ABSTRACT

Title of Thesis: ANALYSIS OF FRAMESHIFTING FREQUENCIES DUE TO HOMOPOLYMERIC NUCLEOTIDE TRACTS IN *NEISSERIA GONORRHOEAE*

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Organisms that are sensitive to the antimicrobial agent nitrofurantoin express nitroreductases, which reduce nitrofuran containing compounds to highly reactive and damaging intermediates. A single nitroreductase, *nfsB*, was identified in *N. gonorrhoeae* FA1090. The sequence of *nfsB* was modified to contain polyguanine tracts of varying lengths. Analysis indicated that mutations yielding nitrofurantoin resistance occurred at a higher rate in strains containing modified *nfsB* genes when compared to wildtype FA1090. The frequency of mutation also increased as polyguanine tract length increased. A polyguanine tract length of 5 residues did not produce elevated mutation frequencies.

The use of PCR and polyacrylamide gel electrophoresis proved to be a reliable method for quickly identifying nitrofurantoin resistant mutants that contained *nfsB* frameshift mutations. When *nfsB* fragments amplified from nitrofurantoin resistant mutants were electrophoresed, the presence of insertions or deletions in the *nfsB* coding sequence was easily detected.

ANALYSIS OF FRAMESHIFTING FREQUENCIES DUE TO HOMOPOLYMERIC NUCLEOTIDE TRACTS IN *NEISSERIA GONORRHOEAE*

by

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Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park in partial fulfillment
of the requirements for the degree of
Master of Science
2006

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“Better really, really, really late than never.”

-RCH

ACKNOWLEDGEMENTS

Although the road was extremely tough and many obstacles had to be overcome, the journey is finally at an end. I owe many people my deepest gratitude for helping me reach the point I'm at now. First, I would like to thank Dr. Stein. Although I wasn't the shining star in his long track record of students, he remained supportive throughout my tenure in his laboratory. He has truly given me a better understanding of how to perform and interpret science. I wish him the best of luck with the remainder of his career.

I would also like to thank the other members of my committee for their contributions. Drs. Song and DeStefano taught courses that gave me an opportunity to see a world outside the realm of bacterial genetics. They were also willing to assist me in any way necessary with the completion of my project.

I would like to thank members of the Stein lab, both past and present, for making my lab experience a memorable one. To Julie Patrone, Ellen O'Connor, Sam Bish, Meredith Davis, Hwalih Han, and Esteban Carrizosa, thank you very much for the memories. I hope that I have been able to make your lab experiences a little more pleasant. To Annie Corriveau, Andrzej Peikarowicz, Derek Braun, and Mac Griffiss, although our time working together was short, I appreciated all of your wisdom and support.

I probably would have thrown in the towel long ago if not for the support from my family. Mom and Dad, thank you for not letting me quit when things seemed hopeless. Mom, I'm sorry I didn't get this project completed sooner. You will always

be in my heart. To my wife Lin, thank you for not giving up on me. You showed great patience during our time in Maryland, and you gave me much emotional support. To my little man Brett, I hope that one day you will realize how much joy you bring to your mom and me.

I would like to thank NASCAR, Jimmie Johnson, Playstation 2, NASCAR Thunder 2003, and Hooters for all being cool as hell!!

And lastly, I would like to thank *Neisseria gonorrhoeae* FA1090. You little bastard, you made for some long days and frustrating nights, but you taught me a lot about bacteriology. Fortunately, the doctor gave me a prescription for penicillin, and your infection is clearing nicely.

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LIST OF ABBREVIATIONS

α = Alpha
β = Beta
bp = Base Pair
°C = Degrees Celsius
CDC = Centers for Disease Control and Prevention
cfu = Colony Forming Units
CO_2 = Carbon Dioxide
Δ = Change or Deletion
Gal = Galactose
GCK = Gonococcal Base with Kellogg's Supplement
Glc = Glucose
GalNAc = N-acetyl Galactosamine
GlcNAc = N-acetyl Glucosamine
Hep = Heptose
Kan = Kanamycin
Kan^r = Kanamycin Resistant
kb = Kilobase
L = Liter
LB = Luria Broth
LOS = Lipooligosaccharide
MIC = Minimum Inhibitory Concentration
(-) = Negative
Nitro = Nitrofurantoin
$Nitro^r$ = Nitrofurantoin Resistant
Opa = Opacity Associated Protein
PCR = Polymerase Chain Reaction
PEA = Phosphoethanolamine
polyG = Polyguanine
(+) = Positive
STD = Sexually Transmitted Disease
Spc = Spectinomycin
Spc^r = Spectinomycin Resistant

Introduction

Neisseria gonorrhoeae is an obligate human pathogen responsible for causing the disease gonorrhea. These organisms are facultatively anaerobic, gram (-) cocci that are generally arranged in pairs (diplococci), with flattened sides adjacent to each other. They are catalase (+), oxidase (+), and non-motile. The bacterium utilizes carbohydrates oxidatively. *N. gonorrhoeae* normally colonizes the mucosal surfaces of humans, predominantly the urethra, cervix, and other parts of the urogenital tract. Ocular infections can occur due to infant passage through an infected birth canal.

Gonorrhea is one of the most common sexually transmitted diseases. The disease usually manifests itself in males as an acute anterior urethritis, accompanied by the onset of dysuria and discharge of a purulent exudate. Rarely, the primary infection can spread locally, leading to conditions such as epididymitis, seminal vesiculitis, and prostatitis (Hart and Rein 1976). Female gonococcal infection usually results in increased vaginal discharge, increased urinary frequency, and uterine bleeding. In ~15% of women infected with *N. gonorrhoeae*, the primary infection will lead to further complications, such as endometritis and pelvic inflammatory disease (PID). Unfortunately, PID frequently leads to sterility and contributes to ectopic pregnancies (Curran, Rendtorff et al. 1975; Hook and Holmes 1985).

N. gonorrhoeae has a significant impact on human health. In 2001, over 350,000 cases of gonorrhea were reported in the United States (CDC 2002). Health economists estimate that costs associated with treatment of these gonococcal

infections approach 1.1 billion dollars per year (NIAID 2002). Also, there is evidence suggesting that gonococcal infections facilitate transmission of the human immunodeficiency virus (HIV) (Cohen, Hoffman et al. 1997).

N. gonorrhoeae can infect many anatomical locations, with each niche possessing its own unique physiological properties. In order to successfully establish itself in these different environments, the gonococcus must be able to adapt to its surroundings. The bacteria must produce various cell surface components such that the appropriate components are expressed in the appropriate environments. To facilitate this, *N. gonorrhoeae* has developed genetic mechanisms that allow for high frequency antigenic variation of various cellular components. These include: intramolecular recombination for pili antigenic variation (Meyer, Mlawer et al. 1982); changes in the number of pentameric DNA repeat sequences for opacity associated protein (Opa) expression (Stern, Brown et al. 1986); and changes in the length of homopolymeric tracts for lipooligosaccharide (LOS) variation (Danaher, Levin et al. 1995), *hpuA* expression (Chen, Elkins et al. 1998), *pilC* expression (Jonsson, Nyberg et al. 1991), and *pgtA* expression (Banerjee, Wang et al. 2002).

Pili are cell surface structures that mediate the initial attachment of the gonococcus to host cell surfaces (Swanson 1973). *pilE* encodes the pilin protein, a major subunit of pili. Modified or truncated forms of this gene are found in silent sites clustered throughout the gonococcal genome. Antigenic variation of pili results from the nonreciprocal transfer of partial pilin sequence information from a silent site into

pilE (Hagblom, Segal et al. 1985; Haas and Meyer 1986). Pilin variation can lead to changes in immunoreactivity, posttranslational modification, or adhesive function of the mature pili.

Opa are outer membrane proteins that are important for adhesion and invasion of epithelial cells. The *opa* genes contain multiple copies of the pentameric DNA sequence (CTCTT). Changes in the number of pentameric repeats occurs during DNA replication, producing reading frame changes that result in the phase variable expression of these genes (Stern, Brown et al. 1986). This variation can result in organisms expressing zero, one, or multiple differing Opa proteins (Dehio, Gray-Owen et al. 1998).

Opa variation is biologically important because changes in Opa expression alter its binding specificity. For example, the Opa30 variant has been shown to bind heparin sulfate proteoglycan (HSPG) receptors (van Putten and Paul 1995). Opa50 binds vitronectin, a surface component involved in phagocytosis of apoptotic cells (Dehio, Gomez-Duarte et al. 1998), indicating a possible role for Opa50 in promoting bacterial uptake into host cells via phagocytosis-like mechanisms. Also, some Opa variants bind members of the CD66 family of proteins (Virji, Makepeace et al. 1996; Virji, Watt et al. 1996). Furthermore, changes in Opa alter the type of host cell response induced (Virji, Watt et al. 1996).

The variable expression of several genes can occur due to homopolymeric frameshifting events that occur during DNA replication. An example is *pilC*, which encodes a protein that provides gonococcal pili with its adhesive properties. *pilC* expression is governed by a homopolymeric run of guanines; when translation of *pilC* is shifted out of frame due to changes in the number of guanine residues, functional PilC is not produced. Gonococcal pilus fibers that lack PilC do not support epithelial cell adherence (Rudel, van Putten et al. 1992). Another gonococcal gene, *pgtA*, contains a polyguanine tract within its coding sequence. *pgtA* encodes a galactosyl transferase involved in adding a galactose residue onto the O-linked N-acetylglucosamine (GlcNAc) of pilin glycan. Phase variable expression of *pgtA* appears to play a role in promoting the conversion of uncomplicated gonorrhea to disseminated gonococcal infection (Banerjee, Wang et al. 2002).

Another example of a polyguanine mediated phase variable gene is *hpuA*, a gene that encodes a gonococcal outer membrane protein involved in the binding of hemoglobin. Because mammalian hosts use iron binding proteins and iron sequestering compounds to maintain free iron at a level that is too low to support growth of invading bacterial pathogens (Weinberg 1978), successful colonization requires the ability to adapt to iron replete conditions. Expression of *hpuA* allows gonococci to use hemoglobin as the sole source of iron for growth, eliminating the necessity for scavenging iron from transferrin or lactoferrin compounds. This phase variable receptor is selected for *in vivo* in women during the first half of the menstrual cycle (Anderson, Leone et al. 2001), and it has been hypothesized that phase variation

of this receptor enables efficient utilization of menstrual hemoglobin (Chen, Elkins et al. 1998).

LOS molecules are immunogenic glycolipids found on the surface of the outer membranes of *Neisseria* species. LOS molecules contain three domains: a lipid A region, a core polysaccharide structure, and branched oligosaccharide side chains. LOS closely resembles lipopolysaccharide (LPS) from enterics such as *E. coli* or *Salmonella* species; however, LOS molecules lack an O-antigen. This outer surface component is very important for *Neisseria* pathogenesis, being involved in processes such as immune evasion, attachment, and tissue damage (Ward, Watt et al. 1974; Gregg, Johnson et al. 1981). Each gonococcal strain has the ability to make structurally related forms of LOS, which can be distinguished by the monoclonal antibodies they can bind (Gibson, Webb et al. 1989).

Many genes involved in LOS biosynthesis have been cloned and characterized (Gotschlich 1994). Synthesis of the LOS α chain is initiated by *lgtF*, which encodes a glycosyl transferase that adds a glucose residue to the first heptose molecule in the LOS structure. The *lgt* gene cluster, consisting of genes *lgtA*, *lgtB*, *lgtC*, *lgtD*, and *lgtE*, is responsible for the addition of various sugars to the LOS α chains. *lgtA*, *lgtC*, and *lgtD* contain polyguanine tracts within their coding sequences. The number of guanine residues in each gene determines if it will encode a functional protein. Since sugars are added to a growing oligosaccharide, the loss of function of a protein early in the biosynthetic process will produce truncated LOS molecules. *lgtG*, which is

responsible for initiating β chain synthesis, contains a polycytosine tract. When this gene is out of frame, the bacterium does not add a β chain onto its LOS.

LOS phenotypic variation occurs at a frequency of ~10^{-3}/cell/generation, suggesting that replicational frameshifting should occur at this frequency (Apicella, Shero et al. 1987; Schneider, Hammack et al. 1988). However, no studies have been performed to correlate genotypic and phenotypic variations in *Neisseria*. Furthermore, the influence of the length of a polynucleotide run on frameshifting is unknown.

The addition of bases due to frameshifting leads to the affected genes being expressed out of frame and resultant protein products that are not functional. For example, if the glycosyl transferases produced by the *lgt* gene cluster are not functional, truncated structures of LOS are formed. Figure 1 is a diagram of the composite structure of *N. gonorrhoeae* LOS. If *lgtA* is out of frame, termed “off,” the glucose (Glc) and galactose (Gal) molecules proximal to the first heptose molecule (Hep1) can be added to the LOS α chain correctly, but an N-acetyl glucosamine (GlcNAc) molecule cannot be added due to the lack of LgtA. This truncated structure represents lactosyl LOS. If *lgtC* is “on” in the absence of functional LgtA, an alternative α chain is formed that contains a glucose molecule and two adjacent galactose molecules. If *lgtA* is “on” and *lgtD* is “off,” a lacto-N-neotetraose structure of LOS will be formed.

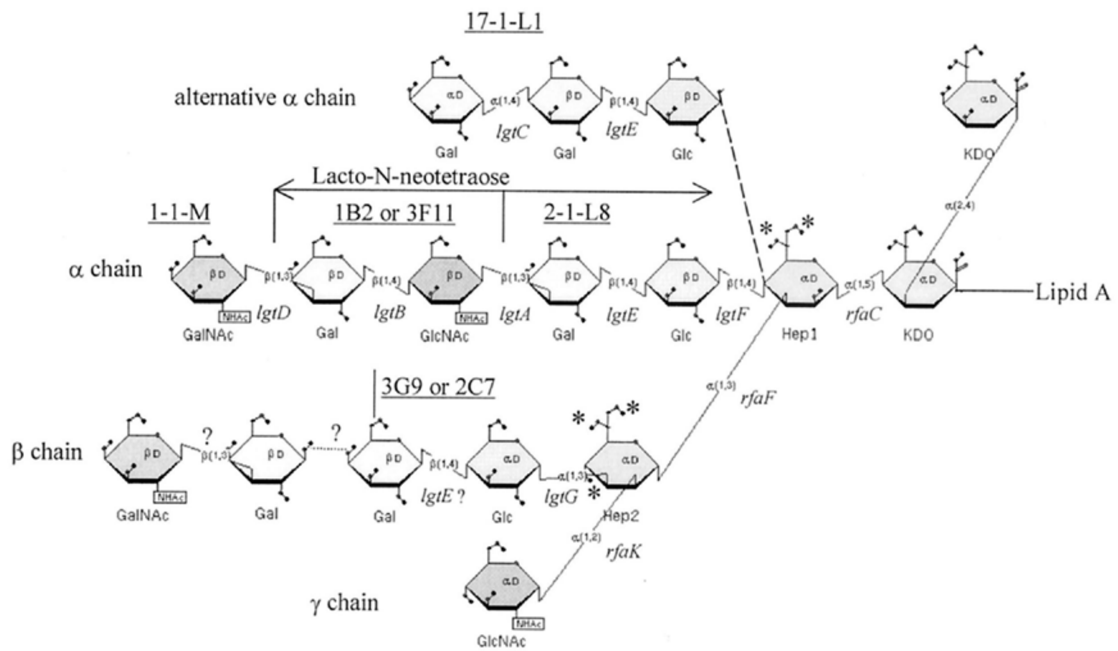

Figure 1 – Composite structure of *Neisseria gonorrhoeae* LOS. Genes involved in LOS biosynthesis are indicated. Monoclonal antibody reactivities are underlined. Dotted lines represent alternative LOS structures. *, represents sites where PEA or phosphate can be added.(Tong, Reinhold et al. 2001)

The various forms of LOS appear to be associated with differing levels of gonococcal pathogenicity. For example, Schneider et al. (1995) demonstrated that a gonococcal variant expressing the lacto-N-neotetraose form of LOS was more infectious than a gonococcal variant exhibiting a lactosyl LOS (Schneider, Cross et al. 1995). Tong et al. (2002) showed that *N. subflava* 44 expresses a limited amount of lacto-N-neotetraose LOS, while *N. gonorrhoeae* PID2 produces numerous LOS structures related to the lacto-N-neotetraose LOS molecules (Tong, Arking et al. 2002). Also, induction and persistence of symptomatic urethral gonorrhea require that the infectious strain antigenically shift to a variant that produces a high molecular weight LOS structure (Schneider, Griffiss et al. 1991). The aforementioned genes are by no means the only phase variable genes in the gonococcus, but this sampling shows that phase variability can have a major impact on the pathogenicity of *N. gonorrhoeae*.

Because the majority of these variation mechanisms require the occurrence of DNA mismatches, it suggests that *N. gonorrhoeae* might be defective in DNA repair. DNA repair mechanisms have been well characterized in *E. coli*, but very little is known about these repair mechanisms in the pathogenic *Neisseria*. The availability of both gonococcal and meningococcal genome sequences has facilitated the identification and comparison of potential Neisserial DNA repair genes to the *E. coli* paradigm (Kline, Sechman et al. 2003).

One of the most important DNA repair systems in bacteria is the SOS response. The *E. coli* SOS system is induced in response to DNA damage, such as

thymine dimers resulting from UV irradiation. In an uninduced cell, LexA acts as a repressor of more than 20 genes, including the *recA* and *lexA* genes, by binding to specific operator sequences upstream of these genes. Although *recA* and *lexA* are considered repressed, a small level of each of these proteins is produced during this repressed state. During DNA damage, regions of single-stranded DNA are produced, and the binding of RecA to these single-stranded regions appears to convert RecA to an "activated" form (RecA*). RecA* interacts with LexA, resulting in proteolytic cleavage of LexA and the increased expression of the various SOS genes, such as the *umuDC* operon. UmuD' (a modified form of UmuD resulting from RecA-facilitated self-cleavage), UmuC, and RecA are thought to work in concert with DNA polymerase III to carry out translesion synthesis, enabling replication over DNA lesions that would otherwise be strongly blocking (Friedberg, Walker et al. 1995; Smith and Walker 1998). There is no known SOS system in the *Neisseria*, as no SOS boxes or LexA homologues have been found in the Neisserial genomes (Roe, Clifton et al. 1997; Black, Fyfe et al. 1998).

E. coli possesses a nucleotide excision repair mechanism that appears to counter many different types of DNA lesions with differing chemical and structural properties (Rupp 1996). The protein products of *uvrA* and *uvrB* form a complex that recognizes and binds to many types of bulky DNA adducts. A third protein, UvrC, interacts with UvrB and both of these proteins make DNA incisions that facilitate the removal of a small region of DNA that contains the DNA damage being repaired. DNA polymerase synthesizes a new strand of DNA to replace the excised fragment.

All of the genes encoding components of the nucleotide excision repair pathway (*uvrA*, *uvrB*, *uvrC*, *uvrD*, and *mfd*) are present in the sequenced genomes of *Neisseria* (Kline, Sechman et al. 2003).

The base excision repair pathway in *E. coli* begins when a damaged base is removed by a DNA *N*-glycosylase that severs the bond linking the base to the sugar-phosphate backbone of DNA. This now abasic site is removed through nuclease activity, and the resulting gap is filled with new DNA by DNA polymerase. Base excision repair pathway homologues (*nth*, *tag*, *mutMTY*, *xthA*, *ogt*, and *fgp*) can be found in the *Neisseria* genomes; however, the *Neisseriae* lack homologues of the endonuclease *nfo* and the glycosylase *nei*. It is thought that molecules with redundant functions may allow for the absence of these two genes or lesions recognized by Nfo and Nei may be rare (Kline, Sechman et al. 2003).

Photoreactivation is a process used by *E. coli* to repair pyrimidine dimers without having to excise any portion of DNA. The photoreactivating enzyme photolyase attaches to pyrimidine dimers that form during UV irradiation of DNA. Upon exposure to light, methylenetetrahydrofolate absorbs energy from the light and transfers it to reduced flavin adenine dinucleotide. This intermediate transfers an electron to the dimer to split it. Further electron transfers restore the original pyrimidines and regenerate the DNA photolyase enzyme (Sancar 1994). Although *N. gonorrhoeae* has been reported to lack a photoreactivation system (Campbell and

Yasbin 1979), a potential homologue to the photolyase enzyme encoded by the *phr* gene is present in the sequenced genomes of both *N. gonorrhoeae* and *N. meningitidis*.

VSP (very short patch) repair in *E. coli* functions to correct thymine-guanine mismatches in the context of the sequence -CC(A/T)GG-. If the second cytosine in this sequence is methylated at position 5 by Dcm, it can spontaneously deaminate to form a thymine, resulting in a thymine-guanine mismatch. The *vsr* gene product, Vsr endonuclease, creates a nick just upstream of the mispaired pyrimidine to initiate the repair process. This strand specific nicking is followed by conventional DNA polymerase I-dependent excision repair (Hennecke, Kolmar et al. 1991). Homologues of genes involved in VSP repair can be identified in the *Neisseria* genomes (Kline, Sechman et al. 2003).

The most studied DNA repair system in the *Neisseriae* is the recombinational repair system. Recombinational repair can be mediated in *E. coli* by either the RecBCD or RecF pathway of homologous recombination (Kowalczykowski, Dixon et al. 1994). The RecBCD pathway is the major pathway for recombination in wildtype *E. coli*. The RecF pathway facilitates recombination events in *recBC* mutants in the presence of *sbcBC* suppressor mutations (Kowalczykowski, Dixon et al. 1994). The *Neisseriae* contain many genes from both of these pathways, and many of these genes have been shown to play a role in DNA repair (Koomey and Falkow 1987; Mehr and Seifert 1998; Stohl and Seifert 2001; Skaar, Lazio et al. 2002). Although homologues of certain RecF pathway components are present in *Neisseriae*, a RecF homologue has

not been identified, leading to the designation of a RecF-like pathway in these organisms (Mehr and Seifert 1998). Based on a lack of *sbcBC* homologues in *N. gonorrhoeae* and the decreased repair phenotypes of RecF-like pathway mutants, it has been suggested that the gonococcus behaves similarly to an *E. coli sbcBC* mutant such that the RecF-like pathway is active in wildtype cells (Mehr and Seifert 1998), probably due to the fact that recombinational repair pathways are more important for *Neisseriae*, which lack an SOS response (Kline, Sechman et al. 2003).

Mismatch repair (MMR) is a mechanism that processes mismatches between 2 undamaged normal bases that cannot form classic Watson-Crick base pairs (Rupp 1996). The majority of our understanding of mismatch repair comes from studies performed on the methyl-directed MMR pathway of *E. coli*. There are three basic steps involved in *E. coli* mismatch repair: recognition of a DNA mismatch, excision of the misincorporated base(s) and DNA surrounding the mismatch, and replacement of excised DNA. To begin, a DNA mismatch is recognized by the MutS protein. The MutL protein, together with the MutS-DNA mismatch complex, stimulate strand scission by MutH, which makes nicks opposite a *dam*-methylated GATC parental DNA sequence. Because newly synthesized strands of DNA will not be fully methylated immediately, making nicks in DNA opposite a *dam*-methylated sequence ensures that the integrity of the parental strain will remain intact. After nicking of the DNA, exonuclease reactions are catalyzed by either ExoVII or RecJ from one side of the mismatch or Exonuclease I from the other side of the mismatch. Finally, DNA polymerase III and SSB proteins replace the excised DNA with new sequence

(Grilley, Griffith et al. 1993; Modrich 1994). The pathogenic *Neisseriae* possess homologues to genes known to be involved in *E. coli* methyl-directed mismatch repair, including *mutS* and *mutL* but lacking *mutH* (Richardson and Stojiljkovic 2001). Although pathogenic *Neisseriae* lack MutH, which is responsible for the initial nick of a newly synthesized, unmethylated strand of DNA, it has been hypothesized that the strand recognition system in *Neisseriae* may be more similar to eukaryotic organisms and could be directly linked to replication apparatus (Holmes, Clark et al. 1990; Petranovic, Vlahovic et al. 2000).

Although some information about gonococcal repair systems has been obtained, DNA repair in *N. gonorrhoeae* has not been well characterized. Understanding gonococcal repair systems would enhance our knowledge about prokaryotic DNA repair in general as well as give insight into the understanding of gonococcal pathogenesis. Although many genes in *N. gonorrhoeae* contain homopolymeric nucleotide repeats, which should represent possible hot spots of mutation, this organism does not appear to be hypermutable. In fact, observations indicate that this organism is hypomutable (Campbell and Yasbin 1984), with mutation frequencies occurring about 10 fold less often than what is seen in *E. coli* (Baquero, Nilsson et al. 2004). However, neisserial *mutY* mutants have been shown to have a hypermutable phenotype in both meningococci and gonococci when inactivated (Davidsen, Bjoras et al. 2005). This paradox implies that the gonococcus has a novel method of maintaining genome stability and repairing DNA mismatch events.

The study of homopolymeric frameshifting in *N. gonorrhoeae* has been hampered by the lack of a selection method for measuring mutation frequencies. Schneider et al used immunoelectron microscopy and monoclonal antibody binding patterns to demonstrate that *N. gonorrhoeae* strain 4505 was able to vary its LOS expression (Schneider, Hammack et al. 1988). Although this phenotypic screen yielded an observed LOS variation frequency, the variation in LOS expression could not be directly attributed to changes in the homopolymeric nucleotide repeats found in the phase variable LOS biosynthetic genes. Furthermore, because the scoring of phase variation is a subjective measure, it is possible that Schneider et al. could have over- or under-estimated the true phase variation frequency.

The goal of the research presented here was to use molecular biology techniques to study and assess frameshifting frequencies in *N. gonorrhoeae* FA1090. To accomplish this, a reporter system was created that could allow for positive selection of frameshifting events while maintaining the viability of the affected organism. This reporter system involved the manipulation of a gonococcal nitroreductase gene and its effects on gonococcal resistance to nitroaromatic compounds.

Nitroreductases have been identified in a wide variety of microorganisms. They were originally studied because they play a role in the reductive activation of various nitroaromatic antimicrobials, such as nitrofurantoin, nitrofurazone, and furazolidone. Although the biological role of nitroreductases in bacteria is unknown,

strains lacking nitroreductases are more resistant to nitroaromatic compounds (Whiteway, Koziarz et al. 1998). The antimicrobial actions of nitrofurantoin, nitrofurazone, and furazolidone are all dependent on the reduction of the nitro groups found in these compounds. Although the starting and ending products of this nitroreduction lack antimicrobial activity, it is believed that the reactive oxygen intermediates produced during this reaction can damage DNA and may inhibit bacterial protein synthesis (McOsker and Fitzpatrick 1994; Greenwood 2000).

Because the loss of gene function results in resistance to an antimicrobial agent, these genes provide an ideal starting point for studying spontaneous mutation. Database searches were used to identify potential nitroreductases in *N. gonorrhoeae*. The biochemical properties of these putative nitroreductases were verified, and a nitrofurantoin-based reporter system was developed that allowed for the determination of spontaneous mutation frequencies due to homopolymeric tracts in *N. gonorrhoeae*.

Materials and Methods

Bacterial Strains, Plasmids, and Oligonucleotides

All bacterial strains, plasmids, and oligonucleotide primers used in this study are listed in Table 1, Table 2, and Table 3.

Bacterial Growth and Storage Conditions

All media and solutions used in this study are described in Table 4. All *Neisseria* strains were grown in GCP broth plus growth supplements (White and Kellogg 1965) and 0.042% sodium bicarbonate or on GCK agar plates plus growth supplements (Difco, Detroit, MI) in a 37°C, 6% CO_2 incubator. *E. coli* strains were grown in LB broth or on LB agar plates. When needed, antimicrobials were added to the growth media as follows: kanamycin, 50μg/mL; spectinomycin, 50μg/mL; and nitrofurantoin, 3μg/mL. When screening for β-galactosidase activity, X-gal was added to the medium (35μg/mL). All strains were stored at -80°C in GCP or LB broth supplemented with 20% glycerol. Optical densities of gonococcal and *E. coli* cultures were determined using a Klett-Summerson colorimeter fitted with a green filter. One Klett unit corresponds to a culture density of approximately 5×10^6 CFU/mL.

Chemicals, Reagents, and Enzymes

Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA). All chemicals used in this study were reagent grade or better and were purchased from Sigma (St. Louis, MO) unless otherwise specified.

Table 1 – Bacterial Strains Used		
Strain	**Phenotype**	**Source**
Neisseria gonorrhoeae FA1090		P. Frederick Sparling
N. gonorrhoeae FA19		William Shafer
N. gonorrhoeae FA853		P. Frederick Sparling
N. gonorrhoeae F62		P. Frederick Sparling
N. gonorrhoeae MS11		Herman Schneider
N. gonorrhoeae PID2		
FA1090 *nfsB* (mod) polyG-5	Spc^r; contains 5-residue polyG tract	This study
FA1090 *nfsB* (mod) polyG-6	Spc^r; contains 6-residue polyG tract	This study
FA1090 *nfsB* (mod) polyG-7	Spc^r; contains 7-residue polyG tract	This study
FA1090 *nfsB* (mod) polyG-8	Spc^r; contains 8-residue polyG tract	This study
FA1090 *nfsB* (mod) polyG-9	Spc^r; contains 9-residue polyG tract	This study
FA1090 *nfsB* (mod) polyG-10	Spc^r; contains 10-residue polyG tract	This study
FA1090 *nfsB* (mod) polyG-11	Spc^r; contains 11-residue polyG tract	This study
FA1090 *nfsB* (mod) polyG-12	Spc^r; contains 12-residue polyG tract	This study

Table 2 – Plasmids Used		
Plasmid	**Properties**	**Source**
pK18	Gonococcal suicide vector; Kan^r	RD Pridmore
pK18-*nfsB*	FA1090 *nfsB* cloned into pK18	This study
pK18-*nfsB*-polyG10	10-residue polyG tract within *nfsB*	This study
pNFSB-polyG10	Spc^r cassette cloned downstream of *nfsB* in pK18-*nfsB*-polyG10	This study
pNFSB-polyG5	5-residue polyG tract within *nfsB*; Spc^r	This study
pNFSB-polyG6	6-residue polyG tract within *nfsB*; Spc^r	This study
pNFSB-polyG7	7-residue polyG tract within *nfsB*; Spc^r	This study
pNFSB-polyG8	8-residue polyG tract within *nfsB*; Spc^r	This study
pNFSB-polyG9	9-residue polyG tract within *nfsB*; Spc^r	This study
pNFSB-polyG11	11-residue polyG tract within *nfsB*; Spc^r	This study
pNFSB-polyG12	12-residue polyG tract within *nfsB*; Spc^r	This study

Table 3 – Primers Used	
Primer	**Sequence (5’ > 3’)**
NP1	AAAGGATCCCATGAACGCCATTGCAGACG
NP2	GGGGGATCCAGAAGATACCATACGCCTCT
G string 1	AAAAACCGGTTTAAGCAAAGAGCAGGTTCTATCCG
G string 2 (polyG-10)	AAAAACCGGTCCCCCCCCCCATCGTGCATCCTTTTATTACTTTGAT
polyG-5	AAAAACCGGTACCCCCACCCATCGTGCATCCTTTTATTACTTTGAT
polyG-6	AAAAACCGGTACCCCCCATCGTGCATCCTTTTATTACTTTGAT
polyG-7	AAAAACCGGTCCCCCCCATCGTGCATCCTTTTATTACTTTGAT
polyG-8	AAAAACCGGTACCCCCCCCACCCATCGTGCATCCTTTTATTACTTTGAT
polyG-9	AAAAACCGGTACCCCCCCCCATCGTGCATCCTTTTATTACTTTGAT
polyG-11	AAAAACCGGTACCCCCCCCCCCACCCATCGTGCATCCTTTTATTACTTTGAT
polyG-12	AAAAACCGGTACCCCCCCCCCCCATCGTGCATCCTTTTATTACTTTGAT
OmegaABC	TCAGATGGCGCGCCTGTACATCGATGGTGATTGATTGAGCAAGCTTTATGC
dwnstrmF	AAAATGTACAATTTGCCGGGCGGCAGCCTGC
dwnstrmR	AAAATGTACAGGCGTTATCTCGCTCCCGGCG
GampF	ATCAATTTGCAATCAAAGTAATAAAAGG
GampR	TGCGGATAGAACCTGCTCTTTGC
GampR +1	ATGCGGATAGAACCTGCTCTTTGC
GampR -1	GCGGATAGAACCTGCTCTTTGC

Table 4 – Media Composition

GCK (1L)

Component	Amount
Gonococcal Base Medium	36.5g
Bacto-Agar	5.0g
Kellogg's Supplement (100X)	10.0mL
HPLC H_2O	

GCP (1L)

Component	Amount
Protease Peptone #3	15.0g
Soluble Starch	1.0g
KH_2PO_4	1.0g
K_2HPO_4	4.0g
NaCl	5.0g
HPLC H_2O	

100X Kellogg's (1L) – filtered

Component	Amount
Glucose	400.0g
Glutamine	5.0g
Ferric Nitrate	0.6g
Thiamine Pyrophosphate	0.02g
HPLC H_2O	

Luria Broth (1L)

Component	Amount
Bacto-Tryptone	10.0g
Yeast Extract	5.0g
NaCl	5.0g
HPLC H_2O	

Luria Agar (1L)

Component	Amount
Bacto-Tryptone	10.0g
Yeast Extract	5.0g
NaCl	10.0g
Bacto-Agar	15.0g
HPLC H_2O	

Polymerase chain reaction reagents were purchased from Roche Molecular Biochemicals (St. Louis, MO) or Invitrogen (Carlsbad, CA).

Polymerase Chain Reactions

Polymerase chain reaction amplifications were generally performed using Platinum PCR Supermix (Invitrogen) or the Expand Long Template PCR kit (Roche). Oligonucleotide primers were purchased from Bioserve Biotechnologies (Laurel, MD) or Integrated DNA Technologies (Coralville, IA). PCR products were purified using the Qiaquick PCR Purification kit (Qiagen, Valencia, CA).

Restriction Endonuclease Reactions

Restriction endonuclease digestions were performed according to manufacturer's recommendations for each enzyme used. Reaction mixtures were incubated at the appropriate temperature for 2-24 hours. Enzymes were heat inactivated according to the instructions from New England Biolabs. If heat inactivation was not possible, phenol chloroform extractions were used to inactivate the enzymes prior to purification procedures.

T4 DNA Ligase Reactions

DNA ligation reactions were carried out at 4°C or at room temperature. DNA to be ligated was added to 1µL of T4 DNA ligase and the appropriate amounts of T4 DNA ligase buffer and water. Ligations were incubated for 2-24 hours.

Transformation of *E. coli*

Competent cells of *E. coli* DH5αMCR were prepared by the method of Inuoe (Inoue, Nojima et al. 1990). The introduction of DNA into *E. coli* was done using competent cells prepared as described above using the standard heat shock protocol (Cohen, Chang et al. 1972). Cells were allowed to express the transforming DNA for 30-60 minutes prior to plating on appropriate selective agar.

Transformation of *N. gonorrhoeae*

N. gonorrhoeae transformations were done using a tube transformation procedure. Piliated gonococcal cells were suspended in 1mL GCP broth supplemented with growth supplements, 0.0042% sodium bicarbonate, and 10 mM $MgCl_2$ to a cell density that gave slight turbidity. DNA (1-10µL) was added to the culture, and the bacterial suspension was incubated with shaking at 37°C for 2-4 hours. Bacteria were plated onto GCK agar plates supplemented with the appropriate antibiotic and incubated at 37°C, in 6% CO_2 for 24-48 hours.

Plasmid DNA Isolations

Plasmid isolations were carried out using an alkaline lysis protocol (Birnboim and Doly 1979). The nature of the obtained product was determined by analysis using restriction digestions and agarose gel electrophoresis.

Isolation of Gonococcal Chromosomal DNA

Chromosomal DNA was isolated from *N. gonorrhoeae* by suspending a small amount of gonococcal cellular material in 5µL 0.5M NaOH. This solution was neutralized by the addition of 5µL 1M Tris-HCl, pH 7.5. This suspension was diluted into 90µL of purified water. DNA prepared in this way is suitable for PCR amplification.

Nitrofurantoin Plating Procedure

Gonococcal cells were suspended in 4mL of GCP broth until a Klett reading of 100 was achieved. 10-fold dilutions were carried out to a final dilution of 10^{-6}. 100µL aliquots from the 10^{-1}, 10^{-2}, and 10^{-3} dilution tubes were plated on GCK agar plates supplemented with 3µg/mL nitrofurantoin. Aliquots from the 10^{-5} and 10^{-6} dilution tubes were plated on GCK agar. Plates were incubated at 37°C, 6% CO_2 for 24-48 hours. The number of colonies arising on these plates was determined, and the mutation frequency obtained was calculated by dividing the number of colonies on the selective medium by the total number of colonies obtained.

Agarose Gel Electrophoresis

DNA fragments were separated by agarose gel electrophoresis using 1% agarose TBE gels. The location of the DNA within these gels was visualized by staining with ethidium bromide (0.5µg/mL), which was added to agarose prior to pouring of the gel. Gels were run at a constant 100 volts for 15-60 minutes.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gels were electrophoresed using the method developed in our laboratory. Gels were prepared by pouring 18mL of liquid 15% polyacrylamide between two 20cm vertical glass plates. TBE-saturated butanol was layered on top of the acrylamide immediately after pouring it. The acrylamide was allowed to polymerize for 1 hour. The butanol layer was discarded, and the top of the acrylamide gel was washed multiple times with hot 1X TBE buffer. A comb was inserted between the glass plates so as not to disturb the integrity of the acrylamide gel. Hot 1% agarose was added on top of the acrylamide, and it was allowed to harden for 30 minutes. The comb was then gently removed. Polyacrylamide gels were run at constant voltage (1000 volts) for 2-2.5 hours. Gels were stained in a solution containing ethidium bromide (10 mg/mL) for 15 minutes, and destained in TBE buffer.

DNA Sequencing

DNA sequencing was performed by Macrogen, Inc. (Seoul, Korea). PCR products to be sequenced were purified using a Qiaquick PCR Purification kit (Qiagen, Valencia, CA) and diluted to a final concentration of ~50ng/µL. 20µL of PCR product were sent per sequencing reaction. Plasmids to be sequenced were purified using an alkaline lysis procedure (Birnboim and Doly 1979) and diluted to a final concentration of ~100ng/µL. 20µL of purified plasmid were sent per sequencing reaction. Primers to be used for sequencing reactions were diluted to 5pmol/µL, and

5µL of primer were sufficient for completing 10 sequencing reactions. Further information can be obtained from Macrogen's website (www.macrogen.com).

Results

I. Identification of a Putative Reporter System

Identification of Potential Nitroreductase Genes

Two nitroreductases have been identified in *E. coli*; *nfsA* and *nfsB* (Zenno, Koike et al. 1996; Zenno, Koike et al. 1996). The amino acid sequences of these two genes were used to search the gonococcal genomic DNA sequence database. Although no similarities to *nfsA* were obtained, an open reading frame (ORF) with some homology to *nfsB* was found. Figure 2 is an alignment of proteins that showed significant homology to the gonococcal NfsB homolog. All of these proteins have nitroreductase activity. The gonococcal sequence shows 25% identity and 44% similarity to the *E. coli* NfsB protein, suggesting that the gonococcal chromosome may encode a nitroreductase. The amino acid sequence of the gonococcal *nfsB* was used to search the GenBank database, and ORFs that possessed significant homology to it were identified. Significant sequence variation was observed in the immediate amino terminal end of the protein. The *E. coli* gene appeared to be missing the first 5 amino acids that were contained within the coding sequence for the other genes, suggesting that the amino acid sequence at the start of the gene is highly variable and not necessarily essential for gene function.

MIC/Spontaneous Mutation Frequency Studies

If *N. gonorrhoeae* possesses a nitroreductase, it should be sensitive to antimicrobial agents that are activated by nitroreductases and it should be possible to isolate mutants that become resistant to these activated antimicrobials due to the

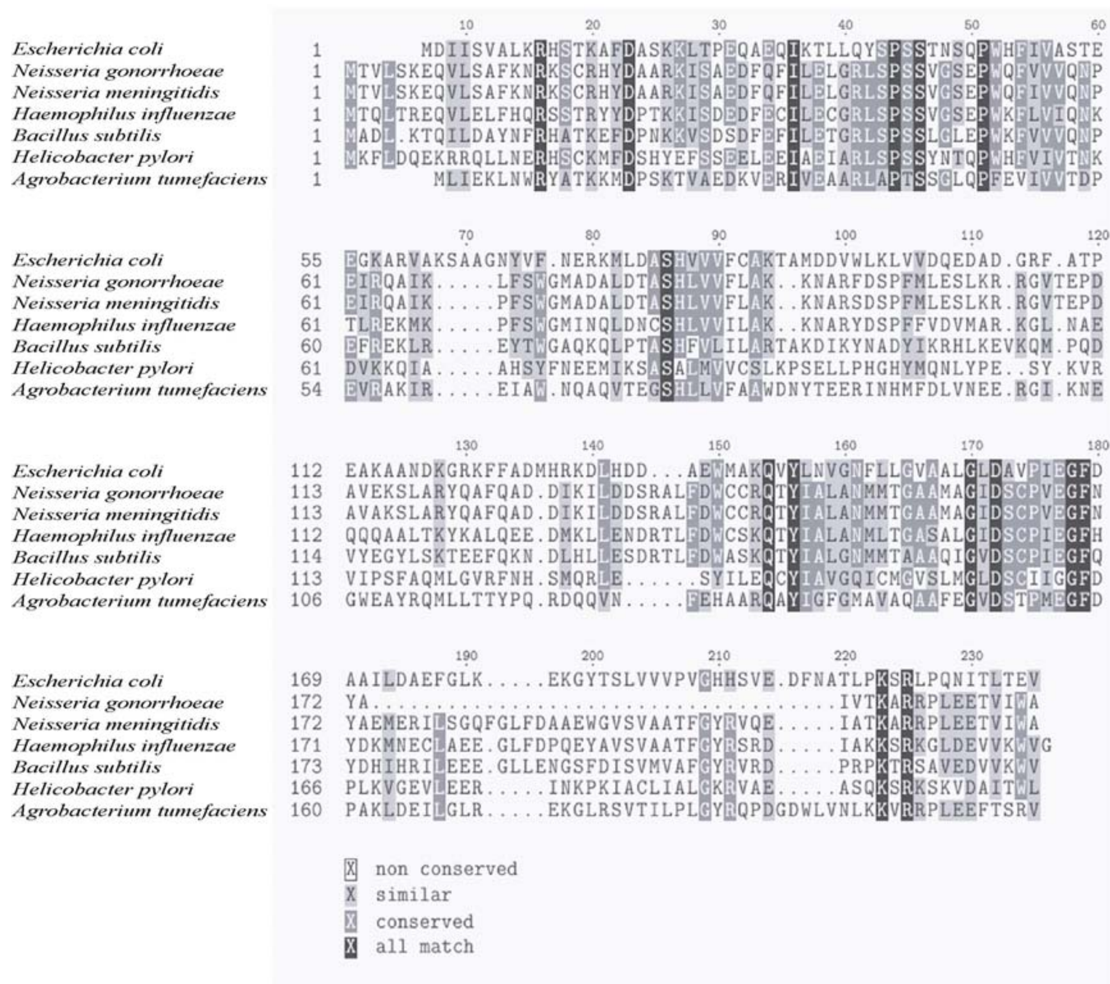

Figure 2 – Amino acid sequence alignments of various nitroreductase genes from select organisms. The amino acid sequence of *E. coli* NfsB (accession number BAA05004) was used to search the genomic databases using a Blast search. Proteins with significant homology to BAA05004 were aligned. The amino acid sequences used were derived from the following strains: *Neisseria gonorrhoeae* FA1090, NC002946; *Neisseria meningitides* MC58, NP273846; *Haemophilus influenzae*, Q57431; *Bacillus subtilis*, O34475; *Helicobacter pylori*, AAL37284; and *Agrobacterium tumefaciens* str. C58, NP_534964.

organism's loss of nitroreductase activity. The minimum inhibitory concentration (MIC) of nitrofurantoin for several strains of *N. gonorrhoeae* was determined by a plate dilution method. Briefly, approximately 10^8 gonococci were spotted onto media containing various amounts of nitrofurantoin. The inoculated plates were incubated overnight, and the MIC was defined as the amount of nitrofurantoin needed in the plate to completely inhibit the growth of the organisms in 24 hours. This analysis indicated that the MIC for nitrofurantoin was approximately 2μg/mL. The plates were incubated for an additional 24 hours, and colonies that arose after this incubation step were presumptive nitrofurantoin resistant mutants. The spontaneous mutation frequency for various gonococcal strains was measured. The frequencies ranged from 6.16×10^{-7} to 6.17×10^{-9} (Table 5; done in conjunction with Esteban Carrizosa), indicating that the mutation frequency associated with this antimicrobial agent varied among the different strains of the gonococcus. Since it was possible to isolate mutants that readily grew on media containing levels of nitrofurantoin above the MIC, we hypothesized that the mutation responsible for this phenotype was in the coding sequence for the putative gonococcal nitroreductase gene.

Evaluation of Nitroreductase Potential in *N. gonorrhoeae*

In *E. coli*, resistance to nitroheterocyclic compounds occurs in a stepwise manner. Step one is inactivation of *nfsA*, which produces resistance to low levels of nitro compounds. Step two, resulting in the combined inactivation of *nfsA* and *nfsB*, yields considerably higher nitroaromatic resistance (Whiteway, Koziarz et al. 1998). Attempts were made to isolate second-step mutants in *N. gonorrhoeae* by taking

Table 5 - Spontaneous mutation frequencies to nitrofurantoin resistance for various gonococcal strains.

Strain	Spontaneous Mutation Frequency*
F62	6.17×10^{-9}
FA19	2.92×10^{-8}
FA1090	3.36×10^{-9}
MS11	6.16×10^{-7}
PID2	9.72×10^{-8}

*Gonococcal cultures were suspended to approximately 10^9 CFU/mL. Serial dilutions of each culture were plated on media containing 2µg/mL nitrofurantoin, and the number of viable cells for each experiment was determined.

isolates resistant to 3µg/mL nitrofurantoin and plating them on GCK agar plates containing 4µg/mL nitrofurantoin. No isolates were obtained that could grow on the higher level of nitrofurantoin, suggesting that the *nfsB* homolog is the only nitroreductase in the gonococcus able to reduce nitrofurantoin. Nitrofurantoin resistant derivatives were isolated from all strains tested. Two nitrofurantoin resistant derivatives of FA19 were chosen for further biochemical study, FA19(M2) and FA19(M3).

Detection of Nitroreductase Activity in *N. gonorrhoeae*

If gonococci become resistant to nitrofurantoin because of the loss of a nitroreductase, it should be possible to demonstrate a lack of nitroreductase activity in these mutants. An indirect spectrophotometric assay was performed to measure nitroreductase activity in parent and mutant strains of *N. gonorrhoeae*. If a bacterial strain possesses a nitroreductase, that protein's reduction of a nitroaromatic substrate should be coupled with a consumption of NADPH. The conversion of NADPH to NADP could be monitored by measuring a decrease in absorbance at a wavelength of 340nm.

Mutants resistant to nitrofurantoin were identified. The amount of nitroreductase present in these mutants was compared to that found in the parent strain. The OD_{340} remained relatively constant in FA19(M2) and FA19(M3), indicating a lack of nitroreductase activity in these strains (Figure 3; done by Esteban Carrizosa). Conversely, the OD_{340} of the wildtype strain decreased over 2 minutes to a

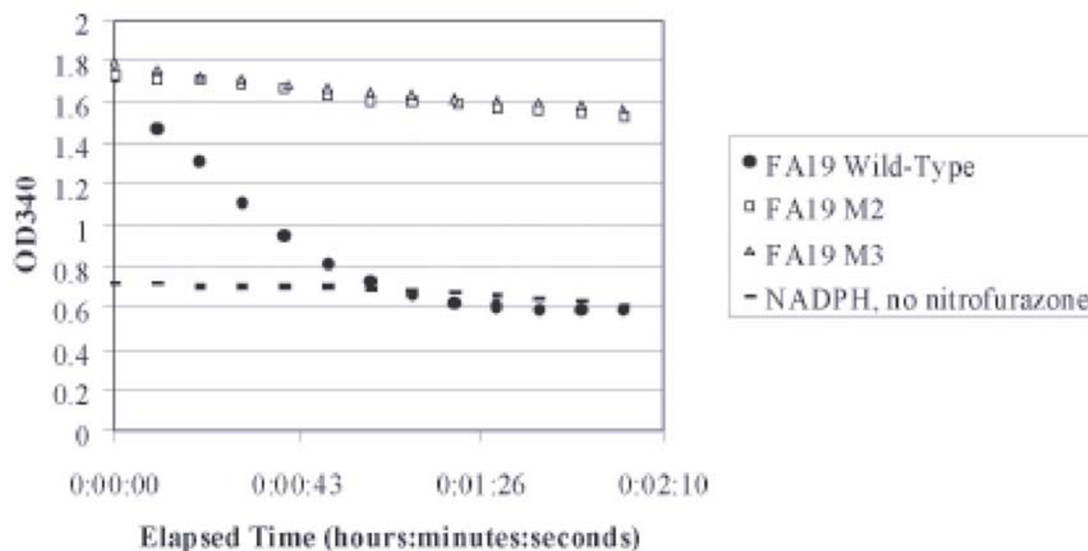

Figure 3 – Biochemical analysis of nitroreductase activity in wildtype and mutant lysates. Wildtype cultures possess nitroreductase activity, mutants in *nfsB* lack nitroreductase activity. Closed circles, wildtype *N. gonorrhoeae* FA19; open squares, mutant with a frameshift mutation in *nfsB*; open triangles, mutant with a point mutation in *nfsB*; black dashes, reaction carried out in the absence on nitrofurazone (FA19 wildtype lysate and 0.2mM NADPH present).

level equal to the control sample lacking NADPH, indicating the presence of nitroreductase activity. The inability to isolate mutants with increased resistance to nitrofurantoin and the lack of measurable nitroreductase activity in FA19(M2) and FA19(M3) suggest that the gonococcus contains a single nitroreductase.

The DNA sequence of the gonococcal *nfsB* homolog was determined from two nitrofurantoin resistant mutants, FA1090(nitro 1) and FA1090(nitro 2). Primers NP1 and NP2 were used to amplify the *nfsB* region from these organisms, and DNA sequencing was carried out on the PCR products. The data indicate that FA1090(nitro 1) possessed a single base change that introduced a premature stop codon into the coding sequence of *nfsB*. FA1090(nitro 2) possessed an insertion of a single adenine nucleotide in the beginning of the *nfsB* coding sequence, resulting in a frameshift mutation. This sequence information suggests that the nitrofurantoin resistant phenotype is due to the loss of nitroreductase activity.

Analysis of the *nfsB* Coding Sequence

The *nfsB* DNA sequences for *N. gonorrhoeae* strains 853, F62, FA19, MS11, and PID2 were determined by sequencing PCR products amplified from gonococcal chromosomal DNA. Sequence analysis indicated that the *nfsB* gene was highly conserved, with nucleotide variation limited to two independent single base substitutions identified in strains 853 and PID2. These single base changes resulted in amino acid substitutions in NfsB. Since these proteins were essentially identical, the

variability in spontaneous mutation frequencies observed in these strains could reflect the different DNA repair capacities of each strain.

II. Identification of the Genetic Basis of Spontaneous Nitrofurantoin Resistant Mutants

**The following work was done collaboratively by Esteban Carrizosa and Robert Holder*

Analysis of Spontaneous Mutation in Nitrofurantoin Resistant Mutants

Numerous FA1090 nitrofurantoin resistant mutants were isolated, and the DNA sequence of *nfsB* in these strains was determined. A majority of these mutants possessed the insertion of an adenine residue in a run of 5 adenines, suggesting a bias for frameshifting during DNA replication at this position. To eliminate the frameshifting bias, this DNA sequence was modified to alter the polyA tract while preserving the amino acid sequence. To accomplish this, the *nfsB* gene was cloned into pK18, resulting in plasmid pK18-*nfsB*. Using PCR, the polyA sequence was altered to AAGAA, giving rise to plasmid pEC1. The Ω fragment from pHP45Ω was inserted into this construct to yield pEC3.

A tube transformation using pEC3 as transforming DNA was done to introduce the modified *nfsB* into the FA1090 chromosome. Transformation mixtures were plated on GCK agar containing 50µg/mL spectinomycin, resulting in strain FA1090 NfsB-BsmI-Ω. The Ω fragment coding sequence was removed from this strain via the spot transformation procedure (Gunn and Stein 1996), resulting in FA1090-

NfsB(mod). PCR using primers NP1 and NP2 was done to amplify the modified *nfsB* regions from spot transformants. Primers S1 and S2 were used for sequencing of these amplicons. Analysis of the obtained sequences indicates that FA1090-NfsB(mod) contained the desired sequence modification. Nitroreductase assays done with this strain demonstrate that it possesses wildtype levels of NfsB activity (data not shown).

The genetic basis of numerous spontaneous nitrofurantoin resistant mutants of strain FA1090-NfsB(mod) was determined. This strain was plated on GCK agar containing 3μg/mL nitrofurantoin, and 107 independent mutants that arose from this plating were isolated. Primers NP1 and NP2 were used to amplify the *nfsB* region from these mutants, and primers S1 and S2 were used to determine the DNA sequence of these PCR amplicons. This approach should allow for the identification of 5 different types of mutations: missense mutations, nonsense mutations, insertions, deletions, and mutations that affect nitrofurantoin uptake.

The data in Table 6 summarize the types of mutations identified by analysis of the PCR amplicon sequences. About 50% of the mutants harbored point mutations, 25% possessed insertions, and 25% contained deletions. The largest insertion mutant was 13bp in length and the largest deletion was 4bp in length. None of the multiple base insertions greater than 3bp appeared to be the result of duplications in the native coding sequence, and none of the deletions appeared to eliminate repeated sequences or sequences that contained obvious secondary structure.

Table 6. Genetic basis of nitrofurantoin resistance.

Point					Frameshift				Other[a]
Nonsense			Missense		Insertions (single site)		Deletions (single site)		
	observed	potential[b]			Single base	22	Single base	16	3
CAA->TAA	7	2	Transitions		Multiple bases	4	Multiple base	9	
CAG->TAG	11	8	C->T	7					
TCG->TAG	9	4	G->A	1					
GAG->TAG	5	4							
TGG->TGA	1	5							
Total:	33	23	Total:	20	Total:	26	Total:	25	

Transitions/transversions generated by missense mutants		Transitions/transversions generated by nonsense mutants	
			Total
C->T	7	19	26
G->A	1	0	1
Total # transitions[c]			27
Transversions			
T->A	3		3
G->C	2		2
T->G	5		5
A->C	2	14	16
Total # of transversions[d]			27

[a]Other are mutations that occurred outside of the coding sequence for *nfsB*.

[b]Potential is the number of times this nonsense mutation could occur within the *nfsB* coding sequence.

[c]Total # of transitions is the sum of the number of transitions obtained from nonsense mutations plus the number obtained from missense mutations.

[d]Total # of transversions is the sum of the number of transversions obtained from nonsense mutations plus the number obtained from missense mutations.

III. Utilization of the Nitrofurantoin Reporter System

**The following work was done by Robert Holder*

Construction of *N. gonorrhoeae* FA1090 *nfsB*(mod) polyG-10

The overall goal of this project was to construct a reporter system that could be used to directly measure phase variation frequencies. This study focused on the gonococcal *nfsB* gene because of how the presence of this gene affects an organism's sensitivity to an antimicrobial agent. With antibiotics such as kanamycin and spectinomycin, the presence of the genes *aph(3')-Ia* and *aad9*, respectively, allow an organism to be resistant to these antibiotics. Genetically interrupting the coding sequences of these genes to study phase variation would result in bacteria unable to grow in the presence of kanamycin or spectinomycin, which is an immeasurable phenotype. However, the expression of the *nfsB* gene confers sensitivity to nitrofuran antimicrobials. The insertion of potentially phase variable sequences into the coding sequence of this gene would allow for the measurement of phase variation frequency because a phase variation event would result in nitrofuran resistant organisms, a phenotype that can be measured.

In order to develop this reporter system, a construct was made that had a polyG tract inserted into the coding sequence of *nfsB*, taking special care to maintain the correct reading frame of *nfsB*. After constructing this modified gene, it needed to be incorporated into the chromosome of a test strain and then the nitrofurantoin sensitivity of this strain needed to be verified. After performing these control experiments, this reporter system could be used to measure frameshifting frequencies.

Frameshifting events should result in cells that contain an out-of-frame *nfsB* and nitrofurantoin resistant phenotype. By plating a known amount of modified gonococcal cells on media containing a nonpermissive level of nitrofurantoin, frequencies associated with frameshifting could be assessed by determining the number of nitrofurantoin resistant colonies that arise.

The first step in assessing frameshifting frequencies due to homopolymeric nucleotide tracts was to create gonococcal strains containing modified *nfsB* genes. To do this, primers NP1 and NP2 were used to amplify the *nfsB* region from *N. gonorrhoeae* FA1090. This amplicon was cloned into the BamHI site of the pUC-based plasmid pK18, resulting in the plasmid pK18-*nfsB*. pK18-*nfsB* was transformed into *E. coli* DH5αMCR, and plasmids were extracted from kanamycin resistant transformants using an alkaline lysis procedure (Birnboim and Doly 1979). Extracted plasmids were digested with DrdI to verify the presence of the correct insert; digestion with DrdI produced the expected banding pattern when digestion products were run on a 1% agarose TBE gel. Figure 4 is a schematic of the procedure followed. Figure 5 is a gel image of DrdI digestions that demonstrate that the desired modifications were introduced into the plasmid.

The next step in plasmid construction was to insert the desired polyG tract into the coding sequence of *nfsB*. Amino acid sequence alignments suggested that the first few amino acids of NfsB could be modified without hindering protein function (Whiteway, Koziarz et al. 1998). Using pK18-*nfsB* as template, primers Gstring 1 and

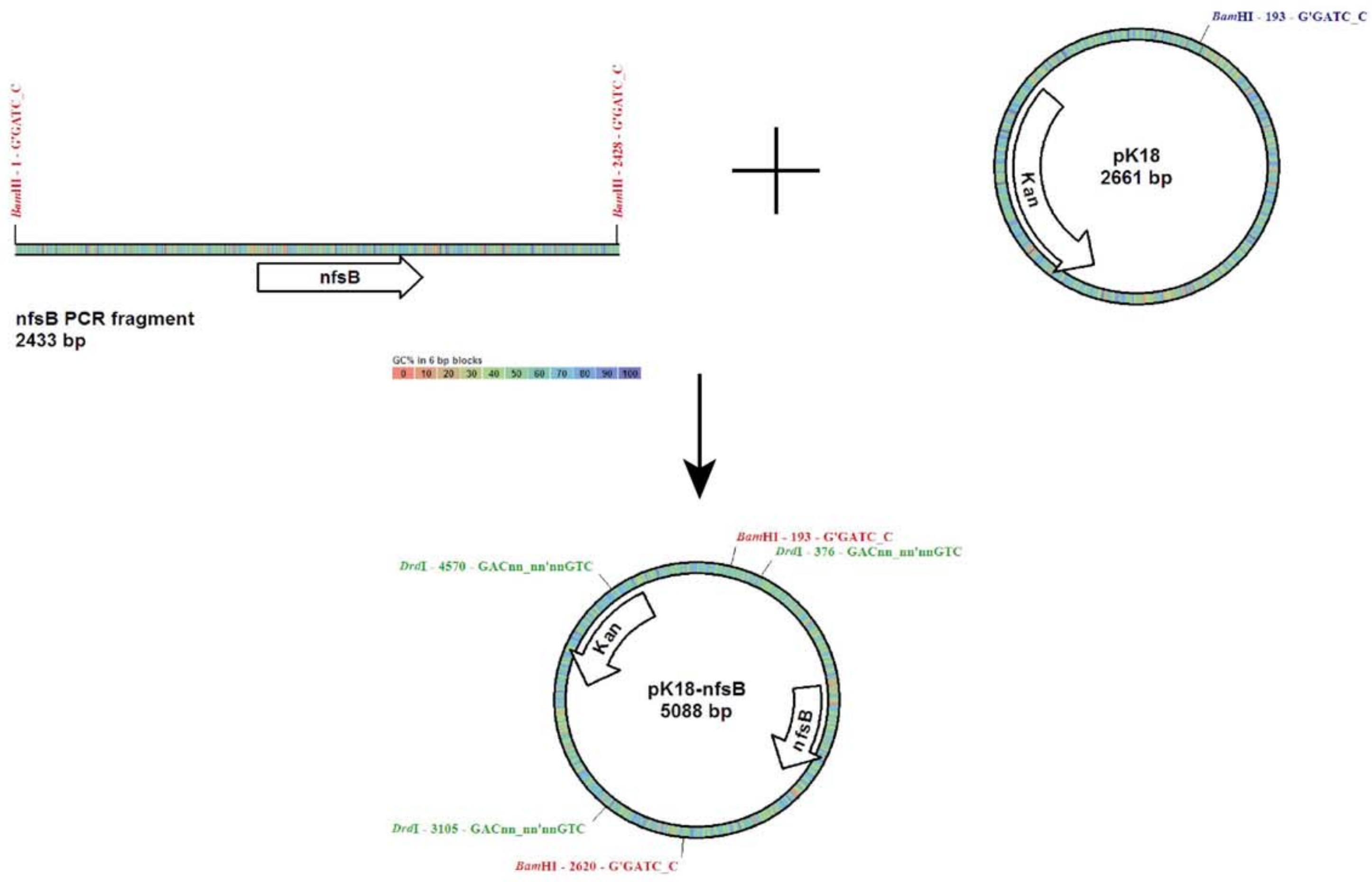

Figure 4 - Cloning of *nfsB* into pK18.

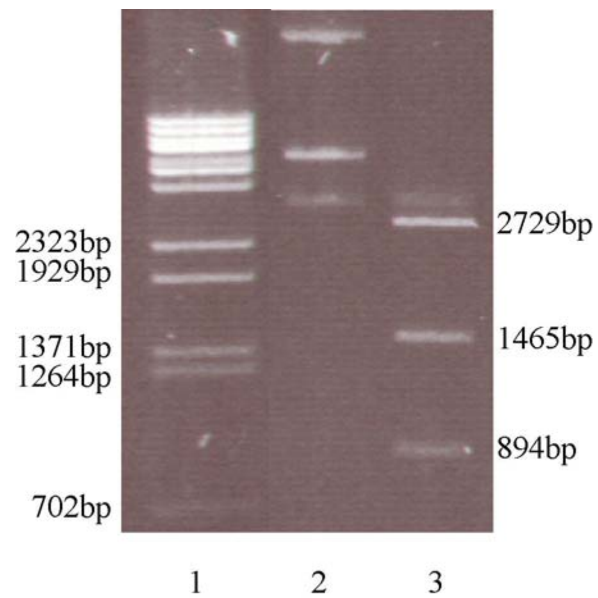

Figure 5 – Digestion verification of pK18-*nfsB*. Plasmids isolated from kanamycin resistant transformants were digested with DrdI to verify the presence of the *nfsB* gene. The expected digested fragment sizes are 2729bp, 1465bp, and 894bp. Lane 1, Lambda DNA-BstEII digest ladder; Lane 2, pK18-*nfsB* undigested; Lane 3, pK18-*nfsB* digested with DrdI.

Gstring 2 were used to amplify the entire sequence of this construct. These two primers were designed to anneal immediately after the start codon of the *nfsB* sequence and amplify in opposite directions, thus amplifying the entirety of pK18-*nfsB*. These primers were also designed to incorporate a 9-residue guanine tract and a unique AgeI site immediately downstream of the *nfsB* start codon. Figure 6 is a schematic showing the construction scheme. Figure 7 shows the *nfsB* modifications introduced. The introduction of a 9-residue guanine tract actually results in a tract length of 10 residues due to the guanine of the start codon. Digestion of this PCR product with AgeI and ligation of the digested product upon itself yielded pK18-*nfsB*-polyG10. This construct was used to transform *E. coli* DH5αMCR, and plasmids were extracted from kanamycin resistant transformants. The length of 10 guanine residues was chosen because phase variable genes in *N. gonorrhoeae* contain as few as 10 guanine residues.

Although the pK18 portion of pK18-*nfsB*-polyG10 does contain a kanamycin resistance marker, this portion of the construct does not get incorporated into the gonococcal genome during transformation; pK18 does not contain an origin of replication that will allow it to replicate in a gonococcal background. For expression of any gene contained on pK18 to occur in the gonococcus, a double crossover event must transpire and the gene to be expressed must be transferred into the gonococcal chromosome. To provide for a way of selecting for the desired transformation event, the Ω fragment from pHP45Ω (Prentki and Krisch 1984) was amplified using the primer OmegaABC. The Ω fragment confers spectinomycin resistance. Primers

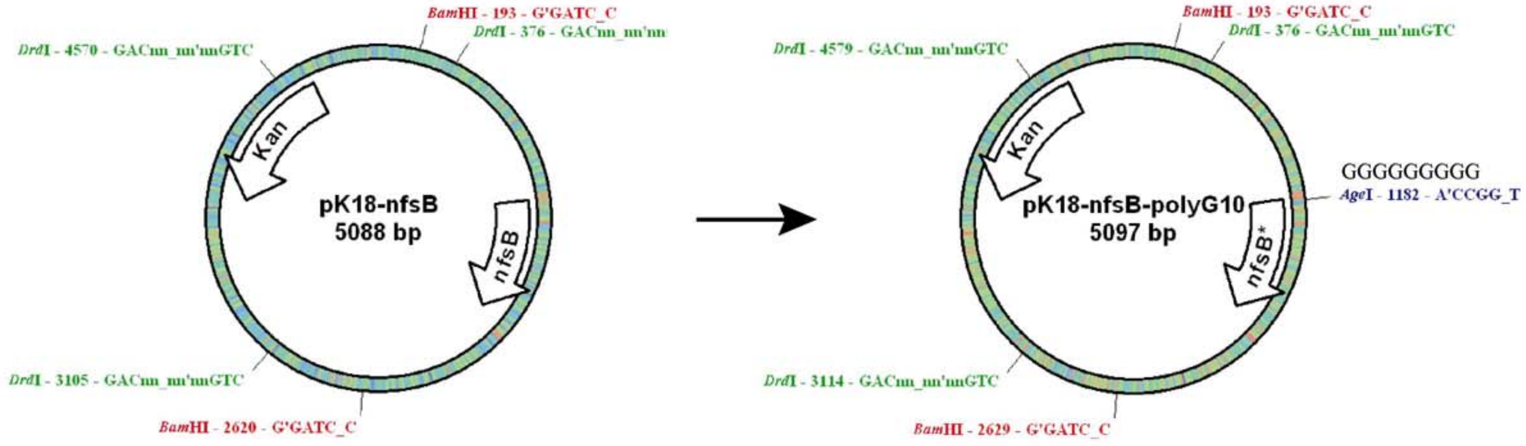

Figure 6 - Construction of pK18-*nfsB*-polyG10.

TGCACGATGACAGTATTAAGCAAAGAGCAG

M T V L S K E Q

TGCACGATGGGGGGGGGG*ACCGGT*TTAAGCAAAGAGCAG

M G G G T G L S K E Q

Figure 7 – *nfsB* sequence modification. Inserted guanine residues are underlined. The novel AgeI site introduced is in italics.

dwnstrmF and dwnstrmR were used to amplify the entire sequence of pK18-*nfsB*-polyG10, introducing a unique BsrGI site 86bp downstream from the stop codon of the *nfsB* sequence. The amplified Ω fragment was cloned into this new BsrGI site, resulting in pNFSB-polyG10. This construct was used to transform *E. coli* DH5αMCR, and plasmids were extracted from spectinomycin resistant transformants. Figure 8 is a schematic showing how this plasmid was constructed. Figure 9 is a gel image verifying that the Omega fragment had been inserted into this plasmid.

The modified *nfsB* coding sequence had to be inserted into a gonococcal background. To attain this, a gonococcal tube transformation was done. Piliated FA1090 cells were suspended into 1 ml of GCP broth supplemented with Kellogg's growth supplement, $MgCl_2$, and sodium bicarbonate until the tube became slightly turbid. 2-3µL of pNFSB-polyG10 were added to the tube, and the tube was incubated in a rolling bin at 37°C for 4 hours. After this incubation, 100µL and 250µL aliquots were plated onto GCK agar containing 50µg/ml spectinomycin. Transformants were detected after 36-48 hours of incubation at 37°C, 6% CO_2. Figure 10 is a schematic that indicates how the transformation occurs.

Verification of Polyguanine Tract Insertion

The success of the gonococcal transformation relied on the occurrence of a double recombination event between the FA1090 chromosome and the pNFSB-polyG10 transforming DNA. Because the recombination event could occur in many places on the FA1090 chromosome, the presence of the desired polyguanine addition

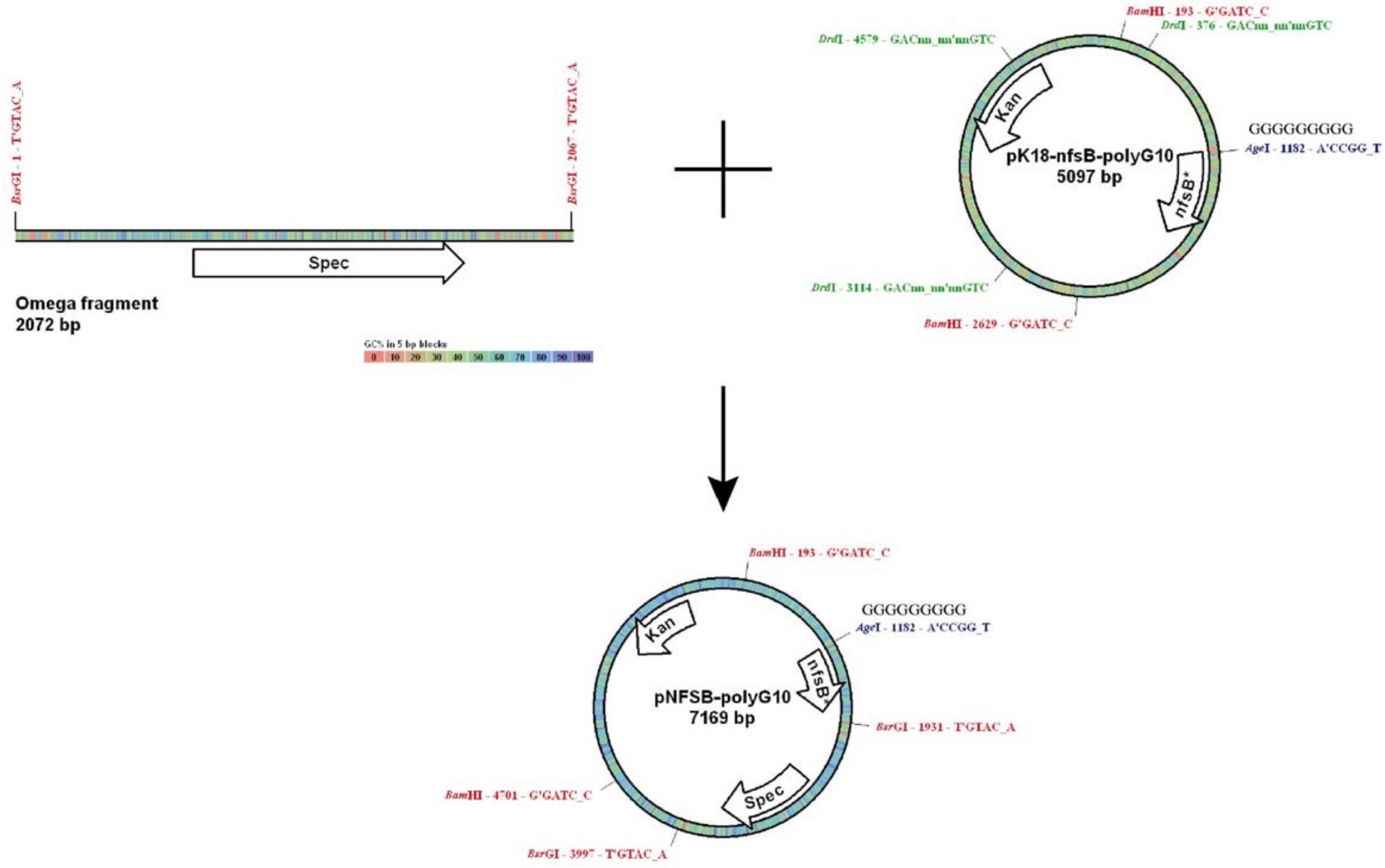

Figure 8 - Construction of pNFSB-polyG10

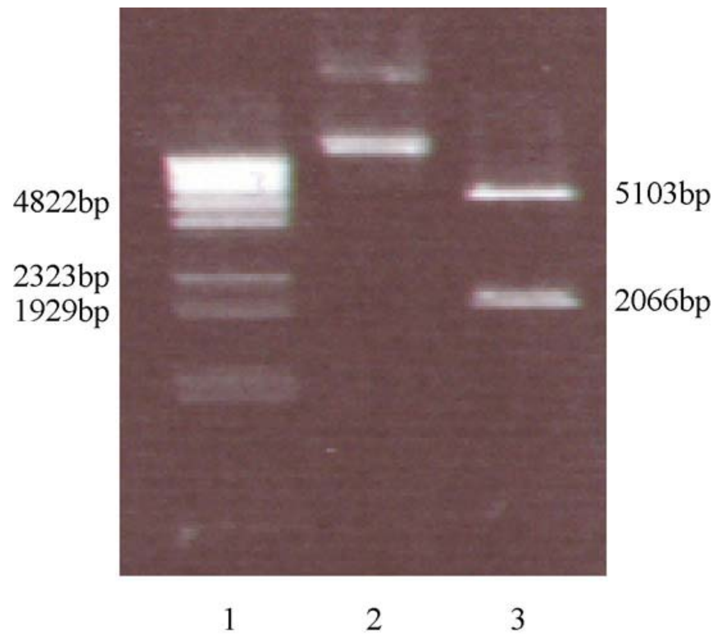

Figure 9 – Digestion verification of pNFSB-polyG10. Plasmids isolated from spectinomycin resistant transformants were digested with BsrGI to verify the presence of the Omega fragment. The expected digested fragment sizes are 5103bp and 2066bp. Lane 1, Lambda DNA-BstEII digest ladder; Lane 2, pNFSB-polyG10 undigested; Lane 3, pNFSB-polyG10 digested with BsrGI.

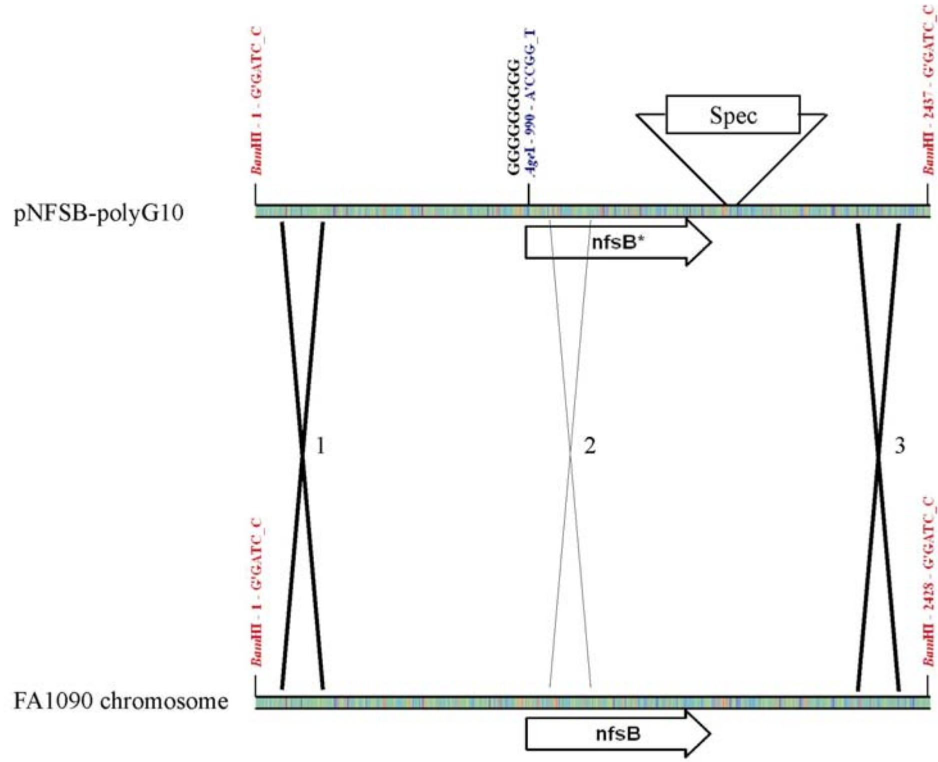

Figure 10 - Possible transformation recombination outcomes. Large X's represent possible sites of homologous recombination. A double recombination event at sites 1 and 3 would result in the modified *nfsB* gene and the spectinomycin resistance cassette being inserted into the FA1090 chromosome; this is the desired result. A double recombination event at sites 2 and 3 would result in an FA1090 chromosome that contained the wildtype *nfsB* sequence and the spectinomycin resistance cassette. A double recombination event at sites 1 and 2 would result in a spectinomycin sensitive FA1090 chromosome containing the modified *nfsB* sequence.

had to be verified in all transformants. Primers NP1 and NP2 were used to amplify the *nfsB* gene from several spectinomycin resistant gonococcal transformants, and these PCR products were digested with AgeI. An AgeI restriction site should only be present if the polyguanine tract had been successfully inserted. Figure 11 shows an AgeI digestion of these *nfsB* fragments. The data show that the *nfsB* PCR fragments obtained from our transformants were digested with AgeI, indicating the presence of the desired polyguanine addition.

Assessing the Frameshifting Frequency Due to a 10-residue Guanine Tract

A small number of spectinomycin resistant transformants were streaked for confluency onto GCK agar plates. After streaking, GCK plates were incubated less than 24 hours at 37°C, 6% CO_2. The incubation period was held to under 24 hours to ensure that the majority of gonococcal cells on these plates were viable at the time of their use. Each transformant was suspended into 3-4 ml of GCP broth and optical densities of each culture were determined using a Klett-Summerson colorimeter. Cells were suspended in GCP until a Klett reading of 100 was achieved. This value was equated previously with ~$5x10^8$ CFU/mL.

After acquiring the desired starting cell concentration, aliquots were plated onto GCK agar and GCK agar containing 1, 2, and 4 μg/mL nitrofurantoin. Plates were allowed to dry and were then incubated for 48 hours at 37°C, 6% CO_2. Wildtype FA1090 was also plated as a control using this same procedure. After the incubation period, colony counts were made and CFU/mL values for each sample determined.

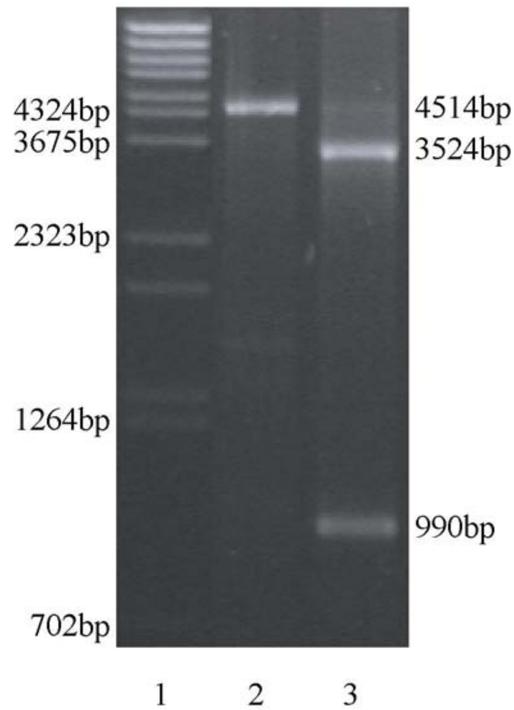

Figure 11 – Digestion verification of gonococcal spectinomycin resistant transformant *nfsB* fragments. The *nfsB* gene was amplified from gonococcal spectinomycin resistant transformants and digested with AgeI. The expected undigested PCR fragment size is 4514bp. The expected digested fragment sizes are 3524bp and 990bp. Lane 1, Lambda DNA-BstEII digest ladder; Lane 2, mutant *nfsB* undigested; Lane 3, mutant *nfsB* digested with AgeI.

By comparing the number of colonies obtained after growth on GCK agar plates with the number of colonies obtained after growth on GCK/nitrofurantoin plates, it was possible to determine a mutation frequency associated with a polyG tract of 10 residues. Using this plating procedure, a mutation frequency of 9.2×10^{-4} was observed.

Construction of *N. gonorrhoeae* FA1090 *nfsB*(mod) polyG-5...12

After demonstrating that a nitrofurantoin plating procedure could be used successfully to determine mutation frequencies, various parameters associated with frameshifting were investigated. The FA1090 *nfsB* gene was modified to contain polyG tracts of 5-12 residues, in order to measure the effect of tract length on mutation frequency. Since we had preliminary data that indicated that a 5-residue polyA tract within *N. gonorrhoeae nfsB* has a slightly elevated mutation frequency (Carrizosa, unpublished data), a tract length of 5 residues was chosen as the shortest polyG tract to be investigated. To determine if polyG tract lengths longer than 10 residues contribute to higher frameshifting frequencies, constructs containing 11 and 12-residue polyG tracts were also constructed and tested.

pNFSB-polyG10 was used as a template. Primers polyG-5, polyG-6, etc., were used in conjunction with the primer Gstring 1 to amplify the entirety of the pNFSB-polyG10 sequence. However, the polyG primers were designed so that the 10-residue polyG template sequence would be replaced with a polyG tract of the desired length. The same PCR/transformation procedure was employed for the

construction of each construct, as described above in Figure 6. Table 7 is a summary of all of the plasmid constructs that were made and shows the *nfsB* sequence modifications. Primers were designed so that they maintained the intact reading frame of *nfsB* after modifications were in place. PCR amplicons were digested with AgeI and religated upon themselves to create intact plasmid DNAs. These ligation mixtures were used to transform *E. coli* DH5αMCR, and plasmids were extracted from spectinomycin resistant colonies using an alkaline lysis procedure (Birnboim and Doly 1979). The plasmids produced in this step are referred to as pNFSB-polyG5, pNFSB-polyG6, etc., in this work. DNA sequencing was performed on each construct to verify that the desired modification had been incorporated. These plasmids were transformed into *N. gonorrhoeae* FA1090 using the tube transformation procedure described previously, resulting in strains *N. gonorrhoeae* FA1090 *nfsB*(mod) polyG-5 through *N. gonorrhoeae* FA1090 *nfsB*(mod) polyG-12.

Assessing Frameshifting Frequencies of Remaining FA1090 *nfsB*(mod) Strains

Of the remaining FA1090 *nfsB*(mod) strains to be tested, FA1090 *nfsB*(mod) polyG-12 and FA1090 *nfsB*(mod) polyG-8 were randomly selected to be tested first. The mutation frequencies observed with these strains were $\sim 1.2 \times 10^{-3}$ and $\sim 6.5 \times 10^{-5}$, respectively. These values represent the average of ≥3 independent experiments plated in triplicate. The mutation frequencies observed with polyG tracts of 8, 10, and 12 residues suggested that there was a direct relationship between polyG tract length and mutation frequency. This information prompted the testing of FA1090 *nfsB*(mod) polyG-5, FA1090 *nfsB*(mod) polyG-6, and FA1090 *nfsB*(mod) polyG-7 in an attempt

Wildtype FA1090 (5' to 3')		# Bases
ATCAATTTGCAATCAAAGTAATAAAAGGATGCACGATG	ACAGTATTAAGCAAAGAGCAGGTTCTATCCGCA	71
FA1090 *nfsB*(mod) polyG-5		
ATCAATTTGCAATCAAAGTAATAAAAGGATGCACGATGGGTGGGGGT	ACCGGTTTAAGCAAAGAGCAGGTTCTATCCGCA	80
FA1090 *nfsB*(mod) polyG-6		
ATCAATTTGCAATCAAAGTAATAAAAGGATGCACGATG GGGGGT	ACCGGTTTAAGCAAAGAGCAGGTTCTATCCGCA	77
FA1090 *nfsB*(mod) polyG-7		
ATCAATTTGCAATCAAAGTAATAAAAGGATGCACGATG GGGGGG	ACCGGTTTAAGCAAAGAGCAGGTTCTATCCGCA	77
FA1090 *nfsB*(mod) polyG-8		
ATCAATTTGCAATCAAAGTAATAAAAGGATGCACGATGGGTGGGGGGGGT	ACCGGTTTAAGCAAAGAGCAGGTTCTATCCGCA	83
FA1090 *nfsB*(mod) polyG-9		
ATCAATTTGCAATCAAAGTAATAAAAGGATGCACGATG GGGGGGGGT	ACCGGTTTAAGCAAAGAGCAGGTTCTATCCGCA	80
FA1090 *nfsB*(mod) polyG-10		
ATCAATTTGCAATCAAAGTAATAAAAGGATGCACGATG GGGGGGGGG	ACCGGTTTAAGCAAAGAGCAGGTTCTATCCGCA	80
FA1090 *nfsB*(mod) polyG-11		
ATCAATTTGCAATCAAAGTAATAAAAGGATGCACGATGGGTGGGGGGGGGGGTACCGGTTTAAGCAAAGAGCAGGTTCTATCCGCA		86
FA1090 *nfsB*(mod) polyG-12		
ATCAATTTGCAATCAAAGTAATAAAAGGATGCACGATG GGGGGGGGGGGTACCGGTTTAAGCAAAGAGCAGGTTCTATCCGCA		83

Table 7 - Modifications to the *nfsB* coding sequence. The start codon of the *nfsB* coding sequence has been underlined. The polyG tract has been shaded. Expected PCR fragment sizes using primers GampF and GampR are depicted in the "# Bases" column.

to determine the minimum polyG tract length required for frameshifting. Using the modified nitrofurantoin plating procedure described above, it was observed that polyG tracts of 5 residues and 6 residues don't appear to give significantly elevated frameshifting frequencies. Four independent spectinomycin resistant isolated transformants were used for each of these experiments, with no nitrofurantoin resistant colonies arising from either of these samples. The observed mutation frequency of a 7-residue polyG tract was calculated to be ~$6.3x10^{-3}$.

Identification of the Genetic Basis of Generated Nitrofurantoin Resistant Mutants

Because the minimum polyG tract length required for frameshifting appeared to be 7 residues, the remainder of the project was focused on FA1090 *nfsB*(mod) polyG-6 and FA1090 *nfsB*(mod) polyG-7. The general approach for this portion of the project was to amplify the *nfsB* region from the nitrofurantoin resistant mutants as well as their parent cells and compare amplicon fragment sizes on a polyacrylamide gel. The expectation was that fragments obtained from nitrofurantoin resistant mutants would appear 1 or 2 base pairs larger than fragments obtained from their parent cells after electrophoresis due to the insertion of extra guanine residues during cell replication.

Size markers were produced so comparisons could be made between parent and mutant PCR fragments. To achieve this, using pNFSB-polyG7 as a template, primers GampR-1 and GampR+1 were both used in conjunction with the primer

GampF to amplify fragment sizes that were 1bp smaller (GampR-1, resulted in 76bp fragment) or 1bp larger (GampR+1, resulted in 78bp fragment) than the expected fragment size obtained from parent cells. This design was used in hopes that fragments obtained from parent cells, when viewed on a polyacrylamide gel after electrophoresis, would show mobility in between these two markers. Fragments from mutant cells were expected to show mobility equal to one of these two markers, depending on if the outcome of the frameshift was insertion or deletion of a base. It should be noted that primers GampF and GampR were used to amplify a 77bp marker as well. Figure 12 is a diagram of a mock gel image that shows the expected fragment mobilities.

Of the FA1090 *nfsB*(mod) polyG-7 parent cell PCR fragments tested, all showed mobility corresponding to PCR fragments obtained from wildtype unmodified *nfsB* genes, meaning that they ran lower on the polyacrylamide gel than expected. Because wildtype *nfsB* genes lack the polyG tract, PCR fragments from wildtype cells will be smaller and run faster during electrophoresis. This phenomenon was also observed with the FA1090 *nfsB*(mod) polyG-6 parent cells that were tested. As for the FA1090 *nfsB*(mod) polyG-7 mutant cells tested, fragments appeared to be a mixture of 77bp and 78bp fragment sizes, corresponding to the expected parent fragment size as well as the expected +1 frameshift fragment size. Figures 13 and 14 are gel images of these tested fragments.

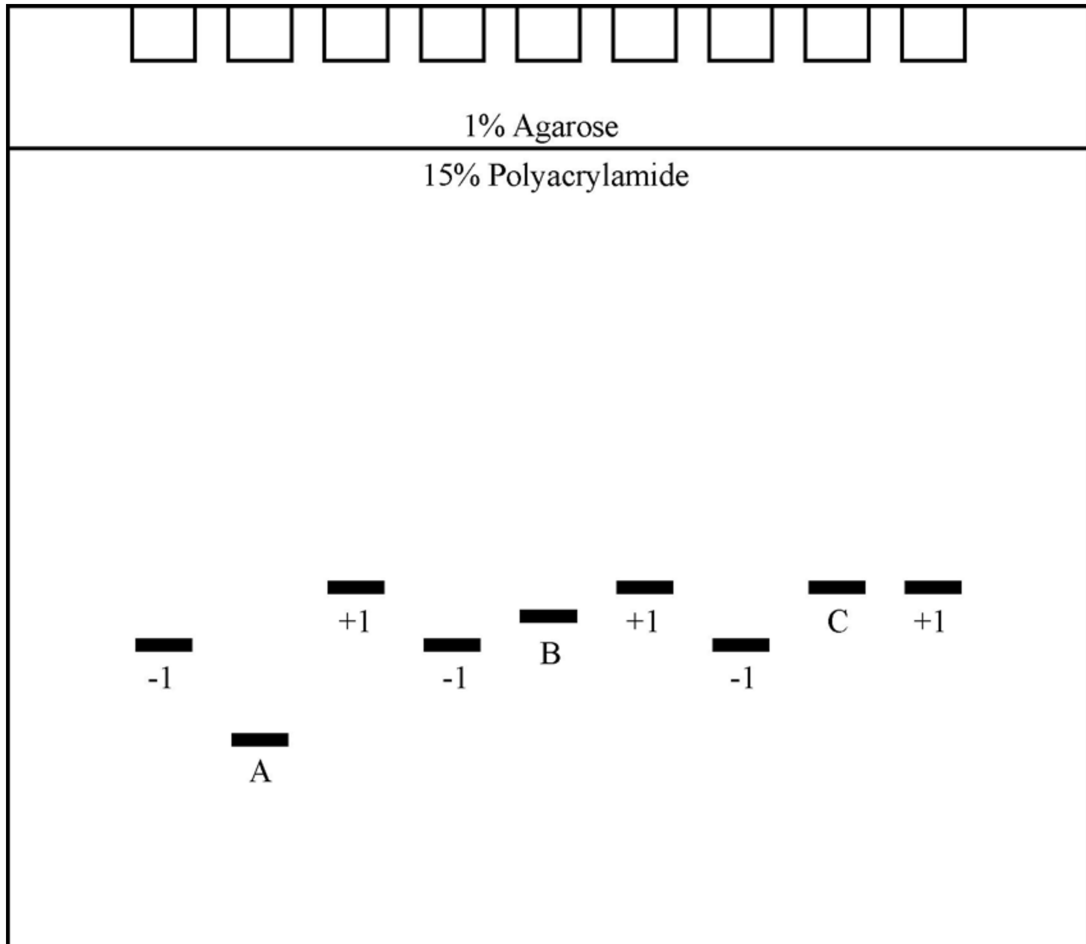

Figure 12 – Mock gel of expected mutant *nfsB* fragment mobilities. The diagram depicts the expected results of wildtype and mutant 5' *nfsB* PCR analysis. -1, size marker representing 76bp; +1, size marker representing 78bp; A, theoretical 5' wildtype FA1090 *nfsB* PCR fragment (lacking polyguanine addition; expected size of 71bp); B, theoretical 5' FA1090 *nfsB*(mod) polyG-7 parent *nfsB* PCR fragment (expected size of 77bp); C, theoretical 5' FA1090 *nfsB*(mod) polyG-7 mutant *nfsB* PCR fragment (containing +1 frameshift mutation; expected size of 78bp).

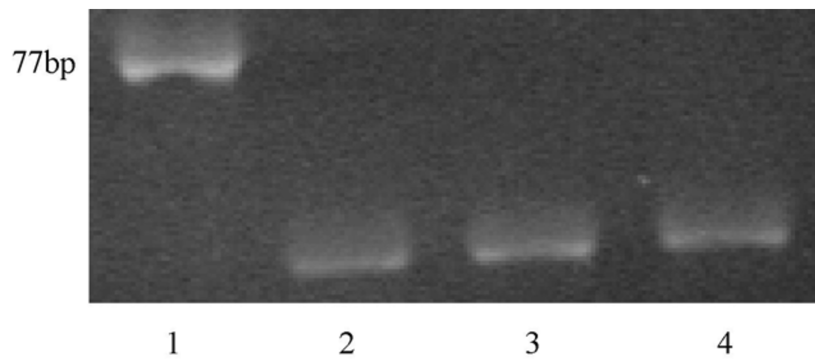

Figure 13 – FA1090 *nfsB*(mod) polyG-6 5' *nfsB* fragment analysis. Primers GampF and GampR were used to amplify the 5' portion of *nfsB* from FA1090 *nfsB*(mod) polyG-6 parent cells. Lane 1, size marker representing 77bp; Lanes 2, 3, and 4, FA1090 *nfsB*(mod) polyG-6 parent *nfsB* fragments. The expected PCR fragment size is 77bp.

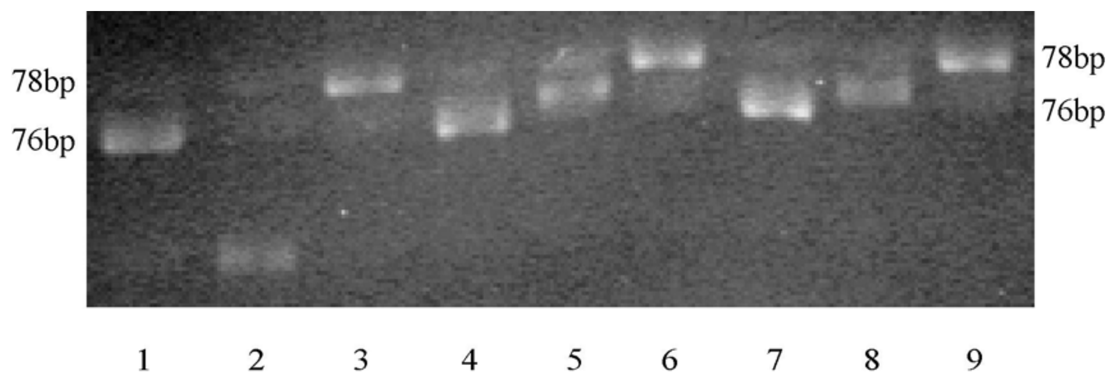

Figure 14 – FA1090 *nfsB*(mod) polyG-7 5' *nfsB* fragment analysis. Primers GampF and GampR were used to amplify the 5' portion of *nfsB* from FA1090 *nfsB*(mod) polyG-7 parent and mutation (nitrofurantoin resistant) cells. Lanes 1, 4, and 7, size marker representing 76bp; Lanes 3, 6, and 9, size marker representing 78bp; Lane 2, FA1090 *nfsB*(mod) polyG-7 parent *nfsB* fragment; Lanes 5 and 8, FA1090 *nfsB*(mod) polyG-7 mutant *nfsB* fragments.

Analysis of FA1090 *nfsB*(mod) polyG Parent and Mutant Cells

Because the parent cells didn't appear to be correct genotypically, it was necessary to investigate the reasons behind this. Using apparent wildtype cells for the nitrofurantoin plating procedure should not have yielded mutation frequencies similar to those observed with phase variation. The nitrofurantoin plating procedure was modified so that parent cells used had been verified as correct through digestion procedures. Gonococcal tube transformations were done using pNFSB-polyG6 and pNFSB-polyG7. 20 isolated spectinomycin resistant colonies from each transformation (total of 40) were picked and restreaked for isolation onto GCK agar containing 50µg/mL spectinomycin and incubated for 24 hours at 37°C, 6% CO_2. After incubation, 10 isolated colonies from each of the 40 isolation streaks (total of 400 colonies) were picked and patch plated onto GCK agar containing 50µg/mL spectinomycin and again incubated overnight.

Patched cells were placed in groups of 10, and crude chromosomal DNA extractions were done with each group. To achieve this, a toothpick was used to pick up cells from the desired patch position and place them onto a glass slide. After all ten patches had been placed onto the same slide, the cellular material on the slide was mixed together. Another toothpick was used to pick up a small portion of this mixed cellular material and suspend it into 5µL of 0.5M NaOH. 5µL of 1M Tris-HCl pH 7.5 were then added to neutralize the NaOH. Finally, 90µL of water were added to bring the final volume to 100µL. This process was repeated until 40 "pooled" DNA extractions had been completed.

Using these pools as templates, primers NP1 and NP2 were used to amplify the *nfsB* region from these cell mixtures. The resulting amplicons were digested with AgeI to verify the presence of the modified *nfsB* sequence, and digestion products were run on a 1% agarose gel. Just fewer than 50% of the pools tested contained the desired modification, evident by the digestion of the PCR fragment with AgeI. 3 of the correct pools for FA1090 *nfsB*(mod) polyG-6 and 3 of the correct pools for FA1090 *nfsB*(mod) polyG-7 were used for further study. 5 individual patches from within each of the correct pools were chosen, and crude chromosomal DNA extractions were done with these samples. Again, using these DNA extractions as template, primers NP1 and NP2 were used to amplify the *nfsB* region from these now individual cells. These amplicons were digested with AgeI, and digestion products were run on a 1% agarose gel. All individual samples tested digested with AgeI, suggesting that they all contained the desired modified *nfsB* sequence. Figure 15 is a gel image of the individual AgeI digestions.

The previously described steps ensured that samples being tested using the nitrofurantoin plating procedure contained the desired mutation to be tested. 3 individual samples containing a 6-residue polyG tract and 3 individual samples containing a 7-residue polyG tract were chosen for nitrofurantoin plating. For this procedure, aliquots from the 10^{-1}, 10^{-2}, and 10^{-3} dilution tubes were plated onto GCK agar containing 3μg/mL nitrofurantoin. The amount of nitrofurantoin was increased from 2μg/mL because the higher concentration of nitrofurantoin yields a higher percentage of truly nitrofurantoin resistant colonies (Carrizosa, personal

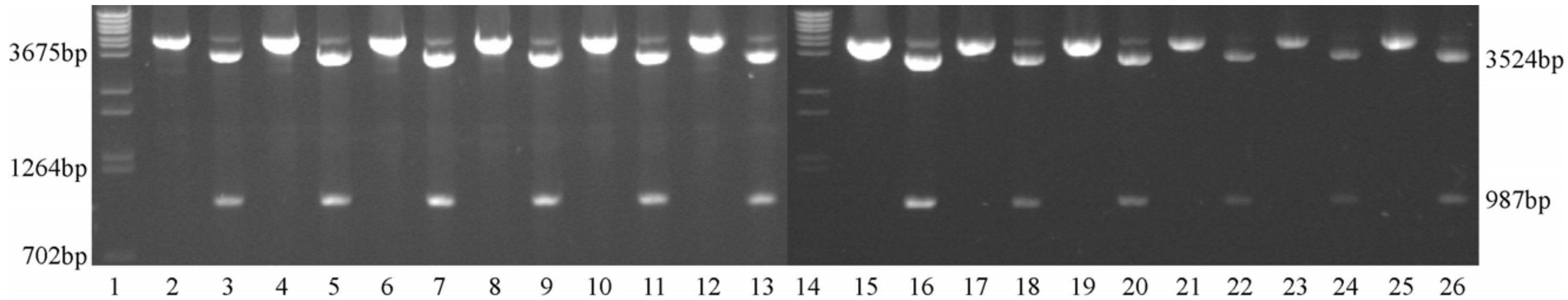

Figure 15 - Digestion verification of "individual" FA1090 *nfsB*(mod) polyG-6 and polyG-7 *nfsB* PCR fragments. Primers NP1 and NP2 were used to amplify the *nfsB* gene of individual FA1090 *nfsB*(mod) polyG-6 and polyG-7 colonies. These individual colonies were selected from colony groups that had been shown to contain a polyguanine tract insertion. Lanes 1 and 14, Lambda DNA-BstEII digest ladder; Lanes 2, 4, 6, 8, 10, and 12, FA1090 *nfsB*(mod) polyG-6 parent *nfsB* PCR fragments undigested; Lanes 3, 5, 7, 9, 11, and 13, FA1090 *nfsB*(mod) polyG-6 parent *nfsB* PCR fragments digested with AgeI; Lanes 15, 17, 19, 21, 23, and 25, FA1090 *nfsB*(mod) polyG-7 parent *nfsB* PCR fragments undigested; Lanes 16, 18, 20, 22, 24, and 26, FA1090 *nfsB*(mod) polyG-7 parent *nfsB* PCR fragments digested with AgeI.

communication). Aliquots from the 10^{-5} and 10^{-6} dilution tubes were plated onto GCK agar alone. The mutation frequencies observed with FA1090 *nfsB*(mod) polyG-6 and FA1090 *nfsB*(mod) polyG-7 were ~$9.09x10^{-7}$ and ~$2.65x10^{-3}$, respectively.

After calculating mutation frequencies, it was necessary to analyze the cause for these mutations. 6 nitrofurantoin resistant colonies from the FA1090 *nfsB*(mod) polyG-6 plating experiment and 18 nitrofurantoin resistant colonies from the FA1090 *nfsB*(mod) polyG-7 plating experiment were chosen for further study. Primers GampF and GampR were used to amplify the *nfsB* region from these nitrofurantoin resistant cells as well as their parent cells. These amplicons were analyzed on a 1% agarose/15% polyacrylamide combination gel. Figure 16 is a gel image of some of these samples. Fragments from parental cells show the same mobility as a 77bp fragment, the expected parental cell fragment size. The majority of fragments obtained from nitrofurantoin mutants also appear to be 77bp in length. One fragment, obtained from an FA1090 *nfsB*(mod) polyG-6 nitrofurantoin mutant, appears to be 78bp in length.

To better interpret the outcomes of the mutation events that occurred, the *nfsB* regions from each of the samples tested were sequenced. Using crude chromosomal DNA extractions, primers NP1 and NP2 were used to amplify the *nfsB* regions from the desired samples. PCR products were purified using a Qiaquick PCR Purification kit (Qiagen, Valencia, CA), and purified samples were sent for sequencing (Macrogen).

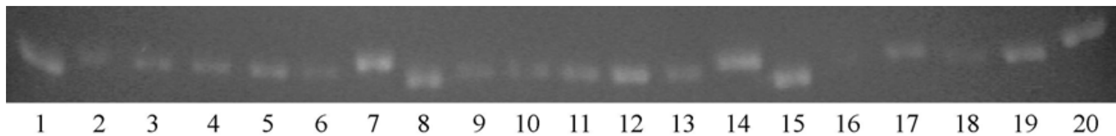

Figure 16 – FA1090 *nfsB*(mod) polyG-6 and polyG-7 5' *nfsB* fragment analysis. Primers GampF and GampR were used to amplify the 5' portion of *nfsB* from FA1090 *nfsB*(mod) polyG-6 and polyG-7 parent and mutant cells. Lanes 1, 8, and 15, size marker representing 76bp; Lanes 7, 14, and 20, size marker representing 78bp; Lane 2, FA1090 *nfsB*(mod) polyG-7 parent *nfsB* fragment; Lanes 3, 4, 5, 6, 9, 10, 11, 12, and 13, FA1090 *nfsB*(mod) polyG-7 mutant *nfsB* fragments; Lane 16, FA1090 *nfsB*(mod) polyG-6 parent *nfsB* fragment; Lanes 17, 18, and 19, FA1090 *nfsB*(mod) polyG-6 mutant *nfsB* fragments.

Several of the samples sequenced contained mutations in the coding sequence of *nfsB* that could lead to nitrofurantoin resistance. Four FA1090 *nfsB*(mod) polyG-6 mutants contained single transition mutations: three mutants contained a C→T transition at nucleotide position 59, resulting in a serine→leucine amino acid substitution, and one mutant contained a G→A transition at nucleotide position 154, resulting in a glutamic acid→lysine amino acid substitution. The serine residue affected is conserved in *E. coli* K12 *nfsB*, and the glutamic acid residue affected aligns with a conserved putative FMN binding region of *nfsB* (Whiteway, Koziarz et al. 1998). One FA1090 *nfsB*(mod) polyG-6 mutant (Figure 16, lane 17) contained a +1 frameshift mutation in the inserted 6-residue polyguanine tract. This frameshift results in a premature STOP codon at amino acid position 16. All the FA1090 *nfsB*(mod) polyG-7 mutants sequenced contained an intact, in frame *nfsB* coding sequence.

Discussion

Neisseria gonorrhoeae has the ability to colonize many distinct anatomical regions, including the urogenital tract, the oropharynx, and the conjunctiva. The organism relies on the phase variation of certain genes to allow for the expression of appropriate surface components in a given physiological niche. Phase variation can also enhance gonococcal survivability, facilitating the processes of immune evasion through molecular mimicry and disease dissemination. However, the study of phase variation in *N. gonorrhoeae* has been impeded by the lack of a positive selection system for measuring mutation frequencies. The goal of this research was to design a positive selection based reporter system to study and assess mutation frequencies in *N. gonorrhoeae*. The analysis of genes whose loss of function would provide for positive selection would allow for an unbiased comparative analysis of spontaneous mutations. The study of spontaneous mutation in these genes would yield information for future studies on factors that might effect antigenic variation. Furthermore, the system provides a model for which questions concerning changes in gene expression as a result of frameshifting or other replicational errors can be studied.

A single nitroreductase, *nfsB*, was identified in *N. gonorrhoeae*. The protein product of this gene shows 26% amino acid identity and 46% amino acid similarity to the *E. coli* NfsB protein. Deletion of this coding sequence and biochemical analysis of spontaneous nitrofurantoin resistant mutants confirmed that this gene encoded a nitroreductase. Amino acid sequence alignment analysis of various bacterial nitroreductases indicated that all of the domains that have been predicted to be

important for enzymatic activity are present in gonococcal NfsB. The nature of spontaneous mutation to nitrofurantoin resistance was determined by analyzing where mutations occurred in *nfsB*. The results of the spontaneous mutation frequency plating experiments and the subsequent genetic analysis showed that nitrofurantoin resistance could be a target for analyzing gonococcal mutation because the gene exhibited a low inherent spontaneous mutation frequency. This system was modified to measure the effect of the length of polynucleotide runs on phase variation by inserting polyguanine tracts within the gonococcal *nfsB* coding sequence.

The data indicate that mutations yielding nitrofurantoin resistance occurred at a higher rate in strains containing modified *nfsB* genes. The frequency of mutation also increased as the length of the polyguanine tract increased. A homopolymeric run of 5 guanines did not produce elevated mutation frequencies, while a tract length of 6 guanines resulted in higher mutation frequencies and frameshifting mutations. The measured mutation frequency of a 12-residue guanine tract was very similar to the mutation frequencies observed by Schneider et al in LOS biosynthetic genes containing 11-17 guanine residues (Schneider, Hammack et al. 1988). This similarity suggests that the maximal mutation frequency associated with homopolymeric runs is about 10^{-3}. Given that polyguanine tracts as short as 6 nucleotides have elevated mutation frequencies, many gonococcal genes may be subject to phase variation due to frameshifting.

The use of PCR and polyacrylamide gel electrophoresis proved to be a reliable method for quickly identifying nitrofurantoin resistant mutants that contained *nfsB* frameshift mutations. When *nfsB* fragments amplified from nitrofurantoin resistant mutants were electrophoresed, the presence of insertions or deletions in the *nfsB* coding sequence was easily detected.

During the analysis of nitroreductase mutation frequencies, it was observed that strains containing a 7-residue guanine tract within *nfsB* exhibited a mutation frequency similar to that observed with a 12-residue guanine tract. This frequency failed to follow the observed trend that mutation frequency increased as polyguanine tract length increased. Also, sequence analysis of nitrofurantoin resistant 7-residue guanine mutants yielded no mutations within the coding sequence of *nfsB*. This result suggests that second site mutations are responsible for the nitrofurantoin resistant phenotype. This reasoning is supported by the fact that mutations outside of the *nfsB* coding sequence were identified during spontaneous mutation frequency analysis of *N. gonorrhoeae* FA1090 (Table 5). It can be hypothesized that the proposed second site mutations occur at a higher frequency than frameshift mutations within the 7-residue guanine tract. The presence of a second site mutation that results in nitrofurantoin resistance would negate the necessity for the *nfsB* coding sequence to be mutated to yield a resistant phenotype.

Frameshifting did not account for all of the mutations identified during analysis of mutants containing 6-residue guanine tracts within *nfsB*. Similar to the 7-

residue guanine *nfsB* example, some of the 6-residue guanine mutants contained intact *nfsB* coding sequences, suggesting that mutation occurred at a secondary site. Furthermore, two unique transition mutations were identified, both of which resulted in amino acid changes in NfsB, and a nonsense frameshift mutation was identified.

The identification of a nitrofurantoin resistant mutant containing a frameshift mutation validates the use of the described nitrofurantoin reporter system as a method for studying frameshifting mutations in *N. gonorrhoeae*. The sample containing the frameshift mutation exhibited a more slowly migrating band upon polyacrylamide electrophoresis when compared to parent samples, and this banding pattern was supported by sequencing data obtained for this sample. Also, the samples containing point or second site mutations yielded banding patterns that were supported by sequencing data. Importantly, whether a frameshifting mutation was present or not, polyacrylamide electrophoresis and sequencing gave equivalent results, indicating the accuracy attainable with this novel nitrofurantoin reporter system.

This system can be adapted to study mutation frequencies associated with a variety of systems generating genetic variation. While this work focused on mutation frequencies associated with polyguanine tracts, simple modifications to the reporter system could allow for the study of the effects of polyadenine, polycytosine, and polythymine tracts on mutation frequencies. Also, this novel system can be used to study antigenic variation not governed by homopolymeric runs. For example,

insertions of the pentameric repeat sequence CTCTT could facilitate the investigation of mutation frequencies associated with Opa protein variation.

More experimentation needs to be done to optimize the conditions for use with this reporter system. By analyzing a larger number of 6-residue and 7-residue polyguanine tract nitrofurantoin mutants, a more accurate assessment of mutation frequencies could be achieved. Also, sequence analysis could be expanded to include the *nfsB* promoter region in these mutants, which could give insight into the nature of some of the second site mutations that have yet to be identified in the current study. The importance of further experimentation with this process has already been recognized. In work done since the completion of this project, it has been determined that a 5-residue homopolymeric tract results in an elevated mutation frequency to nitrofurantoin resistance. Also, the system has been modified to include the use of denaturing gradient gel electrophoresis (DGGE) as a better method of quickly identifying frameshift mutations.

In summary, the use of *nfsB* as a novel reporter system has proven successful in measuring gonococcal mutation frequencies. Elevated mutation frequencies have been observed in *nfsB* mutants containing a homopolymeric run as short as 5 nucleotides, suggesting that any gene containing a short polynucleotide run has the potential to phase vary. The nature of gonococcal mutations identified with this reporter system can be effectively studied with polyacrylamide electrophoresis and sequencing analysis.

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