using the *E. coli* strain, XL1-Red. Mutation of the plasmid DNA was verified by blue/white screening. Afterwards, the mutants were screened for temperature-sensitivity by replica plating and growth at the permissive (30°C) and non-permissive (42°C) temperatures. A total of 2,400 colonies were screened, and two temperature-sensitive plasmids were isolated. However, further analysis by replica plating showed that the two plasmids were temperature-sensitive for antibiotic resistance, not replication.

These plasmids cannot be removed from *Brucella* by raising the temperature. Hence, it is not yet clear whether the proposed method can generate knockout mutants of *Brucella*. Future studies should mutate pBBR1 more extensively, or apply the mutagenesis procedure to a different plasmid, such as pGL10.

DEDICATION

I dedicate this thesis to my parents. I realize that I can never fully repay them for all of their love, guidance, and support. However, I hope that this thesis will provide them with proof that their son turned out "OK."

ACKNOWLEDGMENTS

I would not have been able to produce this thesis were it not for the constant support I received from those around me. I would like to recognize these people in thanks for all the help they gave me.

First, I would like to thank Dr. Thomas Ficht for agreeing to serve as my mentor. He gave me free reign to plan and carry out this project and had confidence in my ability to succeed. I have learned incredible amounts of practical knowledge by working in his laboratory, knowledge that I know I will use later in my career. I can think of no better place to train as a fledgling scientist, and I thank him once more for giving me the opportunity to work in his lab.

Of course, a great lab consists of great people. Thus, thanks are due to my labmates: to Carol Turse for teaching me the basics and sterile technique; to Josh Turse for helping me through equipment troubles and for always laughing at my jokes; to Melissa Kahl for giving me advice on how to design my project; to Kristen and Jenni for holding great conversations between experiments; and, finally, to my fellow undergraduate workers (David, Amanda, Sruti, Shuo, and Troy) for sharing stories, ideas, chemical solutions, and bench space.

I would also like to thank Dr. Finnie Coleman, Dr. Amy Earhart, and Donna O'Connor for their work in organizing and running the Fellows program. Dr. Coleman was instrumental in motivating all Fellows to succeed in the program; Dr. Earhart, in providing invaluable advice on how to format the thesis; and, Donna O'Connor, in sending a wealth of timely e-mails without which many a deadline would have been missed.

I must also thank Dr. Gunn for making me aware of the Fellows program and encouraging me to apply.

Finally, I would like to thank my family and friends for proofreading my thesis and keeping my writing relatively free of scientific jargon.

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I. INTRODUCTION

Brucellosis: Disease & Treatment

Brucellosis is an infectious disease that primarily affects animals, most frequently cows and goats[1]. However, humans can become infected as well if they drink unpasteurized milk contaminated with the bacteria, or handle an infected animal. Hence, brucellosis, like rabies, is a zoonosis. In animals, the disease results in abortion, orchitis, and sterility. Humans, however, experience a range of less severe symptoms: headaches, undulant fever, and loss of appetite. Any combination of these can persist for three to twelve months and recur afterwards.

Bacteria classified in the genus *Brucella* cause this disease. Though all of these organisms are gram-negative, facultative, and non-motile, different species of *Brucella* cause brucellosis in different animals[2]. Cows, for example, are susceptible to *Brucella abortus*, while goats succumb to *Brucella melitensis*. Some other species include *B. suis* and *B. canis* which infect pigs and dogs, respectively. All four of these species can cause disease in humans. However, *B. melitensis* is the most virulent and responsible for the majority of severe cases of human brucellosis[3].

Once inside the body, *Brucella* penetrates the mucosa and triggers a natural immune response[4]. Macrophages and neutrophils residing in the submucosa ingest the bacterium through phagocytosis. These immune cells, however, are unable to destroy *Brucella*. The pathogen somehow evades the killing mechanisms of these phagocytes and proliferates within them. Bacteremia develops within 1-3 weeks of exposure, after which *Brucella* localizes to organs rich in reticuloendothelial tissue – e.g., the lymph nodes, spleen, and liver[5]. If left untreated, brucellosis can be fatal[6].

Serological agglutination tests are widely used to diagnose brucellosis in both animals and humans[6]. Human brucellosis patients are treated with combinations of antibiotics such as rifampin and doxycycline. Unfortunately, a human vaccine for brucellosis does not exist today. For animals, however, three vaccines are available:

This thesis follows the style and formatting of *Nature*.

B. abortus S19, *B. abortus* RB51, and *B. melitensis* REV.1. The first two are used to immunize cattle, while the latter protects goats and sheep. Neither type meets the current standards for safety and efficiency that apply to human vaccines[7].

Nonetheless, a human vaccine for brucellosis needs to be developed. One reason why is because there is growing concern that microorganisms, such as *Brucella*, will mutate to gain antibiotic resistance. This event would render the current treatment for the disease ineffective. Also, since the pathogen survives well in aerosols and resists drying, it may be used as a biological weapon[5]. Actually, American researchers attempted to develop *Brucella* into a biological weapon in 1942 and, in fact, succeeded[8]. In 1954, it became the first bioweapon ever made, and, one year later, the United States was producing cluster bombs filled with *B. suis*. For these reasons, the Centers for Disease Control and Prevention recently classified *Brucella* as a select agent[9]. This fact alone highlights the need for a human vaccine for brucellosis.

To have the greatest chance of success, such a vaccine must be designed, not isolated by chance. It must be developed based on an understanding of how *Brucella* causes disease. However, the basis of *Brucella*'s virulence has long defied description[7]. The only well-defined virulence factor is the O-antigen of the lipopolysaccharide coat. Otherwise, *Brucella* lacks the virulence factors – such as toxins and fimbria – commonly found in other pathogens. The recent release of *Brucella*'s genomic sequence verified the absence of such virulence factors[10]. Interestingly, genes controlling the survival and persistence of *Brucella* are the main features of its genome.

Therefore, one may assume that the pathogenesis of *Brucella* depends upon its ability to survive inside the host. *Brucella*, of course, initially resides in the host's white blood cells[4]. These macrophages use low pH and reactive oxygen intermediates to break down the organisms they engulf. *Brucella* avoids this degradation by transcribing heat shock and other stress response genes. It is hypothesized that *Brucella* mutants produced from knocking out these genes will have reduced virulence and make good vaccine candidates.

Candidate Knockout Genes

The specific genes of interest in this study are: asp24, dnaK, aapJ, and htrA. The expression of asp24, for example, is optimal at pH values below 4.0[11]. This directly correlates with a period of bacterial survival in the acidic environment of macrophages. Thus, the gene is thought to be responsible for *Brucella* resisting intracellular elimination within macrophages. The expression of dnaK is also increased in response to acid pH, which leads to higher levels of the molecular chaperone protein, DnaK. Without this protein, *Brucella suis* cannot replicate inside macrophages[12]. Transcription of aapJ is increased in B. melitensis in response to heat shock[13]. AapJ is a periplasmic protein that is involved in amino acid binding. Presumably, it helps Brucella adjust to the host cell's amino acid pool. Another gene that is upregulated due to heat shock is htrA. The latter produces a serine protease that helps Brucella abortus resist destruction from phagocytes[14]. The genes selected are summarized below in Table 1.

Table 1. Candidate knockout genes.

Gene	Protein Size (kDa)	Function	Upregulation
AapJ	37.1	Amino acid binding	Heat Shock
Asp24	20.4	Calcium binding	Acid pH
DnaK	68.7	Chaperone	Acid pH
HtrA	60	Protease	Heat Shock

Gene Knockout Methods in *Brucella*

It is incredibly difficult to create *Brucella* knockout mutants using the current procedures available. One must first replace the gene of interest with an antibiotic resistance marker through homologous recombination and then remove this cassette. The deletion of the marker gene is crucial. Otherwise, not only has one introduced foreign

DNA into the pathogen, but conferred antibiotic resistance to it as well. This is not suitable if one hopes to test these mutants as vaccine candidates.

There are two techniques available to remove this antibiotic marker. In the first method, one replaces the marker with sacB, a gene that confers sucrose intolerance[15]. The bacteria are then grown on a medium containing sucrose, an environment that promotes the spontaneous loss of sacB. Bacteria that have lost the gene are easy to identify – they grow on the sucrose-containing medium. The second technique involves using a plasmid-encoded enzyme – flippase – to excise the antibiotic resistance gene[16].

Both methods have drawbacks. Rather than being lost, the sacB gene may mutate and become inactivated. In this case, the bacterium would appear to be sucrose-resistant – and, so, an unmarked knockout – when, in truth, it would still contain sacB. Furthermore, the sacB method is inefficient: one study analyzed 200 colonies only to find 2 unmarked knockouts[15].

In contrast, flippase removes the kanamycin marker at high frequencies – near 100%[17]. Unfortunately, the plasmid used to introduce flippase into *Brucella* remains inside the bacterium after the excision. Thus, one still faces the problem of foreign DNA and antibiotic resistance – as encoded by the plasmid – in the *Brucella* knockouts.

Fortunately, this obstacle can be overcome if one uses a temperature-sensitive plasmid. Such a plasmid is stable at low temperatures (~ 30°C) but very unstable at higher temperatures (~ 42°C)[18]. This characteristic provides an easy way to remove the plasmid from *Brucella*: grow the bacteria at a high temperature. In these conditions, the plasmid is unstable and will be lost from the cell.

This study proposes to use this novel flippase/temperature-sensitive plasmid procedure to create *Brucella* knockout mutants. The first step, then, is to create a temperature-sensitive plasmid.

II. MUTAGENESIS & SCREENING OF PLASMID DNA

Introduction

To make a plasmid temperature-sensitive, one must first mutate the plasmid's DNA. There are many different mutagens one can use to accomplish this – e.g., ultraviolet light, a chemical such as hydroxylamine, or PCR, to name a few. However, some of these mutagens are more effective than others at generating temperature-sensitive mutations.

Previous work in the lab had shown that mutagenesis with ultraviolet light did not consistently mutate plasmid DNA (Farooqi, 2002, unpublished data). Thus, a different mutagen was desired. A literature search revealed that hydroxylamine had been used to produce a temperature-sensitive plasmid[19]. Unfortunately, this chemical is very hazardous[20], making it difficult for an undergraduate student to use it openly in the lab. In addition, treatment of DNA with hydroxylamine would require subsequent dialysis – a highly laborious and expensive procedure.

A safer and simpler method for mutating DNA was sought. It was found in the *E. coli* strain XL1-Red[21]. This cell line is deficient in three primary DNA repair genes – mutS, mutD, and mutT – prompting its mutation rate to be approximately 5,000-fold higher than normal. Furthermore, it has been used to generate temperature-sensitive plasmids and alleles[22, 23]. Thus, XL1-Red was selected to mutate plasmid DNA.

Materials & Methods

Mutagenesis. The broad-host range plasmid pBBR1 was transformed into XL1-Red cells (Stratagene, La Jolla, CA) following the manufacturer's instructions[21]. The plasmid pUC18 was used as a positive control and transformed in parallel. The transformants were grown on LB plates containing 30 μg/mL of chloramphenicol (Cm@30) for 3 days at 37°C. Of the resulting colonies, two hundred were picked into 10 mL LB/Cm@30 broth and grown at 37°C for an additional four days. The plasmid was recovered from the XL1-Red cells via a mini-prep (Sigma, St. Louis, MO) on the fourth, fifth, sixth, and

final day of mutagenesis. Agarose gel electrophoresis was used to verify that the DNA recovered was, indeed, that of pBBR1. Plasmid DNA isolated on the seventh day was used for the rest of this experiment.

Blue/White Colony Screen. To ensure that the plasmid had been mutated, a blue-white colony screen was performed. Both the stock plasmid and the mutated plasmid were transformed into the chemically competent E. coli strain, Top10 (Invitrogen, Carlsbad, CA). The transformants were plated on LB/Cm@30 plates, on which 32 μL of X-Gal had been spread.

Screening for Temperature-Sensitivity. The mutated plasmid DNA was then transformed into XL1-Blue – a supercompetent E. coli strain (Stratagene, La Jolla, CA) – and plated on LB/Cm@30 plates. The colonies were then screened for temperature-sensitivity.

Individual bacterial colonies were picked into 96-well dishes at one colony per well. Each well held 200 μL LB/Cm@30 broth. One dish was then stamped onto four LB/Cm@30 plates: the first 48 wells on two plates, and the remaining 48 wells on two other plates. One plate in each pair was placed at the permissive temperature (30°C) and the other at the non-permissive temperature (42°C).

Results

Figure 1 shows that the plasmid recovered after the mutagenesis was indeed pBBR1.

Figure 1. Plasmid DNA as analyzed by gel electrophoresis. Lane 1 contains stock pBBR1 DNA. Lanes 2-5 contain plasmid DNA recovered from XL1-Red cells via miniprep on days 4, 5, 6, and 7.

The results of the blue/white colony screen are given in Table 2. The normal stock of plasmid contained no mutations. However, after growth in XL1-Red, mutations were seen in the plasmid's lacZ gene, which gave rise to white colonies.

Table 2. Blue-White colony count.

Plasmid	Blue Colonies	White Colonies	Total	% White
PBBR1 Stock	151	0	151	0.00
pBBR1 Mutated	264	46	310	14.84

Approximately 240 colonies were assessed before two candidates for temperature-sensitivity – designated A10 and C12 – were found. Another candidate, A2, was found after screening an additional 140 colonies. Figure 2 shows two of the three candidates.

These three colonies were plated once more to confirm temperature-sensitivity. Figure 3 shows that two of three colonies – A2 and A10 – are indeed temperature-sensitive while C12 is not. However, A10 shows very poor growth at 30°C and no growth at 42°C. In contrast, A2 shows a much stronger phenotype by growing well at 30°C but not growing at all when moved to 42°C.

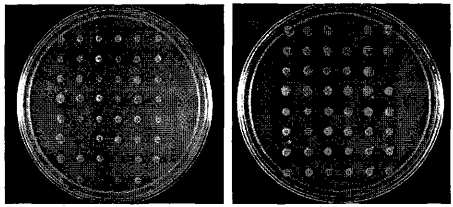

Figure 2. Two temperature-sensitive colony candidates. The plate on the left was grown at 30°C, while the one on the right was grown at 42°C. The 4th colony in the 1st row (termed A10) and the last colony in the third row (termed C12) show a temperature-sensitive phenotype.

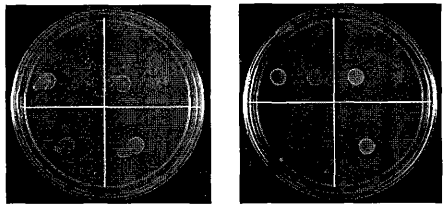

Figure 3. Spot check of temperature-sensitive candidates. The plate on the left was grown at 30°C, while the one on the right was grown at 42°C. The top left quadrant contains colony C12. The left spot is a control; it holds bacteria that grow equally well at both temperatures. The right spot consists of bacteria from well C12 of the 96-well culture dish. The top right quadrant contains colony A10. Again, the left spot is a control, and the right spot contains the colony being tested. The bottom left quadrant is colony A2. In this case, the right spot is the control. Colonies A2 and A10 were confirmed to be temperature-sensitive.

III. CHARACTERIZATION OF THE MUTANT PLASMIDS

Introduction

Of course, there are two different mutations that could give rise to such a temperature-sensitive phenotype. One possibility is that the mutation occurs in the plasmid's replication gene (Rep). The other is that it occurs in the plasmid's antibiotic resistance gene (CmR). In the first case, the plasmid is unable to replicate at 42°C (Ts^{REP}). As a result, bacterial cells produced after replication no longer contain copies of the plasmid. These bacteria, then, cannot grow in the presence of the antibiotic. In the second case, the plasmid makes an antibiotic resistance protein that is functional at 30°C, but not at 42°C (Ts^{CmR}). Here, the bacterium retains the plasmid, but is still unable to grow in the presence of the antibiotic. However, if the antibiotic is removed, both the plasmid and the bacterium are able to replicate at 42°C.

Materials & Methods

To distinguish between these two possibilities, colonies A2 and A10 were grown at 42°C on an antibiotic-free LB agar plate. This plate was then replica-plated onto an LB/Cm@30 plate, and it was grown at 30°C. If the plasmid was Ts^{REP}, it would be lost from the bacterium as it grew at 42°C (the first step). Therefore, this bacterium would be unable to grow at 30°C in the presence of chloramphenicol (the second step). However, if the plasmid was Ts^{CmR}, it would remain inside the bacterium as it grew at 42°C. This bacterium would still grow in the presence of chloramphenicol at 30°C. This method of differentiation is summarized in Figure 4.

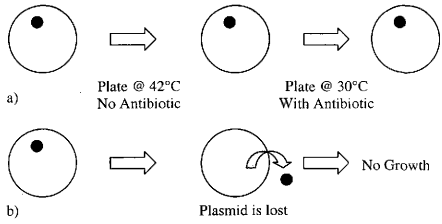

Figure 4. Differentiating between a ts^{CmR} plasmid (a) and a ts^{REP} plasmid (b).

Results

When carried out, the procedure showed that the two plasmids were temperature-sensitive for chloramphenicol resistance. The final replica plate is shown in Figure 5.

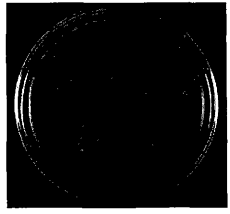

Figure 5. Replica plate (30°C, LB/Cm@30) of the 42°C, no chloramphenicol plate.

Following this result, an additional 960 colonies of bacteria were screened for temperature-sensitivity. There were no positive findings. This prompted the addition of another step in the procedure.

IV. ENRICHMENT OF PLASMID DNA

Introduction

A search of the literature revealed a procedure termed 'enrichment.' Bernard Davis, a scientist who wanted to isolate biochemically deficient bacteria, developed this method[24]. Bacteria that can grow only in the presence of a certain nutrient, such as histidine, are an example of the type of cells Dr. Davis wished to isolate.

In this case, mutant bacteria (His^- cells) and normal bacteria (His^+ cells) can both grow in a culture containing histidine. However, if no histidine is present, only His^+ cells can replicate. If one takes such a culture – one where only normal cells are dividing – and adds an antibiotic that specifically kills growing cells (like penicillin), then there will be a marked decrease in the concentration of His^+ cells. As a result, the frequency of His^- cells in the culture will become much greater relative to His^+ cells.

This principle was applied to the case of temperature-sensitive bacteria. At 42°C, only bacteria containing normal plasmids will grow. Cells containing a temperature-sensitive plasmid will remain dormant. The addition of a bacteriostatic antibiotic at this point should enrich for cells with a temperature-sensitive plasmid.

Materials & Methods

The mutated plasmid was transformed into XL1-Blue cells according to the manufacturer's instructions. 500 μL of the transformation culture were used to inoculate 50 mL of LB/Cm@30 broth. The culture was aerated overnight at 30°C and 250 rpm. 500 μL from the overnight culture were used to inoculate 10 mL of LB/Cm@30 broth. This culture was grown at 42°C, 250 rpm, until the optical density (at 550 nm) increased 4 to 5-fold (approximately 2.5 hours). At this point, ampicillin was added to give a final concentration of 40 μg/mL. Then, the culture was aerated at 42°C, 250 rpm, once more until the OD_{550} decreased (approximately 30 minutes). Afterwards, the cells were recovered via centrifugation at 12,000 rpm for 10 minutes. The bacteria were washed

twice with 1XPBS and then resuspended in LB/Cm@30 broth. Finally, the enrichment method was repeated to increase the efficiency of the procedure.

Results

The 'enriched' colonies were plated on LB/Cm@30 plates and screened for temperature-sensitivity as previously described. Not one colony out of the 864 assessed showed the desired phenotype. The lack of a positive result prompted a revision of the experimental procedure.

V. REVISED EXPERIMENTAL PROCEDURE

Introduction

Previously, the plasmid DNA was mutated using XL1-Red cells following to the manufacturers' instructions. These directions called for the transformants to be grown at 37°C. This poses a problem since this temperature may select against the generation of temperature-sensitive mutations. However, it should be noted that the scientists who used XL1-Red to make a temperature-sensitive plasmid grew the bacterial cells at 37°C following the manufacturer's directions[22]. Nevertheless, to ensure that no selection against temperature-sensitive mutations occurred, the temperature was reduced to 30°C.

Another cause for concern is that the enrichment procedure has never been applied to temperature-sensitive cells, much less plasmids. The method should work with temperature-sensitive bacteria, since these cells would probably remain dormant at 42°C. However, the case of a temperature-sensitive plasmid is quite different. Rather than remain dormant, a normal bacterium containing a temperature-sensitive plasmid would probably lose the plasmid when grown at 42°C. Since it is the plasmid that is temperature-sensitive, the enrichment procedure may actually force the host cell to lose it. Again, to prevent any selection against a temperature-sensitive plasmid, the enrichment step was removed from the experiment.

Materials & Methods

The transformation of pBBR1 into XL1-Red cells was carried out as before, except all remaining steps after the 'heat shock' were carried out at 30°C. Again, the plasmid pUC18 was used as a positive control and transformed in parallel. The transformants were grown on LB/Cm@30 plates for 9 days at 30°C. The mutated plasmid was recovered from the XL1-Red cells on the 9th day by a miniprep.

To see how effectively the plasmid was mutated, a blue/white screening was performed using both pBBR1 and pUC18 (the positive control). Both the stock plasmid and mutant plasmid were transformed into Top10 cells. The transformants were plated

on LB/Cm@30 plates – LB/Amp@100 plates for the pUC18 transformants – containing 32 μL of X-Gal.

Afterwards, the pBBR1 transformants were screened for temperature-sensitivity as previously described.

Results

Table 3 presents the colony count of the blue/white colony screen. The normal stock of plasmid contained no mutations. However, after growth in XL1-Red, both pBBR1 and pUC18 contained mutations – proven by the presence of white colonies.

Table 3. Blue/White screening.

Plasmid	Blue Colonies	White Colonies	Total	% White
pBBR1 Stock	172	0	172	0.00
pBBR1 Mutated	146	25	171	14.62
pUC18 Mutated	61	71	132	53.79

A total of 384 colonies were screened, and no temperature-sensitive candidates were found.

VI. CONCLUSIONS AND FUTURE WORK

A novel method of creating *Brucella* knockout mutants was proposed. It involved using a plasmid-encoded enzyme, flippase, to precisely excise a DNA cassette. It also called for a temperature-sensitive plasmid, because the latter could subsequently be removed from *Brucella*. This method offered several advantages over the current knockout procedure. First, it could be applied to a wide variety of genes. Second, knockouts would be produced at a much higher frequency relative to the sacB procedure. And finally, the knockout mutants would contain no gene markers, allowing the mutants to be used to develop a human vaccine for brucellosis.

Unfortunately, the creation of a temperature-sensitive plasmid proved to be incredibly laborious and difficult. This was unexpected. One possible explanation is that temperature-sensitive mutations occurred in the plasmid's replication protein but were masked. Such an event would occur if the plasmid forsook its own protein and used the host cell's replication protein. However, pBBR1 is a broad-host range plasmid, which means that it depends very little on the host cell for replication. Thus, such a masking event is very unlikely.

Another way temperature-sensitive mutations could be masked is if there were multiple plasmids in one cell. In this scenario, the mutant plasmid is lost from the cell at 42°C, but any normal plasmid present allows the bacterial cell to survive. To reduce this possibility, the plasmid was removed from XL1-Red cells and then retransformed into second bacterial strain. This approach has previously been used to reduce the problem of multiple plasmids in cells[19].

A more likely explanation as to why it was so difficult to find a temperature-sensitive plasmid is that pBBR1 may not have been mutated very efficiently. As shown in Table 3, a mutant lacZ gene was found in pBBR1 at a frequency of 14.6%. Comparatively, a lacZ mutation was much more frequent in pUC18 (nearly 53.8%). Of course, pUC18 is smaller in size relative to pBBR1 (2686 bp and 4707 bp, respectively). The former is also a high-copy plasmid, as opposed to pBBR1, which is low-copy. These qualities may explain the large difference between mutational rates of the two plasmids.

Another factor may be that pBBR1 replication is more precise, making the plasmid less apt to mutate. Perhaps a different plasmid, such as pGL10, should be used instead.

In any case, further studies should use blue/white screening as a tool to measure the effectiveness of the mutagenesis procedure, not only as a control. A target mutational frequency should be set, and pBBR1 should remain in XL1-Red cells until that goal is reached. A mutation rate of 14.6% might be too low since only 1 out of 7 plasmids will contain a mutation in the target gene. Such a rate greatly 'reduces' the actual number of colonies screened. For example, one may assess 1,000 colonies, but, at a mutational rate of 14.6%, the *effective* number of colonies screened would be 143.

Still, two temperature-sensitive^{CmR} plasmids *were* isolated through this procedure. Plus, two other studies have also been able to generate temperature-sensitive mutations. Perhaps this study was sound and all it lacked was the little bit of serendipity that accompanies every successful scientific study.

What other methods besides mutagenesis can one use to make a temperature-sensitive plasmid? Well, one may clone a temperature-sensitive origin/protein into pBBR1 from a known temperature-sensitive plasmid, such as pSC101[58]. However, this would probably cause pBBR1 to lose its broad-host range. Plus, a BLAST search of the pBBR1 origin and replication protein found no homology to any known temperature-sensitive plasmids. Consequently, such an approach is probably not worth pursuing.

As for producing knockout mutants, one might just forego the temperature-sensitive plasmid method altogether and use sacB/sucrose counterselection method, instead. Again, this is less efficient than the flippase method, and the possibility that sacB may mutate remains. But, one can verify loss of sacB by DNA sequencing or a Southern blot, and circumvent this problem.

In spite of this, a temperature-sensitive plasmid operable in *Brucella* would still be a valuable research tool to scientists, since it would allow for a more efficient method of producing knockout mutants. It is regrettable that time, luck, and unanticipated problems prevented the development of a temperature-sensitive^{REP} plasmid in this study. However, this work lays a strong foundation for future progress in this regard.

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VITA

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EDUCATION

Texas A&M University College Station, Texas August 2001-May 2004
- Bachelor of Science in Genetics Cumulative GPR: 4.00

Killeen High School Killeen, Texas August 1997 - May 2001
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RESEARCH

Veterinary Pathobiology College of Veterinary Medicine
- University Undergraduate Research Fellow June 2003 – Present

Explored the use of a temperature-sensitive plasmid to create Brucella knockout mutants.
- Honors Directed Research Project June 2002 – August 2002

Investigated different techniques used to mutate plasmid DNA.

SKILLS

- Emergency Medical Technician – Basic Level
- Bilingual

Fluent in English and Urdu, and can read Arabic.

ACTIVITIES

- Peer Workshop Leader August 2002 – May 2003

Instructed 5 Honors students on how to solve advanced Organic Chemistry problems.
- Genetics & Biochemistry Peer Advisor May 2002 – July 2003

Counseled freshmen on which professors and classes to take, and helped contruct their schedules.

MEMBERSHIPS & AWARDS

- Phi Kappa Phi
- College of Agriculture & Life Sciences Senior Merit Award
- Gamma Sigma Delta Outstanding Senior Award
- Academic Incentive Award
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