ABSTRACT

Title of Thesis: A DNA REPAIR/PHASE VARIATION REPORTER SYSTEM USING A POLY-GUANINE TRACT IN A NEISSERIA GONORRHOEAE NITROREDUCTASE GENE

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Neisseria gonorrhoeae undergoes phase variation to adapt to new environments, increase pathogenesis, and evade the host immune system. This may be due to defects in DNA repair. A reporter system was created to detect phase variation by phenotypic switching from a nitrofuran-sensitive phenotype to a nitrofuran-resistant (Nit^R) phenotype. Strains were created with poly-guanine tracts from 5 to 12 guanines in the coding region of a nitroreductase gene (*nfsB*) that would be susceptible to frame-shifting mutations during DNA replication. The minimum number of consecutive guanines needed to observe increased mutation was 5. A strain expressing 7 guanines *nfsB* possessed nitroreductase activity similar to wild-type and a spontaneous mutation frequency that was increased ~10^4 fold relative to wild-type. Frame-shifting mutations of strain expressing 8 guanines in *nfsB* were observed using denaturing gradient gel electrophoresis (DGGE). Future work with the reporter system could lead to new understanding of phase variation and DNA repair.

A DNA REPAIR/PHASE VARIATION REPORTER SYSTEM USING A POLY-GUANINE TRACT IN A NEISSERIA GONORRHOEAE NITROREDUCTASE GENE

By

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LIST OF ABREVIATIONS

~ = approximately
α = alpha
β = beta
bp = base pairs
C = Celsius
CFU = colony forming unit
^{0} = degrees
Δ = deletion
dH_2O = distilled water
DGGE = denaturing gradient gel electrophoresis
DGI = disseminated gonococcal infection
DNA = deoxyribo nucleic acid
EDTA = Ethylenediaminetetraacetic acid
HIV = human immunodeficiency virus
kB = kilo base
λ = lambda
LOS = lipo-oligosaccharide
MIC = minimum inhibitory concentration
mg = milligram
μg = microgram
μl = microliter
mL = milliliter
MMR = DNA mismatch repair
NAA = nitroreductase activity assay
Nit^S = nitrofuran agent sensitive
Nit^R = nitrofuran agent resistant
Opa = colony associated opacity total
ORF = open reading frame
Ω = Omega Cassette
PBS = phosphate buffered saline
% = percent
PID = pelvic inflammatory disease
PCR = polymerase chain reaction
SMA = spontaneous mutation frequency assay
$Spec^r$ = spectinomycin resistant
TBE = Tris Borate EDTA
Tn = Transposon
V = volts
wt = wild-type
Xgal = 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Introduction

Epidemiology of *N. gonorrhoeae*

Neisseria gonorrhoeae is an obligate-human pathogen responsible for one of the most common sexually transmitted diseases, gonorrhea. This organism is an aerobic, Gram negative cocci-shaped bacterium that is generally arranged in pairs (referred to as diplococci). *N. gonorrhoeae* is catalase positive, oxidase positive, non-motile, and utilizes carbohydrates oxidatively. The bacterium usually colonizes anogenital mucosal membranes, predominately the urethra and the cervix, however, other species of the *Neisseria* genus are found in the mucosal surfaces of the oropharynx and nasopharynx. Members of the *Neisseria* species have potential to colonize or infect most mucosal surfaces throughout the human body. Ocular infections of *N. gonorrhoeae* can occur due to infant passage through an infected birth canal [**1**].

Despite effective antibiotic treatment, gonorrhea is still one of the most commonly reported sexually transmitted diseases (second only to Chlamydia infections). Despite a decrease in occurrence of disease since 1975, the numbers are still high, The Centers for Disease Control (CDC) reported 335,104 new cases of gonorrhea in 2003 in the United States alone [**2**] and an estimated 60 million new gonococcal infections were reported worldwide in 1999 [**3**]. The highest rate of gonococcal disease was reported in 15- to 19-year-old women and 20- to 24-year-old men. The National Institute of Allergy and Infectious Diseases (NIAID) at the National Institutes of Health (NIH) estimates that nearly 1 billion dollars a year is spent on treatment of gonococcal infections [**4**].

Clinical aspects of *N. gonorrhoeae*

Gonorrhea is primarily spread through sexual contact. The disease usually manifests itself in males as an acute anterior urethritis, accompanied by the onset of dysuria and discharge of a purulent exudate. The disease is the result of recruitment of a massive influx of neutrophils to the site of infection. Rarely, the primary infection can spread leading to conditions such as epididymitis, seminal vesiculitis, and prostatitis [**5, 6**].

In women, the disease usually results in increased vaginal discharge, increased urinary frequency, abdominal pain, and uterine bleeding. The symptoms usually pass quickly and are largely ignored; therefore ~15% of women infected have the primary infection lead to further complications, such as endometritis and pelvic inflammatory disease (PID). Inflammation arising from PID can lead to scarring of the fallopian tubes and infertility in as many as 10% of women and also cause ectopic pregnancies [**4, 6, 7**]. Furthermore, 0.1% to 3% of female cases can lead to disseminated gonococcal infection (DGI) [**8**]. Unlike men however, women are usually asymptomatic and can serve as a reservoir or unknowing carrier of the disease. Studies have estimated that anywhere from 25% to 80% of women infected with gonorrhea are asymptomatic carriers and this is one of the reasons for the persistence of *N. gonorrhoeae* in the general population [**9**].

It is also common for patients to be coinfected with *Chlamydia trachomitis*, the causative agent of Chlamydia. Current data have also suggested that gonococcal infection can lead to increased susceptibility to HIV infection, because as gonococcal infection increases so does the population of mononuclear cells within the urogenital tract which the HIV virus infects [**10**].

N. gonorrhoeae cannot resist drying outside of the human reservoir and dies rather quickly; therefore it requires direct host-to-host transmission. In males and females the primary site of infection (and most common) is a colonization of the urogenital epithelium [**38**].

Phase (Antigenic) Variation in *N. gonorrhoeae*

As a sexually transmitted disease, *N. gonorrhoeae* can infect a variety of anatomical locations each possessing unique physiological properties. In order for the bacterium to survive, it must adapt quickly to these new environments and conditions. Gonococci must be able to colonize both the male and female reproductive tract, which are drastically different environments, with the latter constantly changing. The bacteria also evade the immune system of the human host by varying the antigens present in the outer membrane. By developing genetic mechanisms that allow for antigenic and phase variation, *N. gonorrhoeae* is able to adapt quickly to various niches while evading the immune system through variation in the expression of surface components such as opacity proteins, LOS, type IV pili, and iron-utilization receptors.

Opacity proteins, more commonly referred to as "Opa," were named as such because the variation in expression of these proteins causes differing colors and opacities of the gonococcal colonies when grown on translucent medium [**11**]. Opa is important for adhesion to leukocytes and epithelial cells [**12**, **13**]. *N. gonorrhoeae* contains at least eleven of these genes that are constitutively transcribed. Each gene contains multiple copies of a pentameric pyrimidine DNA repeat (CTCTT) within the coding sequence at the 5' end. However, the number of repeats present in the gene is subject to frequent and precise changes during DNA replication. This results in reading frame changes that lead

to translational control of the expression of these proteins [**14, 15**]. These pentameric repeats result in a phase-variable expression of these genes leading to varying phenotypes of gonococcus depending on the Opa protein expressed on the surface.

N. gonorrhoeae express long finger-like structures which form type IV pili, and these are one of the major antigenic determinants on the cell's surface. The pili are composed primarily of identical subunits known as pilin [**16**]. Pili are essential for initial attachment of the gonococcus to human epithelial cells and other host cell surfaces; therefore they are required for infection to occur [**17**]. In addition, pili are also required for natural competence and auto-agglutination of gonococcal cells.

The gene *pilE* encodes pilin, the major subunit of pili. The gonococcal genome contains multiple expressed copies of the pilin gene within two loci, *pilE1 and pilE2*, and silent copies not expressed because of modifications or truncations of the gene, at the *pilS* loci [**18**]. Antigenic variation of pili can occur from intragenic homologous recombination between the *pilS* and *pilE* loci. This can result in a switch from a piliated to a non-piliated variant if the new pilin sequence does not generate a protein or can result in a pilin protein different from the old. The pilin protein contains an N-terminal conserved region, a hypervariable region at the C-terminus, and semi-variable regions in-between. However the pilin genes in the *pilS* loci do not posses the sequence encoding the N-terminal conserved region. This can lead to an antigenic variation mechanism of small cassettes of the variable regions being exchanged in recombination [**19**, **20**]. Pilin variation can lead to changes in immunoreactivity and adhesion functions of the pili, which can be observed occurring frequently throughout the infection process [**21**].

pilE is not the only pilin gene that is subject to phase variation. p*ilC* encodes a protein that is important for assembly and adherence of the type IV pili. The expression of *pilC* is governed by a homopolymeric tract of guanines. Frame shifting due to strand-slippage during DNA replication can occur and functional PilC protein will not be produced. Pilus fibers on the gonococcal surface that lack PilC do not support adherence to epithelial cells [**22**]. Another consequence of non-functional pili besides adherence is that *N. gonorrhoeae* will no longer be naturally competent. *pilC* and *pilE* genes must be expressed for the uptake of DNA into the gonococcus by transformation [**23**].

Related to pilin, the gonococcal gene, *pgtA,* contains a poly-guanine tract within its coding sequence. *pgtA* encodes a galactosyl transferase important for adding a galactose residue onto the O-linked N-acetylglucosamine (GlcNAc) of pilin glycan. Phase variation of this gene appears to play a role in increased pathogenesis which leads to increased virulence of the strain which can cause disseminated gonococcal infection [**24**].

LOS molecules are immunogenic glycolipids found on the outer-membrane surface of *Neisseria* species and are also subject to phase variation. *Neisseria* LOS contains three domains including an outer-membrane-imbedded lipid A molecule, a core polysaccharide molecule, and branched oligosaccharide side chains. This closely resembles LPS found in Gram-negative bacteria, such as enterics like *E. coli* and *Salmonella* species. However LOS is a truncated version that does not contain an O-antigen. LOS is a very important component for *Neisseria* pathogenesis because it is involved in immune evasion, attachment, and host tissue damage [**25, 26**]. Although the precise role of LOS in pathogenesis is still being studied, data have shown that it is

important for invasion of human epithelial cells [**27**]. Also, the different structures of LOS appear to be associated with varying levels of pathogenicity in *Neisseria*. Phase variation of LOS genes can increase or decrease the virulence of the gonococci [**28**-**30**].

LOS biosynthesis is complicated and many genes involved in this process have been cloned and characterized. LOS synthesis is initiated by *lgtF,* encoding a glycosyl transferase that adds glucose to the first heptose molecule in the beginning of the LOS chain. The *lgt* gene cluster, containing *lgtA, lgtB, lgtC, lgtD,* and *lgtE*, is responsible for the addition of various sugars to multiple locations on the growing LOS chain. LOS biosynthesis is also subject to phase variation. For example, in strain F62 lgtA*, lgtC,* and *lgtD* contain poly-guanine tracts of lengths of 17, 10, and 11 base pairs respectively, within their coding sequences. The number of guanine residues in each gene determines if a functional product will be encoded as frame shifting and strand-slippage will cause translation to occur in or out of frame. Since the sugars are added to the LOS chain sequentially, the loss of function of one of these proteins early in the process can lead to truncated versions of LOS [**31**, **32**]. The gene encoding a protein that initiates β-chain synthesis, *lgtG*, contains a poly-cytosine tract. If this gene is out of frame and LgtG is not produced, a beta chain will not be added onto the LOS, serving as yet another form of variation [**33**].

N. gonorrhoeae also contains genes that are involved in iron utilization that are subject to phase variation. FetA is a protein receptor that binds ferric enterobactin and allows for utilization of this compound as an iron source. FetA allows the bacterium to scavenge for iron in low iron concentration environments. FetA is an iron-repressible protein that is highly expressed in iron-limiting environments but expression is repressed

in higher iron environments. The receptor will also phase vary from a high level of expression to a low level of expression or from low to high at a frequency of ~6.9×10^{-3} regardless of environmental conditions. Experimental data show that this phenomenon is due to a poly-cytosine tract between that -10 and -35 promoter sequences. Strand-slippage in this area and addition or subtraction of cytosines in this region can alter the promoter's effectiveness [**34**].

Poly-guanine tracts play a major role in expression of two receptors that have been shown to be imperative for utilization of hemoglobin for a source of iron in *N. gonorrhoeae*. Most gonococcal isolates are unable to utilize human hemoglobin as an iron source and cannot grow if hemoglobin is the sole source of iron. However, a small population of isolates has been recognized that can utilize hemoglobin. The isolates express two outer membrane receptors HpuA and HpuB. The genes that code for these receptors, *hpuA* and *hpuB*, undergo limited phase variation on a translational level by frame-shift mutations in poly-guanine tracts. A one guanine difference in the coding region will either produce a gene that is in frame or out-of-frame, and this will determine if a functional receptor is expressed [**35**]. Phase variation of hemoglobin receptors for iron utilization has also been discovered in *Neisseria meningitidis* where poly-guanine tracts play an important role in the expression of receptors HpuAB and HmbR [**36, 37**]. Utilization of human hemoglobin as an iron source could become very beneficial for gonococcus adapting to survive in the human host.

N. gonorrhoeae can selectively attach to non-ciliated epithelial cells in the human body, cell types found in the urethra, cervix, pharynx, and conjunctiva. Infection initiates when pili produced by the bacteria interact with and attach to specific host cell surface

receptors. Many of the proteins expressed on the gonococcal outer membrane have variable expression levels. These proteins are expressed at differing levels due to phase variation which happens because of pentameric repeats and long homopolymeric nucleotide tracts. Pilin proteins and other proteins involved in attachment in *Neisseria* can be expressed or not expressed due to varied expression of these proteins. These proteins help the bacteria to attach to surfaces, and the variation on the surface helps the bacterium evade immune surveillance.

Opa is a protein expressed on the gonococcal surface that plays an important role in pathogenesis. Opa is important for adhesion to and invasion of epithelial cells. Opa can also bind specifically to receptors on $CD4^+$ T lymphocytes thereby preventing activation and proliferation of these immune cells. LOS molecules are immunogenic glycolipids expressed on the surface of the *Neisseria* species. LOS is an important outer membrane component for *Neisseria* pathogenesis. LOS is involved in immune evasion, attachment, and tissue damage [**25, 26**].

Phase variation has been well studied in pathogenic *Neisseria* and is also known to occur in other pathogenic species. In many cases, phase variation occurs because of slipped-strand mispairing, which occurs during DNA replication when the DNA polymerase ‘slips’ in a tract of repeated nucleotides on the template strand resulting in an addition or deletion of one repeat unit in the daughter [**39**]. Phase variation can occur by repeats located in the open-reading frame of the gene resulting in a frame-shift mutation or the repeats can be located in the promoter region and alter the effectiveness of that promoter when repeats are added or deleted.

DNA Repair Systems in *N. gonorrhoeae*

A majority of slip-stranded mispairing and phase variation result in DNA mismatches, so it was hypothesized that *Neisseria* does not possess a DNA mismatch repair (MMR) pathway. However, it appears that the bacterium does possess the major components of MMR that have been well characterized in *E. coli* (MutS, MutL, and MutH) which can repair most DNA mismatches (except cytosine-cytosine). There are three basic steps in MMR in *E. coli*: recognition of the DNA mismatch, excision of the misincorporated base and DNA surrounding the mismatch, and replacement of the excised DNA. In methyl-directed MMR, MutS forms a clamp around the DNA and scans until it reaches a DNA mismatch. Once MutS has recognized a mismatch, it recruits MutL, in an ATP-dependent manner, to the mismatched DNA site. Next the MutS-MutL complex loops the DNA and scans the nearby DNA until it reaches MutH, a MMR protein that recognizes and binds to hemi-methylated DNA at a sequence of GATC. MutH, aided by the MutS-MutL complex, can then nick the un-methylated strand of DNA. Because the newly synthesized strand will not immediately be methylated, this ensures the integrity of the parental strand. This marks the damaged (or nascent) strand for removal and repair. Helicase II is recruited to unwind the DNA in the direction of the mismatch. Removal of the DNA is catalyzed by ExoVII, RecJ (5' to 3'), or ExoI (3' to 5') depending on the direction of the mismatch from the ummethylated nick in the DNA and removes the nascent (or damaged) strand until reaching MutS still bound to the DNA mismatch. Finally, DNA polymerase III and single-strand binding proteins (SSB) replace the excised DNA using the parental strand as a template [**40, 41**]. Recent studies have shown that not only does *Neisseria meningitidis* possess homologues to MutS, MutL, and

MutH, but knock-out mutants of any of these MMR proteins renders the bacterium susceptible to higher levels of phase variation. Thus, it appears that MMR plays an important role in the modulating phase variation of *Neisseria* [**42**, **43**].

Research of DNA repair in *N. gonorrhoeae* is limited. Some previous studies have demonstrated the various repair pathways that are present or lacking. With the availability of the genomic sequence of a *N. gonorrhoeae* strain, FA1090, a better picture of gonococcal DNA repair is emerging. Studies have established that *N. gonorrhoeae* lacks error-prone repair because it is more sensitive to indirect mutagens that induce a postreplicative repair system that is prone to errors. This system is one of the many damage-inducible repair pathways designated as the SOS response [**44**]. Genes known to encode proteins important in the *E. coli* SOS response were monitored in *Neisseria* and neither *recA*, *uvrA*, or *uvrB* were up-regulated in response to DNA damage from agents like UV and methyl methanesulfonate (MMS). Therefore, it appears that *N. gonorrhoeae* does not possess an SOS-DNA damage inducible repair system [**45**]. However, recent work has giving rise to the idea that *N. meningitidis* may have a non-classical pathway for inducible-DNA repair. A gene with homology to *E. coli's xseB* is part of a regulon that is induced in *N. meningitidis* by contact with host cells. XseB is a subunit, with XseA, of the active exonuclease enzyme ExoVII. The protein not only modifies the ability of the bacterium to deal with DNA damage, but also is responsible for an increase in phase variation during induction from bacteria-host cell contact [**46**]. A non-classical method of inducible DNA repair may yet be discovered in *Neisseria* pathogens.

Survival of ultraviolet-irradiated *N. gonorrhoeae* when exposed to photo-reactivating light did not increase over those incubated in the dark. Therefore,

observations indicate that the gonococcus lacks photoreactivation repair systems [**47**]. Other studies have demonstrated that *N. gonorrhoeae* possesses homologs to genes associated with other DNA repair pathways. *N. gonorrhoeae* has the ability to remove and repair pyrimidine dimers, thus it possesses an active DNA excision repair pathway [**48**]. Genomic data suggest that FA1090 has homologs to genes associated with very-short patch (VSP) repair, but not much else is know in gonococcus. However, studies in *E. coli* show that a protein, Vsr, is responsible for correcting T-G mismatches caused by spontaneous deamination of 5-methylcytosine (created by methylation of cytosine by the protein Dcm) [**49**]. Also, recent studies have demonstrated that *N. gonorrhoeae* has two proteins, pyrimidine dimer glycosylase I and II, responsible for resolution of DNA damage by UV light and oxidative damage [**50**].

N. gonorrhoeae also possesses homologues to two recombination repair pathways, the RecBCD and RecF-like pathways. Studies have shown that the gonococcal genome contains many of the genes needed for these two major repair pathways and that many of them are essential to properly repair damaged DNA in *Neisseria*. Other studies have shown that many of these genes and the encoded proteins are an integral part of phase variation in the gonococcus because phase variation of pili on the surface decreases or is abolished when these genes are removed. A RecF-like pathway, including *recQ, recO,* and *recX*, is involved in altering pilin expression, changing the make-up of the outer membrane pili, and thereby aiding in immune avoidance. The gonococcal RecBCD pathway (genes *recB, recC,* and *recD*) is involved in DNA transformation and DNA repair but not in pilin antigenic variation [**51 – 54**]. Overall, *N. gonorrhoeae* contains the essential genes and pathways for effective recombinational repair.

DNA repair pathways in *N. gonorrhoeae* have not been completely characterized, even though much progress has been made in this area. Increased understanding and insight into gonococcal DNA repair would also enhance our knowledge about prokaryotic DNA repair in general as well as deepen our understanding of gonococcal pathogenesis. Many genes in the gonococcal genome contain homopolymeric nucleotide repeats that are expected to enhance phase variation. The DNA repair pathways keep a check and balance on phase variation; however a significant amount of phase variation occurs and is expected to occur for the gonococcus to survive. Little is known about the control of phase variation, but studies show that it is important for gonococcal pathogenesis and survival and that DNA repair pathways are involved. Unfortunately there is a lack of a proper positive selection method for phase variation and measuring mutation frequencies. The goal of this research is to define such a system in *N. gonorrhoeae* strain FA1090 that maintains the integrity of the organism while allowing a study of phase variation in homopolymeric nucleotide repeats. A reporter system was created that involved the manipulation of a gonococcal nitroreductase gene and conveyed resistance to nitro-aromatic antimicrobials.

Nitroreductase and Nitro-aromatic antimicrobials

Nitroreductases were first studied because of their role in the reduction of nitrofurans, a group of compounds that contain a nitro group attached to an aromatic ring. Nitrofuran derivatives, such as nitrofurantoin, nitrofurazone, and furazolidone, are used as antimicrobials because the reduction by nitroreductases activates these nitro-aromatic compounds and leads to bacterial cell death. Since the starting and end products of this reduction lack antimicrobial activity, it is believed that the reduction of such nitro-

aromatic compounds leads to reactive oxygen intermediates that can damage DNA and inhibit bacterial metabolism and cell wall [**55**, **56**]. The precise biological role of the nitroreductases is unknown, but strains of bacteria that lack a nitroreductase are more resistant to nitrofurans [**57**]. Nitroreductases have been identified and characterized in a wide variety of microorganisms including the pathogens *Salmonella typhimurium* [**58**], *Helicobacter pylori* [**59**], *Vibrio harveyi* [**60**], and *Escherichia coli* [**57**, **61**].

The most characterized nitroreductases are those found in *Escherichia coli*. *E. coli* encodes two types of nitroreductases, oxygen-insensitive (Type I) and oxygen-sensitive (Type II) [**62**]. The *E. coli* genome contains three genes that encode oxygen-insensitive (Type I) enzyme activities. The major component of this activity is encoded by the gene *nfsA*, while two minor components of this activity are encoded by the gene *nfsB* and a gene that has yet to be identified. NfsA, the nitroreductase encoded by *nfsA*, is dependent on NADPH as an electron donor, while NfsB, encoded by *nfsB*, can use either NADPH or NADH as electron donors [**57**, **61, 63**]. Both NfsA and NfsB are flavin-mononucleotide (FMN) containing proteins [**64, 65**]. NfsA shares significant amino acid homology with Frp, a flavin reductase of *Vibrio harveyi*, so much in fact that a single amino acid substitution at position 99 transforms NfsA from a nitroreductase into a flavin reductase as active as Frp [**66**]. NfsA has been implicated as being a part of the *soxRS* regulon, because it is regulated in the same manner and induced with the rest of the genes contained in the regulon. The *soxRS* regulon is induced in response to oxidative stress and contributes to the defense against this oxidative stress. It seems a bit paradoxical that a gene that is part of the defense against toxic oxygen radicals when its action is needed for activation of nitrofuran antimicrobial agents is based on those same radicals [**67**].

The amino acid sequence of NfsB is similar to FRaseI, a *Vibrio harveyi* flavin reductase, and again a single mutation, which changes one amino acid in the least conserved region, will convert NfsB into a flavin reductase similar in activity to FRaseI [**68**].

Mutations that render NfsA non-functional in *E. coli* result in a low-level of resistance to nitrofurans, while mutations that knock-out the function of both NfsA and NfsB result in a much higher level of resistance to these nitro-aromatic antimicrobials [**57**]. The same two-step process of increased resistance with two nitroreductases is also observed in *Helicobacter pylori* with mutations in *rdxA* and *frxA* that render resistance to the prodrug metronidazole, which is reduced by these nitroreductases to a bactericidal agent [**69**].

Because loss of function in genes that encode nitroreductases results in resistance to nitrofuran antimicrobial agents, it is an ideal starting point for creating a reporter system for studying spontaneous mutation. Potential nitroreductases in *Neisseria gonorrhoeae* FA1090 were identified by database searches and biochemical properties of these putative nitroreductases were verified. Next homopolymeric nucleotide tracts were combined with a nitroreductase and out of this a nitrofurantoin-based reporter system was created, providing a valuable tool for investigating the genetic basis for spontaneous mutation, DNA repair, and phase variation in pathogenic *Neisseria*.

Goals of a DNA repair/Phase Variation Reporter System

Thesis Focus: To create and characterize a reporter system using homopolymeric nucleotide tracts within a nitroreductase to further the understanding of phase variation and the systems and proteins that affect it.

Specific Goals:

1. Create variants of *N. gonorrhoeae* FA1090 that contain Poly-Guanine tracts in the nitroreductase in lengths of 5 to 12 guanines.
2. Test each variant for nitroreductase activity after Poly-Guanine insertion and test spontaneous mutation frequency of each variant.
3. Use Denaturing Gradient Gel Electrophoresis (DGGE) to detect frame-shift mutations in nitrofurantoin resistant mutants

Materials and Methods

Bacterial Growth and Storage Conditions

All *Neisseria gonorrhoeae* strains were grown on GCK agar or GCP broth supplemented with Kellogg's solution and sodium bicarbonate (0.042%) at 37°C with 5% CO_2. *E. coli* were grown on LB agar plates or in LB broth. Broth cultures were grown in a rotary shaker at 37°C. When needed, antibiotics were added to the growth media as follows: spectinomycin at 50 μg/mL and nitrofurantoin at 3 μg/mL. Strains were stored at -80°C after suspension in GCP or LB broth with 20% glycerol added. Bacterial strains with poly-guanine tracts were always passaged directly from freezer stock before assay to limit mutation of the poly-guanine tract. Media components are listed in **Table 1** and all bacterial strains are listed in **Table 2**.

Chemicals, Reagents, and Enzymes

Chemicals used in these studies were of analytical grade or better and were purchased from Sigma (St. Louis, MO) unless otherwise specified. Restriction endonucleases were purchased from New England Biolabs (Beverly, MA) and were used with the supplied buffers according to the manufacturer's instruction.

Polymerase Chain Reaction (PCR)

PCR reactions were done using the Expand Long Template PCR System (Roche). PCR reactions were cleaned using the QIAquick PCR Purification Kit (Qiagen). Oligonucleotide primers were purchased from Integrated DNA Technologies (Coralville, IA). All primers used are listed in **Table 3**.

Table 1. Media composition

GCK	
Gonococcal Base Medium (Difco)	36.0 g
Bacto-Agar (US Biologicals)	5.0 g
Kellogg's	10.0 ml
GCP	
Protease Peptone #3 (Difco)	15.0 g
Soluble Starch (Difco)	1.0g
KH_2PO_4 (Sigma)	1.0g
K_2HPO_4 (Sigma)	4.0g
NaCl (US Biological)	5.0g
HPLC H_20 to 1 L	
Kellogg's	
Glucose (Sigma)	400 g
Glutamine (Sigma)	5.0 g
Ferric nitrate (Sigma)	0.5 g
Thiamine pyrophosphate	0.02 g
HPLC H_2O to 1 L	
LB Broth	
LB Broth Base (US Biological)	25.0 g
HPLC H_2O to 1L	
LB Agar	
LB Agar Base	40.0 g
HPLC H_2O to 1 L	

All media was sterilized by autoclaving except Kellogg's which was filter-sterilized.

Table 2. **Bacterial Strains used in these studies**; all strains listed, except DH5α-mcr, are *Neisseria gonorrhoeae*	
Strain name	Description
FA1090	Laboratory strain
M1	FA1090 Δ*nfsB*; G to T point mutation resulting in a premature stop codon at E168; Nitro resistant
MS11	Laboratory strain
F62	Laboratory strain
FA19	Laboratory strain
PID2	Laboratory strain
nfsB G5	FA1090 altered *nfsB* sequence - ATG**GGTGGGGGT**ACCGGT
nfsB G6	FA1090 altered *nfsB* sequence - ATG**GGGGGT**ACCGGT
nfsB G7	FA1090 altered *nfsB* sequence - ATG**GGGGGG**ACCGGT
nfsB G8	FA1090 altered *nfsB* sequence - ATG**GGTGGGGGGGGT**ACCGGT
nfsB G9	FA1090 altered *nfsB* sequence - ATG**GGGGGGGGT**ACCGGT
nfsB G10	FA1090 altered *nfsB* sequence - ATG**GGGGGGGGG**ACCGGT
nfsB G12	FA1090 altered *nfsB* sequence - ATG**GGGGGGGGGGG**ACCGGT
E. coli DH5α-mcr	F$^-$ *mcrA*Δ(*mrr*$^-$*hsdRMS*$^-$*mcrBC*) endA1supE44thi$^-$1*recA1gyrA*; *relA1* Δ(lacIZYA-argF) U169; deoR(Φ89dlacD(lacZ)M15); *Source :* BRL, Life Technologies

Bold are additional nucleotides, and underline is the Poly-G tract.

Table 3. Primers used in these studies	
Primer Name	Primer Sequence
NP1	AAAGGATCCCATGAACGCCATTGCAGACG
NP2	GGGGGATCCAGAAGATACCATACGCCTCT
S1	GAGATGGGTAAAATCCGGGT
S2	CGAACCGGATGCCGTAGAA
*nfsB*G-DGGE-F(gc)	CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCC CGGAGATGGGTAAAATCCGGGT
*nfsB*G-DGGE-R	GCGGTTCTTGAATGCGGATAGAACCTGCTCTTTGCTTAA

Transformation of *N. gonorrhoeae*

Piliated *N. gonorrhoeae* were incubated with plasmid DNA for 2-4 hours in GCP broth supplemented with 1mM $MgCl_2$, 0.042% $NaHCO_3$, and 1% Kellogg's solution, in a rotary shaker at 37°C. Various dilutions were plated onto GCK and GCK + spectinomycin (50 μg/mL) plates and incubated at 37°C for 24-48 hours. Bacteria were transformed with plasmids prepared by Chris Holder in previous work and are listed in **Table 4**.

Transformation of *E. coli*

Competent *E. coli* cells were prepared by the Inoue Method [**70**] and stored at -80°C. For transformation, competent cells were thawed on ice for 20 minutes, DNA was added, and the mixture was incubated for 10 minutes. Cells were heat shocked at 37°C for 2 minutes. To this 950 μl of LB broth was added, and cells were incubated at 37°C in a rotary shaker for 30-45 minutes. Various dilutions were plated onto LB and LB-antibiotic plates [**70**].

Crude Isolation of Gonococcal Chromosomal DNA

Chromosomal DNA was isolated from *N. gonorrhoeae* by suspending a small amount of bacteria in 5 μl of 0.5M NaOH. This solution was neutralized by the addition of 5 μl of 1M Tris-HCl (pH 7.5). The suspension was then diluted using 90 μl of purified water. Prepared DNA could then be used to perform PCR.

Agarose Gel Electrophoresis

DNA fragments were separated using agarose gel electrophoresis using 1% agarose TBE gels. DNA was visualized with Ethidium Bromide, which was added to the gel prior to pouring. Gels were run at constant voltage of 90-100 volts for 45-75 minutes.

Table 4. Plasmids used in these studies	
Plasmid name	Description
pNFSB-polyG5	pK18, *nfsB* gene, unique AgeI site, Ω cassette, 5 guanine tract
pNFSB-polyG6	pK18, *nfsB* gene, unique AgeI site, Ω cassette, 6 guanine tract
pNFSB-polyG7	pK18, *nfsB* gene, unique AgeI site, Ω cassette, 7 guanine tract
pNFSB-polyG8	pK18, *nfsB* gene, unique AgeI site, Ω cassette, 8 guanine tract
pNFSB-polyG9	pK18, *nfsB* gene, unique AgeI site, Ω cassette, 9 guanine tract
pNFSB-polyG10	pK18, *nfsB* gene, unique AgeI site, Ω cassette, 10 guanine tract
pNFSB-polyG11	pK18, *nfsB* gene, unique AgeI site, Ω cassette, 11 guanine tract
pNFSB-polyG12	pK18, *nfsB* gene, unique AgeI site, Ω cassette, 12 guanine tract

NOTE: Plasmids were prepared by Chris Holder in previous work

Sequence Analysis

The *nfsB* coding region was obtained from the *N. gonorrhoeae* sequenced genome found at the University of Oklahoma www.genome.ou.edu/gono.html. Verification of the gene's function was done by a BLAST search, found at www.ncbi.nlm.nih.gov, queried against the *nfsB* gene from *E. coli*. Other genes encoding proteins of similar function were found by BLAST and they were aligned using the ClustalW program from BCM Search Launcher (http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html).

DNA Sequencing

DNA sequencing was performed by Macrogen, Inc. in Seoul, Korea. 20 μl samples of DNA, at a concentration of 50 ng/μl, were sequenced with primers at a concentration of 5 pmole/μl. All *nfsB* sequences were obtained using primers S1 and S2.

Minimum Inhibitory Concentration (MIC)

The MIC of nitrofurantoin for *N. gonorrhoeae* was determined by plating cultures on various concentrations of nitrofurantoin in plated media. Cultures of strain FA1090 were suspended to a Klett = 100 (~5.0×10^8 CFU/mL), then plated onto GCK containing 1, 2, 3, 4, and 5 μg/mL concentrations of nitrofurantoin. Cultures were diluted and plated at 10^{-6} and 10^{-7} to assess spontaneous mutation frequency. Colonies were counted 24-48 hours after incubation and mutation frequency was calculated. The MIC of nitrofurantoin is the lowest concentration that did not allow growth of the gonococcus.

Verification of Poly-Guanine tract inserts

N. gonorrhoeae containing the newly transformed poly-guanine tracts were verified using a three step process. Cultures were re-streaked onto GCK + Spec (50 μg/mL) to test for the insert of the Spec cassette and conferred antibiotic resistance.

Gonococcal DNA was isolated and the *nfsB* region was amplified by PCR with primers NP1 and NP2. Products were examined for an increase in size over the wild-type FA1090 through 1% agarose gel electrophoresis. PCR products of the appropriate size were cleaned up using QIAquick PCR Purification Kit (Qiagen). These potential candidates were then digested with the restriction enzyme AgeI to verify the insertion of the novel restriction site at the beginning of the *nfsB* gene.

Spontaneous Mutation Assay (SMA)

Spontaneous mutation frequencies of gonococcal strains to a nitrofurantoin resistant (Nit^R) phenotype were detected using the following assay. Overnight cultures were suspended to a Klett = 100 (~5.0 x 10^8 CFU/mL) and serial dilutions were made. 100 μl aliquots of FA1090, MS11, F62, FA19, and PID2 were plated at 10^{-7} dilution onto GCK in duplicate to measure original CFU/mL and undiluted aliquots plated on GCK + Nitrofurantoin (3 μg/mL) in duplicate. 100 μl aliquots of all Poly-G variants of FA1090 were plated on GCK at dilutions of 10^{-6} and 10^{-7} in duplicate. Aliquots of *nfsB* G5 and *nfsB* G6 were plated at dilutions of 10^{-1} and 10^{-2} in duplicate onto GCK + Nitro (3 μg/mL). Aliquots of *nfsB* G8 and *nfsB* G9 were plated at dilutions of 10^{-2} and 10^{-3} in duplicate onto GCK + Nitro (3 μg/mL). Aliquots of *nfsB* G7, *nfsB* G10 and *nfsB* G12 were plated at dilutions of 10^{-3} and 10^{-4} in duplicate onto GCK + Nitro (3 μg/mL). Plates were incubated for 24-48 hours, colonies were counted, and spontaneous mutation frequency was calculated (concentration of mutants/concentration of all bacteria).

Nitroreductase Activity Assay (NAA)

Bacteria were inoculated from freezer stocks onto GCK and GCK + Nitro (3 μg/mL) to test for Nitro sensitivity. After overnight incubation, the cultures were

suspended to a Klett = 100 and 1 mL was inoculated into 100 mL of GCP supplemented with 1 mL of 4.2% $NaHCO_3$ and 1 mL of Kellogg's. The GCP was incubated overnight in a shaker at 37°C. Cells were collected by centrifugation for 10 minutes at 5,000 rpm with a Sorvall GSA rotor, and a pinkish pellet collected. The supernatant was discarded and the cells were washed in 20 mL of filtered 1x PBS. Cells were collected again by centrifugation for 10 minutes at 5,000 rpm with a Sorvall GSA rotor. The supernatant was discarded and the pellet resuspended in 5 mL of 100 mM Tris-HCl, pH=7.5. The suspension was transferred to a plastic tube and cells lysed by sonication (Branson Sonifier 250; 20% duty cycle, output control = 4; 45 seconds of sonication, followed by 75 sec of cooling in ice, repeated twice for each sample.) Lysed cellular material was collected by centrifugation at 10,000 rpm for 30 minutes in a Sorvall SS-34 rotor. Supernatant was transferred and the pellet discarded. When performed, protein concentration in the lysate was measured using the BSA protein concentration assay and samples were adjusted to normalize protein concentration across all the samples. 800 μl samples of the supernatant were added to 90 μl of water and placed in a quartz cuvette. The samples were blanked on an Ultraspec 2000 at a wavelength of 400nm. Nitrofurazone was added to the cuvette to a final concentration of 0.1 mM. To start the reaction, NADPH was added to the cuvette to a final concentration of 0.2 mM. Sample was mixed gently, then wavelength readings taken every 30 seconds for 10 minutes.

BSA Protein Concentration Assay

Assay was used to determine the protein concentrations of samples during Nitroreductase Activity Assay. Samples were diluted 1:10 and 1:25 in triplicate. Dye Reagent (Bio-Rad Protein Assay Dye Reagent Concentrate Catalog #500-0006) was

diluted 1:5 then filter sterilized before use. 10 µl of each sample was placed into separate wells on a 96-well plate. BSA standards were added in duplicate at concentrations of 0, 0.05, 0.1, 0.25, 0.4, 0.5 mg/mL. 200 µl of dye reagent was added to each well and incubated at room temperature for 5 to 60 minutes. A plate reader was used to measure the wells at a wavelength of 595nm to determine protein concentration of the samples.

Denaturing Gradient Gel Electrophoresis (DGGE)

Adapted from a protocol by Wang, Watson, and Miller (11/4/04).

DNA for DGGE was amplified using PCR with primers nfsBG-DGGE-F (gc) and nfsBG-DGGE-R to create a ~200 bp product with a 40-mer GC-clamp [**71**]. Amplification products were verified for proper band size and concentration by electrophoresis on a 1% agarose gel then purified using QIAquick PCR Purification Kit (Qiagen). Poly-acrylamide gels were poured between two 20cm vertical glass plates using a gradient maker and 20 mL each of Denaturing Stock Solution A (0%) and Denaturing Stock Solution B (100%) for a 0-100% gradient gel. TBE-saturated butanol was layered on top of the acrylamide gel immediately after pouring. The acrylamide was allowed to polymerize for at least 2 hours. The butanol layer was then removed and a comb inserted between the glass plates as not to disturb the acrylamide gel. A stacking gel was poured using hot 0.7% agarose and allowed to solidify for 45 minutes. The comb was removed and the gel was loaded into the buffer chamber with 1x TBE. The gel contained a gradient ranging from 0% to 100% denaturant (100% denaturant being 7M Urea, 40% deionized formamide) or 40% to 100% denaturant. DGGE was run at constant voltage (70 V) for 15 hours at 60°C unless otherwise stated.

Results

Identification of Nitroreductase genes in *N. gonorrhoeae*

The complete genome of *Neisseria gonorrhoeae* strain FA1090 has been made available on the internet through the University of Oklahoma's Advanced Center for Genome Technologies (www.genome.ou.edu/gono.html). To detect potential nitroreductase genes in the genome of FA1090, the sequences of two *E. coli* nitroreductases, *nfsA* [**64**] and *nfsB* [**65**], were used to search for sequence similarity within the genomic DNA. Although no significant sequence homology to *nfsA* was found, an open reading frame (ORF) with homology to *nfsB* was identified. The gonococcal sequence, named ORF NGO0388 by the University of Oklahoma's Advanced Center for Genome Technologies, has 25% identity and 42% homology with the *E. coli* NfsB protein (**Figure 1**)**.** This gene will be defined as *nfsB* in for the remainder of this thesis. The gonococcal amino acid sequence for NfsB was used to search through GenBank and find ORFs in other bacteria that possessed significant homology. **Figure 2** shows the alignment of gonococcal NfsB with these homologs. All of these proteins have nitroreductase activity and are found in a wide variety of bacteria. Two major conserved regions are observed in the many nitroreductases including the hypothesized FMN-binding box near the N-terminus (residues 43-62) and another stretch of homology closer to the C-terminus (residues 148-171) with no identified function yet. The beginning of these proteins show limited homology which suggests the N-terminus may be a good location to make modifications without disrupting conserved function. In creating a reporter system using this nitroreductase, the first few amino acids may be a good place to make the necessary modifications.

Figure 1 – NfsB BLAST Search of FA1090 Genome

```
Score = 62.0 bits (149), Expect = 2e-11
 Identities = 57/228 (25%), Positives = 98/228 (42%), Gaps = 16/228 (7%)
 Frame = +2

Query: 3      IISVALKRHSTKAFDASKKLTPEQAEQIKTLLQYSPSSTNSQPWHFIVASTEEGKARVAK 62
              ++S    R S + +DA++K++ E  + I  L + SPSS  S+PW F+V    E       +
Sbjct: 381362 VLSAFKNRKSCRHYDAARKISAEDFQFILELGRLSPSSVGSEPWQFVVVQNPE-----IR 381526

Query: 63     SAAGNYVFNERKMLD-ASHVVVFCAKT--------AMDDVWLKLVVDQEDADGRFATPEA 113
               A   + +     LD ASH+VVF AK          ++ +  + V + +  +   A  +A
Sbjct: 381527 QAIKLFSWGMADALDTASHLVVFLAKKNARFDSPFMLESLKRRGVTEPDAVEKSLARYQA 381706

Query: 114    KAANDKGRKFFADMHRKDLHDDA---EWMAKQVYLNVGNFXXXXXXXXXXXXPIEGFDAA 170
                A+D            K L D     +W  +Q Y+ + N             P+EGF+ A
Sbjct: 381707 FQADD----------IKILDDSRALFDWCCRQTYIALANMMTGAAMAGIDSCPVEGFNYA 381856

Query: 171    ----ILDAEFGLKEKGYTSLVVVPVGHHSVEDFNATLPKSRLPQNITL 214
                  +L  +FGL +     + V     + V++    + K+R P   T+
Sbjct: 381857 DMERVLSGQFGLFDAAEWGVSVAATFGYRVQEI---VTKARRPLEETV 381991
```

Fig. 1: TBLASTN results comparing *E. coli* NfsB (Ascension number = BAA35218) (Query) to *N. gonorrhoeae* FA1090 genome (Subject). Only one sequence within the genome produced a significant alignment. The boxed region is the most conserved region of the protein, which may include the FMN binding site [**57**].

Fig. 2: Nitroreductase Amino Acid Sequence Alignment. GenBank was probed for nitroreductases in other microorganisms, particularly pathogens, using the FA1090 amino acid sequence of *nfsB*. The sequences were aligned using ClustalW. Shaded regions show homology within the sequences with the consensus line (`.=homology; *=identity)` at the bottom of each row.

Figure 2 – Nitroreductase Amino Acid Sequence Alignment

```
FA1090            1 ---MTVLSKEQVLSAFKNRRSCRHYDAARKISAEDFQFILELGRLSPSSVGSEPWQFVVV
N.meningitidis    1 ---MTVLSKEQVLSAFKNRRSCRHYDAARKISAEDFQFILELGRLSPSSVGSEPWQFIVV
H.influenzae      1 ---MTQLTREQVLELFHQRSSTRYYDPTKKISDEDFECILECGRLSPSSVGSEPWKFLVI
S.pyogenes        1 ---MDQTIHHQIQQALHFRTAVRVYKEE-KISDEDLALILDAAWLSPSSVGLEGWRFVVL
B.subtilis        1 ----MADLKTQILDAYNFRHATKEFDPNKKVSDSDFEFILETGRLSPSSLGLEPWKFVVV
B.anthracis       1 MTNTNQITKEKIMEAFHFRHACKAFDPMQKISEEDFKFILETGRLSPSSFGYEPWKFVVV
S.typhimurium     1 ---------MDIVSVALQRYSTKAFDPSKKLTAEEADKIKTLLQYSPSSTNSQPWHFIVA
S.typhi           1 ---------MDIVSVALKRYSTKAFDPSKKLTAEEADKVKTLLQYSPSSTNSQPWHFIVA
E.coli            1 ---------MDIISVALKRHSTKAFDASKKLTPEQAEQIKTLLQYSPSSTNSQPWHFIVA
V.cholerae        1 ---------MNIVQASQSRYSTKAFDASRKLSEQQVADLKELVRMSASSVNSQPWHFILA
H.pylori_rdxA_    1 ---MKFLDQEKRRQLLNERHSCKMFDSHYEFSSEELEEIAEIARLSPSSYNTQPWHFVMV
consensus         1          . .... .    *.... ..  .... .. . .......*.**.....*.*...

FA1090           58 QNPEIRQAIKLFSWG----MADALDTASHLVVFLAK--KNARFDSPFMLESLKRRGVTEP
N.meningitidis   58 QNPEIRQAIKPFSWG----MADALDTASHLVVFLAK--KNARSDSPFMLESLKRRGVTEP
H.influenzae     58 QNKTLREKMKPFSWG----MINQLDNCSHLVVILAK--KNARYDSPFFVDVMARKGLNAE
S.pyogenes       57 DNKPIKEEIKPFAWG----AQYQLETASHFILLIAE--KHARYDSPAIKNSLLRRGIKEG
B.subtilis       57 QNPEFREKLREYTWG----AQKQLPTASHFVLLILARTAKDIKYNADYIKRHLKEVKQMPQ
B.anthracis      61 QNKEVREKLRPYSWG----AGGQLATASHFVIVLSRNIKDMHYDAEYIKHMMNDIIELPE
S.typhimurium    52 STEEGKARVAKSAAGNYTFNERKMLDASHVVVFCAKTAMDDAWLERVVDQEDADGRFATP
S.typhi          52 STEEGKARVAKSAAGNYTFNERKMLDASHVVVFCAKTAMDDAWLQRVVDQEDADGRFATP
E.coli           52 STEEGKARVAKSAAGNYVFNERKMLDASHVVVFCAKTAMDDVWLKLVVDQEDADGRFATP
V.cholerae       52 GSDEGKTRIAKATQGGFSFNERKILDASHVMVFCAKTSIDDAYLLSLLDNEDKDGRFANE
H.pylori_rdxA_   58 TNKDLKKQIAAHSYFN----EEMIKSASALMVVCSLK------PSELLPHGHYMQNLYPE
consensus        61  . ... ... ....          . ..*.......  .. ..     .    .  .

FA1090          112 DAVEKSLARYQAFQADDIKILDDSRALFDWCCRQTYIALANMMTGAAMAGIDSCPVEGFN
N.meningitidis  112 DAVAKSLARYQAFQADDIKILDDSRALFDWCCRQTYIALANMMTGAAMAGIDSCPVEGFN
H.influenzae    112 -QQQAALTKYKALQEEDMKLLENDRTLFDWCSKQTYIALANMLTGASALGIDSCPIEGFH
S.pyogenes      111 DGLNSRLKLYESFQKEDMDMADNPRALFDWTAKQTYIALGNMMMTAALLGIDTCPIEGFH
B.subtilis      113 DVYEGYLSKTEEFQKNDLHLLESDRTLFDWASKQTYIALGNMMMTAAQIGVDSCPIEGFQ
B.anthracis     117 DAQKIRYEFFKKFQETDFNLLQSDRAVFDWASKQTYIALGNMMMTSAAQIGIDSCPIEGFD
S.typhimurium   112 EAKAANDKGRRFFADMHRVSLK-DD--HQWMAKQVYLNVGNFLLGVAAMGLDAVPIEGFD
S.typhi         112 EAKAANDKGRRFFADMHRVSLK-DD--HQWMAKQVYLNVGNFLLGVAAMGLDAVPIEGFD
E.coli          112 EAKAANDKGRKFFADMHRKDLH-DD--AEWMAKQVYLNVGNFLLGVAALGLDAVPIEGFD
V.cholerae      112 EAKTKMHGARSYFVNLHRENLN-DA--EHWMQKQVYLNVGTLLLGAAAMGIDAVPIEGFD
H.pylori_rdxA_  108 SYKARVIPS--FTQMLGVRFNHSMQKLESYILEQCYIAVGQICMGVSLMGLDSCIIGGFD
consensus       121 .. . .     ..  .....  .. .... .*.*........... .*.*.....**.

FA1090          172 YADMERVLSGQFGLFDAAEWGVSVAATFGYRVQE-I---VTKARRPLEETVIWA-
N.meningitidis  172 YAEMERILSGQFGLFDAAEWGVSVAATFGYRVQE-I---ATKARRPLEETVIWA-
H.influenzae    171 YDKINECLAEEG-LFDPQEYAVSVAATFGYRSRD-I---AKKSRKGLDEVVKWVG
S.pyogenes      171 YDKINHILAKHN-VIDLEKEGIASMLSLGYRLRDPK---HAQVRKPKEEVISVVK
B.subtilis      173 YDHIHRILEEEG-LLENGSFDISVMVAFGYRVRDP----RPKTRSAVEDVVKWV-
B.anthracis     177 KEKVDSLLRQEG-IIKENNFEVSVMVAFGYRKEEPK---RDKTRQTMDAIVEWIY
S.typhimurium   169 AEVLDAEFGLKE-----KGYTSLVVVPVGHHSVEDFNAGLPKSRLPLETTLTEV-
S.typhi         169 AEVLDAEFGLKE-----KGYTSLVVVPVGHHSIEDFNAGLPKSRLPLETTLTEV-
E.coli          169 AAILDAEFGLKE-----KGYTSLVVVPVGHHSVEDFNATLPKSRLPQNITLTEV-
V.cholerae      169 AQVLNEEFGLTE-----KGFNSVVIVPLGYHSEDDFNAKLPKSRWPAEAVFTEL-
H.pylori_rdxA_  166 PLKVGEILEERIN-----KPKIACLIALGKRVAEAS----QKSRKSKVDAITWL-
consensus       181  . .. ...     .      . . .... .*.. .        ..* .... ....
```

Nitroreductase activity in *N. gonorrhoeae*

If *N. gonorrhoeae* encodes a functional nitroreductase, the bacteria should be sensitive to nitro-aromatic antimicrobial agents, such as nitrofurantoin. It should also be possible then to detect mutations that knock out the function of the nitroreductase by observing acquired resistance to nitrofurantoin. These nitrofurantoin resistant (Nit^R) mutants should lack measurable nitroreductase activity. A spectrophotometric assay was performed to measure nitroreductase activity in *N. gonorrhoeae*. Samples contained cell lysates of FA1090 gonococcal culture mixed with nitrofurazone and the cofactor NADPH. The samples were measured for a decrease in absorbance at 400 nm, which will detect declining concentration of nitrofurazone, hence nitroreductase activity [**57**]. At first, samples were monitored at 340 nm to detect the conversion of the cofactor NADPH to NADP, which would be coupled with nitroreductase activity. However, consumption of NADPH was observed in negative control samples that did not contain nitrofurazone. Therefore, other proteins in the cell lysate may be using NADPH because these are crude lysates and not purified protein. Hence the consumption of NADPH cannot be used as the true measure of nitroreductase activity. The nitroreductase activity of FA1090 is shown in **Figure 3**, while the control of cell lysate with nitrofurazone (nitro) shows that without NADPH, there is no activity. FA1090 possesses nitroreductase activity and has the ability to reduce nitrofurazone. M1 is a spontaneous mutant resistant to nitrofuran antimicrobial agents characterized by Esteban Carrizosa. The nitrofurantoin resistant M1 (FA1090Δ*nfsB*) contains a single point mutation that inserts a premature stop codon at amino acid position 168, a glutamine, and a truncated protein is encoded. Carrizosa showed that M1 has no discernable nitroreductase activity indicating that a loss

Figure 3 – Nitroreductase Activity in FA1090

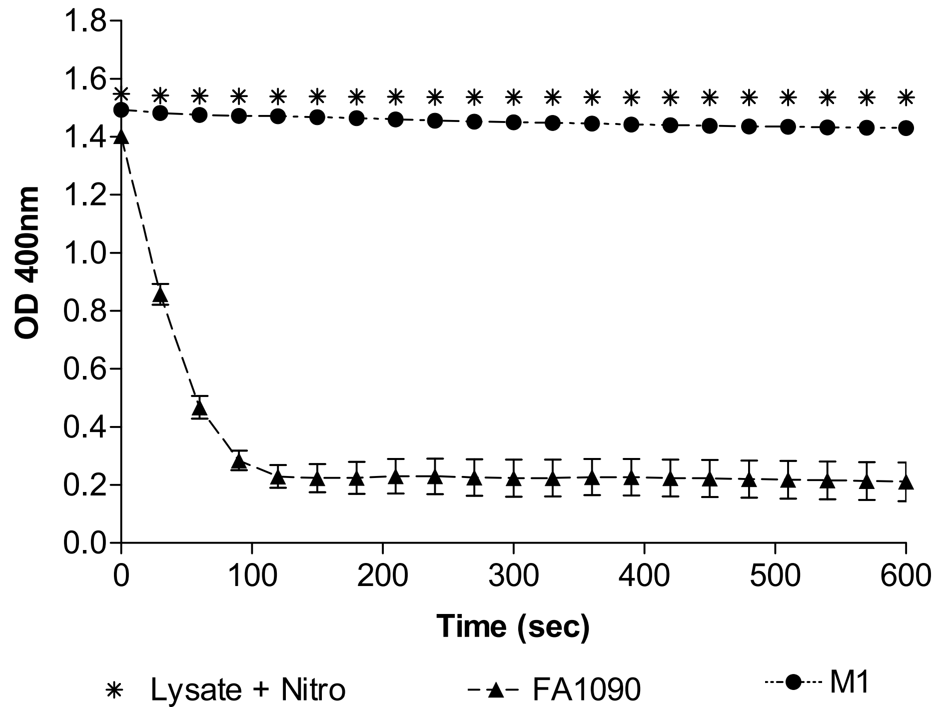


Fig. 3: Nitroreductase Activity in *N. gonorrhoeae* strain FA1090. Samples measured for a loss of absorbance at 400 nm, indicating a reduction of nitrofurazone by an active nitroreductase. FA1090 (closed triangles, ▲) contains an active nitroreductase that requires the cofactor NADPH. Without NADPH there is no activity, shown by the control containing lysate + nitrofurazone (star, *). M1, an *nfsB* mutant of FA1090, does not have a functional nitroreductase and therefore cannot reduce nitrofurazone (closed circles, ●). Samples measured every 30 sec for 10 min. The data represents the average of 7 independent experiments. Error bars were calculated using Standard Error of the Means.

of function mutation in *nfsB* will result in loss of nitroreductase activity and confer resistance to nitrofurans (unpublished work). *N. gonorrhoeae* was found to possess a gene encoding a nitroreductase through searching the genome of FA1090 and showed nitroreductase activity. *E. coli* possesses two nitroreductases that can reduce these nitro-aromatic compounds; *nfsA* and *nfsB*, plus a nitroreductase activity that a gene encoding for the protein has yet to be determined [**57, 61**]. Therefore, it is possible that *N. gonorrhoeae* may possess another gene that confers nitroreductase activity. In *E. coli*, resistance to these nitro-aromatic antimicrobials occurs in a step-wise manner. A mutation that knocks out the function of NfsA yields about three times more resistance, then a second mutation that knocks out the function of NfsB increases resistance to about 10 times the wild-type [**57**]. Earlier attempts made by former lab members to isolate second-step mutants in *N. gonorrhoeae* plated isolates resistant to 3 μg/mL nitrofurantoin onto GCK plates containing 4, 6, 8, 16, and 32 μg/mL nitrofurantoin. No secondary mutants were observed. This suggests that the *nfsB* homolog found in ORF NGO0388 is the only nitroreductase in the gonococcus that is able to reduce nitro-aromatic compounds. The existence of an additional nitroreductase would have resulted in the isolation of secondary mutants upon mutation in the second nitroreductase. The absence of detectable nitroreductase activity in the mutant M1 supports this conclusion.

MIC and Spontaneous Mutation Frequency Studies

Since *N. gonorrhoeae* possesses a nitroreductase, it should be sensitive to the nitro-aromatic antimicrobials, which are activated by the nitroreductase. Therefore, it should be possible to isolate mutants that become resistant to these antimicrobials due to the loss of nitroreductase activity. If nitrofurantoin or nitrofurazone can be used as a

selectable marker for *N. gonorrhoeae* then it would be possible to detect easily whether *nfsB* or the encoded protein is functional**.** This could lead to a reporter system with the nitroreductase being the gene/protein that is detected as being ON or OFF. *N. gonorrhoeae* is sensitive to the nitro-aromatic antimicrobials and the minimum inhibitory concentration (MIC) of nitrofurantoin was determined to be 3 μg/mL (**Table 5**).

Spontaneous mutations occur in the gonococcal genome, and some of those will confer nitrofuran resistance because the mutation knocks-out the function of *nfsB*. If a reporter system is to be created based around this nitroreductase, it is important to know the spontaneous mutation frequency for nitrofurantoin resistance in gonococcal strains. The spontaneous mutation frequency of nitrofurantoin resistance was determined in five strains of *N. gonorrhoeae*, FA1090, F62, FA19, MS11, and PID2. Gonococcal cultures were suspended to approximately 10^9 CFU/mL and serial dilutions were created. The least dilute samples were aliquoted onto GCK plates supplemented with 3 μg/mL nitrofurantoin (to determine the frequency of nitrofurantoin resistant mutants that arise), while the most dilute samples were plated onto GCK to obtain viable cell counts. The results are summarized in **Figure 4** and **Table 6.** Spontaneous mutation frequencies ranged from 4.34×10^{-7} to 1.87×10^{-8}; MS11 was the most mutable of all five strains, while FA1090 had the lowest spontaneous mutation frequency.

Analysis and Manipulation of the *nfsB* coding region

In order to further study *nfsB* and to take steps to create a reporter system, the region had to be analyzed and modified. To accomplish these goals, primers were created to amplify the *nfsB* coding region by polymerase chain reaction (PCR) and another set of primers was created to sequence the length of the coding region. Primers

Table 5. MIC of Nitrofurantoin for FA1090

Plates	10^{-6}	10^{-7}	1 μg/mL	2 μg/mL	3 μg/mL	4 μg/mL	5 μg/mL
Avg. # of colonies	437	41	28	10	0	0	0
CFU/mL	4.1×10^{8}	4.37×10^{8}	280	100	0	0	0
	Avg. = 4.24×10^{8}		-	-	**MIC**	-	-

Note: The average numbers of colonies counted on the specified plates are listed above. The MIC is the lowest concentration of nitrofurantoin that does not allow growth of the Nit^{S} FA1090.

Figure 4 – Spontaneous Mutation Frequency of Gonococcal Strains to Nitrofurantoin

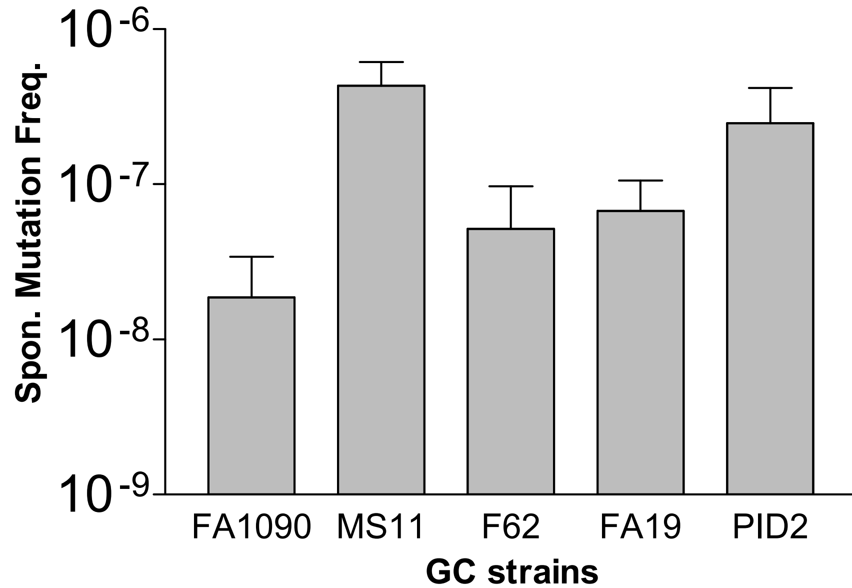


Fig. 4: Spontaneous mutation frequency of various lab strains of *N. gonorrhoeae* is shown by the bar graphs. Mutants that obtained nitrofurantoin resistance were counted from colonies arising on the GCK + Nitrofurantoin (3 μg/mL) plates after 48 hours of incubation at 37ºC, 6% CO_2. Data represent experiments done in triplicate. The most mutable strain was MS11 with a mutation frequency of $4.34 \times 10^{-7} \pm 1.82 \times 10^{-7}$; the least mutable was FA1090 with a frequency of $1.87 \times 10^{-8} \pm 1.53 \times 10^{-8}$. Data values are shown in **Table 6.** Error bars were calculated using Standard Error of the Means.

Table 6. Spontaneous Mutation Frequencies for Nitrofurantoin Resistance

***N. gonorrhoeae* strain**	**Spontaneous Mutation Frequency**
FA1090	$1.87 \times 10^{-8} \pm 1.53 \times 10^{-8}$
FA19	$4.34 \times 10^{-7} \pm 1.82 \times 10^{-7}$
F62	$5.16 \times 10^{-8} \pm 4.54 \times 10^{-8}$
MS11	$6.76 \times 10^{-8} \pm 3.84 \times 10^{-8}$
PID2	$2.48 \times 10^{-7} \pm 1.70 \times 10^{-7}$

NP1 and NP2 (**Table 3**) were used to amplify the length of the coding region and create a PCR product roughly 2,400 base pairs (bp). Primers S1 and S2 (**Table 3**) were designed to sequence the coding strand of the *nfsB* gene. S1 sequences the first half of the gene, while S2 overlaps with the sequencing of S1 and sequences the second half of the gene.

In earlier work in the lab, Esteban Carrizosa and Chris Holder determined the DNA sequence of *nfsB* from five strains of *N. gonorrhoeae*, FA1090, FA19, MS11, F62, and PID2 (unpublished work), by sequencing the PCR products amplified from gonococcal chromosomal DNA using primers NP1 and NP2. The sequence data indicated that all five strains contained identical sequences for *nfsB*. Since the DNA sequence and therefore the amino acid sequences are identical, the variability observed in spontaneous mutation frequency between these strains may be due to differences in DNA repair capabilities amongst these strains.

Earlier work in the lab done by Chris Holder and Esteban Carrizosa involved making subtle changes to the DNA sequence of *nfsB* to enhance its usage as a reporter system. They isolated numerous nitrofurantoin resistant mutants and sequenced *nfsB* to locate the cause for the resistant mutant phenotype. Missense, nonsense, single nucleotide transition, frame-shifting insertion and frame-shifting deletion mutations were all observed (unpublished work). However, a majority of the frame-shifting mutations that were observed involved an insertion of one adenine base into a tract of five adenines. This suggested a bias for frame-shifting in this location and it was determined that this poly-A tract would need to be removed, while preserving the amino acid sequence, to clarify our observations in a future reporter system. In previous work to remove the poly-adenine tract, the *nfsB* gene was cloned into a plasmid (pK18) and modifications were

done using genetic tools in *E. coli*. With the poly-A tract changed, the resulting plasmid was transformed back into *N. gonorrhoeae* FA1090 using spot transformation [**72**] as to not insert a new selectable marker. The subsequent change in the poly-A tract was verified by amplifying the *nfsB* gene by PCR and sequencing the DNA.

The goal was to create a reliable reporter system that could be used to directly measure phase variation, DNA repair, and/or frame-shifting events. The gonococcal *nfsB* gene marks a good starting point for this reporter system, because it is known that we can measure activity of the nitroreductase by sensitivity or resistance to compounds like nitrofurantoin. This can easily be observed on media that contains a nonpermissive level of nitrofurantoin and by growth of the gonococcus on this media. Frame-shifting events in the bacteria could render the *nfsB* gene out-of-frame and the gonococcus would possess a nitrofurantoin resistant (Nit^R) phenotype. The frequencies of this frame-shifting could be assessed by determining the number of nitrofurantoin resistant colonies that arise from a nitrofurantoin-sensitive isolate. Earlier research and studies in DNA repair and phase variation suggest that this could be accomplished by incorporating homopolymeric nucleotide tracts within the *nfsB* gene. The first step to create such a reporter system was to create a gonococcal strain that possessed such a tract in the gene and then assess the gonococcal strain for viability and nitroreductase activity. Poly-guanine tracts play a major role in phase variation and frame-shifting. There are many examples including *pilC* involved in pilin synthesis [**22**], another pili important gene *pgtA* [**24**], LOS biosynthesis genes *lgtA, lgtC, lgtD* [**31, 32**], and the hemoglobin receptors *hpuA* and *hpuB* [**35**], which undergo phase variation apparently due to poly-guanine

tracts. Therefore, work was done in the lab to add a poly-guanine tract in the *nfsB* gene while maintaining the integrity of the gene and the encoded protein.

Again the *nfsB* coding region was amplified using primers NP1 and NP2, the gene was cloned into pK18, and transformed into *E. coli* strain DH5αMCR (**Table 2**). Manipulations to the gene were done by Chris Holder in the *E. coli* background using various primers to add poly-guanine tracts to *nfsB* in varying lengths from 5 guanines to 12 guanines, but the *nfsB* gene remained in frame in all cases with the addition of extra guanines and thymines. All strains with poly-guanines consecutively after the start codon would still be able to encode a functional nitroreductase. Amino acid sequence alignments of nitroreductases suggested that the first few amino acids of NfsB could be modified without effecting changes in protein function (**Figure 2)**; [**57**]. The process of adding the poly-guanine tracts also incorporated a novel AgeI restriction site. To select for transformation of the new *nfsB* gene into the gonococcal chromosome, a **Ω** fragment that confers spectinomycin resistance ($Spec^r$) was added 86 bp downstream from the stop codon of the *nfsB* sequence. The plasmids were extracted from spectinomycin resistant transformants of *E. coli* and the isolated plasmids (**Table 4**) were ready to be transformed into *N. gonorrhoeae* FA1090.

Transformation/Verification of Poly-guanine tract *nfsB* variants

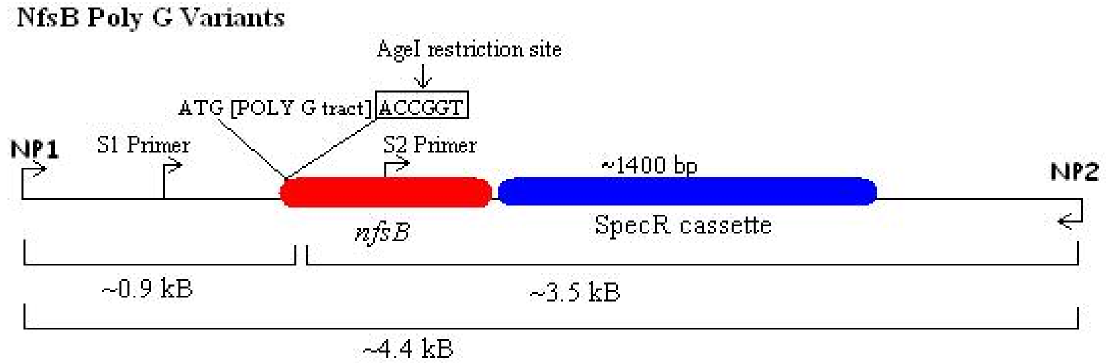
The modified versions of the *nfsB* coding sequence were introduced into the gonococcal genome by transformation. Piliated FA1090 cells were suspended in GCP broth and the appropriate plasmid was added. Cells were incubated for 4 hours in a rotary shaker at 37°C. After incubation, aliquots were plated on GCK agar that contained 50 μg/mL spectinomycin to select for the modified *nfsB* gene. Transformants were

detected after 36-48 hours of incubation at 37°C, 6% CO_2. Separate transformations were done for each poly-guanine length. Various outcomes may have occurred from the transformation that led to the isolated transformant to be selected. Possible outcomes like the Spec cassette inserting into the chromosome alone had to be ruled out and verification that the desired transformation occurred needed to be obtained.

There were three steps to verify the modified *nfsB* gene inserted into the gonococcal chromosome. Taken together, these steps verify transformation of FA1090 with a new nitroreductase gene that includes poly-guanine tracts. **Figure 5** is a schematic illustration of the coding region of *nfsB* in both wild-type (wt) FA1090 and the Poly-G variants after the targeted manipulations. Represented here are the three steps needed for verification. The Poly G variants include a cassette that confers spectinomycin resistance just downstream of the *nfsB* stop codon that makes the Poly-G variants $Spec^r$ while FA1090 (wt) remains spectinomycin sensitive ($Spec^S$). Also this increases the size of the PCR product from primers NP1 and NP2 from 2.4kB to 4.4kB, which can be viewed through gel electrophoresis. Finally, a novel AgeI restriction digest site can be observed in the PCR products of the Poly-G variants.

Step one of verification was rather simple. The selected strains to test for Poly-G insertion in *nfsB* were streaked onto GCK plates containing 50 μg/mL spectinomycin. The original FA1090 (wt) was also inoculated onto GCK + Spec as a negative control for growth. Chromosomal DNA was isolated from the isolates that grew on GCK + Spec plates. For step two, the *nfsB* coding region was PCR amplified with primers NP1 and NP2 from the gonococcal chromosomal DNA. The PCR products for each isolate and FA1090 (wt) were run on a 1% agarose gel for 60 min at 100V to separate based on size

Figure 5 – Schematic of the new *nfsB* Coding Region



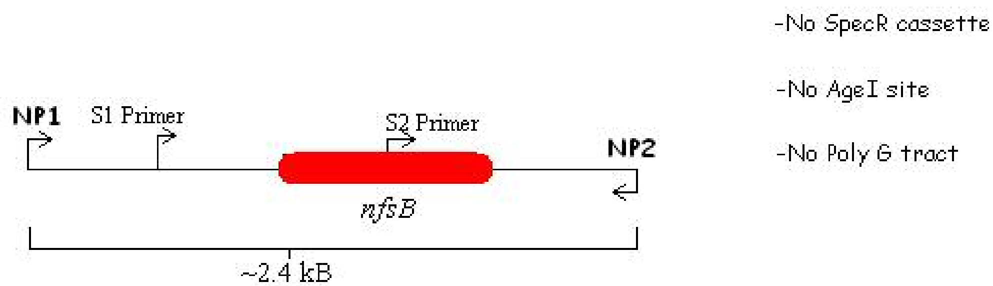


Fig. 5: Schematic illustration comparing the newly modified *nfsB* coding region and the original coding region**.** Sizes are representative of PCR products produced from primers NP1 and NP2. Smaller sizes listed for the modified coding region are indicative after AgeI digestion. The modified coding region is ~2 kB larger due to an inserted $Spec^r$ (Ω) cassette and contains a novel AgeI restriction digest site. Finally the new *nfsB* coding region contains the appropriate length poly-guanine tract.

of the fragments. Poly-G variants are selected based on a shift in band size (~4.4kB) therefore these PCR products do not run as far through the agarose gel. Lambda-DNA ladder (λ) is added to the gel to determine size of bands based on known band sizes. Many isolates were screened in this study but in the end, one Poly-G variant was chosen for each desired poly-guanine tract length, ranging from 5 guanines to 12. The appropriate band sizes and mobility shifts in agarose gel electrophoresis for these variants can be observed in **Figure 6** (lanes 3-8). Isolating a positive variant for a guanine tract of 9 was not as simple, but will be discussed later. Step three checks for the AgeI restriction digest site using the PCR products from each potential variant. PCR products were purified using QIAquick PCR Purification Kit (Qiagen). The DNA was digested using the restriction enzymes AgeI for 4 hours, and then run on a 1% agarose gel to determine restriction patterns. Unmodified FA1090 (wt) does not contain an AgeI site and therefore is not cut (**Figure 7**, lane 3). Modified Poly-G variants that are selected for contain one AgeI restriction site near the poly-guanine tract. The appropriate sizes of the two bands observed after digestion are ~0.9kB and ~3.5kB (**Figure 7, Figure 5**)

One isolate was chosen for each desired length of poly-guanine tract in the *nfsB* gene. These strains were named Poly-G variants of FA1090 and individually named *nfsB* Gx, where x is the length of the poly-guanine tract found in *nfsB* (**Table 2**). No isolates for poly-guanine tract length of 9 passed all three steps of the verification process using the purified plasmids for transformation prepared by Chris Holder. For guanine tracts of 9 nucleotides, the plasmids had to be re-isolated and then retransformed into FA1090 numerous times. Some isolates grown onto GCK + Spec after transformation possessed the desired size of the *nfsB* region, but did not contain the novel AgeI restriction site

Figure 6 – PCR amplification of *nfsB* coding regions in Poly-G Variants

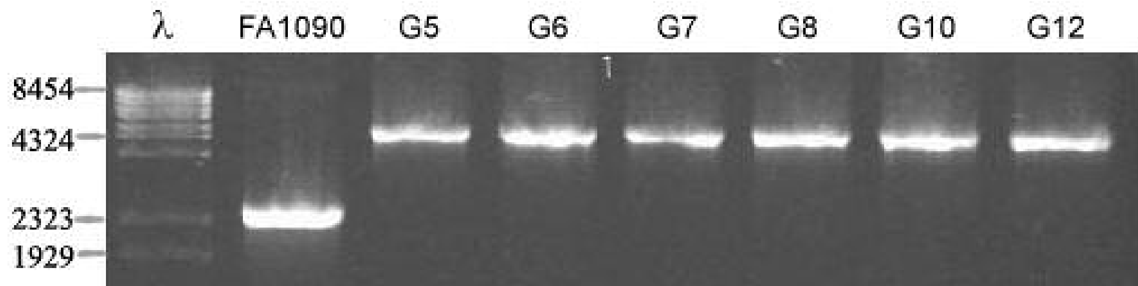


Fig. 6: The *nfsB* coding regions of both FA1090 wild-type and the Poly-G inserted *nfsB* variants were amplified with primers NP1 and NP2. Products run on a 1% agarose gel for 60 min at 100V. Numbers to the left represent sizes of bands of the Lambda (λ) DNA ladder in base pairs. FA1090 without poly-G insert is smaller (~2.4kB), while variants with Poly-G insert contain the Spec resistance (**Ω**) cassette and are bigger products (~4.4kB). G5 = strain *nfsB* G5; G6 = strain *nfsB* G6; G7 = strain *nfsB* G7; G8 = strain *nfsB* G8; G10 = strain *nfsB* G10; G12 = strain *nfsB* G12;

Figure 7 – AgeI Restriction Digest of *nfsB*

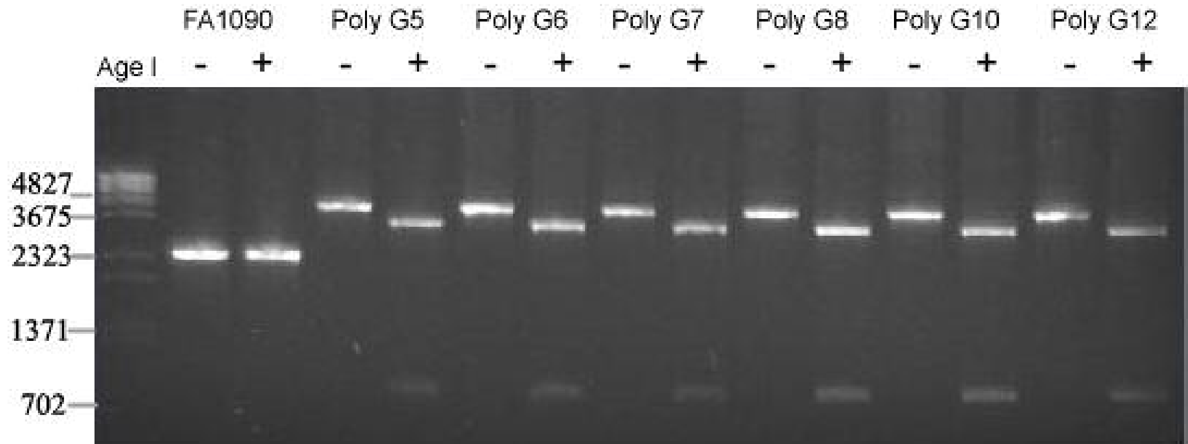


Fig. 7: Amplified PCR products were digested with restriction enzyme AgeI for 4 hours. Samples were run side by side with a sample where AgeI was not added, indicated by the (-) sign. AgeI digested samples are indicated by a (+) sign above the lane. Products were run on a 1% agarose gel for 60 min at 100V. Insertion of the poly-guanine tracts also inserted a novel AgeI restriction site. 2 bands are observed after digestion; ~0.9kB and ~3.5kB. FA1090 (wt) does not contain an AgeI restriction site therefore only one band is observed. Numbers to the left represent sizes of bands of the Lambda (λ) DNA ladder in base pairs. Poly G5 = strain *nfsB* G5; Poly G6 = strain *nfsB* G6; Poly G7 = strain *nfsB* G7; Poly G8 = strain *nfsB* G8; Poly G10 = strain *nfsB* G10; Poly G12 = strain *nfsB* G12;

(**Figure 8**). **Figure 8** shows a very small sample size, illustrating a Poly-G9 transformant that did not digest with AgeI and one transformant that did digest. This transformant was the isolate chosen as the Poly-G variant with a tract of 9 guanines.

After obtaining viable, verified transformants for each desired length of guanine tract, an isolate for each variant was stored as a freezer stock in -80°C. To further verify the presence of the poly-guanine tracts in the *nfsB* gene and to assure other areas of the sequence were kept unchanged, the DNA of the variants and the wild-type (unmodified FA1090) were analyzed. PCR products were amplified from gonococcal chromosomal DNA using primers NP1 and NP2. These DNAs were then purified using a Qiagen kit and 20 μl samples were sent to Macrogen, Inc. in Seoul, Korea for sequencing of the *nfsB* region using primers S1 and S2 (**Table 3**). M1, the Δ*nfsB* mutant, was also sent for sequencing. The *nfsB* sequences of M1 and FA1090 were aligned using ClustalW (**Figure 9**). The M1 sequence was identical to the parent except for a single nucleotide change, a G changed to a T, which creates a premature stop codon (TAA) within *nfsB* for M1. The sequences of all the Poly-G variants and FA1090 were aligned using ClustalW (**Figure 10**). The sequences were virtually identical. The inserted AgeI restriction site (ACCGGT) can be observed in every Poly-G variant, changed from ACAGTA in FA1090. The proper length of guanine tract can be observed in each variant while the coding sequence remains in frame. The sequences returned from Macrogen, Inc, and viewed in **Figure 10** are from the first samples sent of these variants, and the sequences of the Poly-G regions were as predicted.

Figure 8 – Selection of *nfsB* G9 Variant

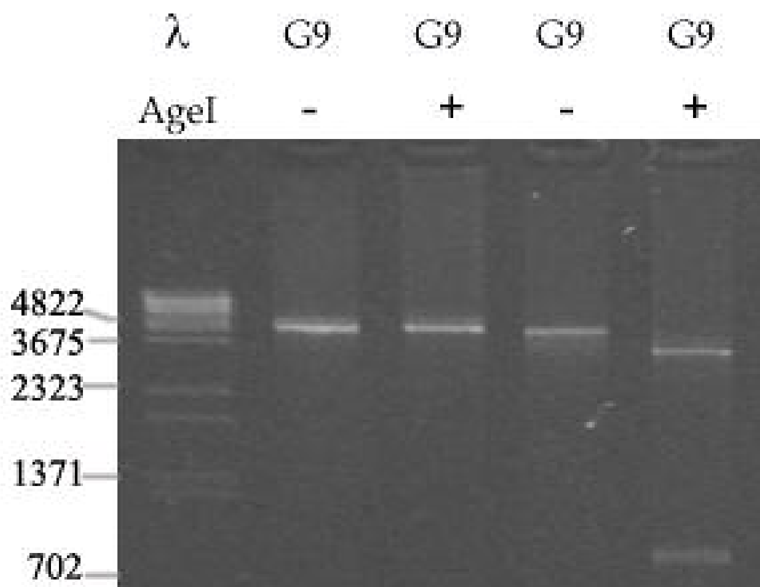


Fig. 8: The proper transformation to obtain the *nfsB* G9 variant was harder to obtain than the others**.** Here Lanes 2 and 3 represent one of the many *nfsB* G9 transformants that did not digest properly with AgeI. Lanes 4 and 5 represent the *nfsB* G9 variant chosen to continue the work. It digested with AgeI to form 2 bands representative of the insertion of the novel AgeI site and the poly-guanine tract. PCR products were digested with AgeI (+) or not digested (-). Numbers to the left represent sizes of bands of the Lambda (λ) DNA ladder in base pairs. G9 = strain *nfsB* G9.

Fig. 9: The coding region of the *nfsB* gene in both FA1090 (wt) and M1 (Nit^R mutant) were amplified with primers NP1 and NP2. Products were purified using the QIAquick PCR Purification Kit (Qiagen). PCR products were sent to Macrogen, Inc. (Seoul, Korea) to be sequenced. Sequences were aligned using ClustalW. Note the start and stop codons of the *nfsB* gene and the single point mutation (G to T) that creates a premature stop codon making M1's encoded nitroreductase non-functional.

Figure 9 – *nfsB* Sequence Alignment of FA1090 and M1 (Nit[R] mutant)

```
FA1090_nfsb    1 ----------------------------------------GAAATCAATTTGCAATCAAA
M1_nfsB        1 CGAGCGGGGNGCTANGGGTGCGGTAGTGCCGTATCGGTTTGAAATCAATTTGCAATCAAA
consensus      1 ........................................********************

                 Start codon
                     ↓
FA1090_nfsb   21 GTAATAAAAGGATGCACGATGACAGTATTAAGCAAAGAGCAGGTTCTATCCGCATTTAAA
M1_nfsB       61 GTAATAAAAGGATGCACGATGACAGTATTAAGCAAAGAGCAGGTTCTATCCGCATTTAAA
consensus     61 ************************************************************

FA1090_nfsb   81 AACCGCAAATCGTGCCGGCATTACGATGCGGCACGTAAAATCAGTGCCGAGGATTTTCAG
M1_nfsB      121 AACCGCAAATCGTGCCGGCATTACGATGCGGCACGTAAAATCAGTGCCGAGGATTTTCAG
consensus    121 ************************************************************

FA1090_nfsb  141 TTTATTTTAGAACTCGGGCGTTTGTCGCCCAGTTCGGTCGGTTCGGAGCCTTGGCAGTTT
M1_nfsB      181 TTTATTTTAGAACTCGGGCGTTTGTCGCCCAGTTCGGTCGGTTCGGAGCCTTGGCAGTTT
consensus    181 ************************************************************

FA1090_nfsb  201 GTTGTGGTTCAAAACCCCGAAATCCGACAGGCAATCAAGCTGTTTTCTTGGGGTATGGCG
M1_nfsB      241 GTTGTGGTTCAAAACCCCGAAATCCGACAGGCAATCAAGCTGTTTTCTTGGGGTATGGCG
consensus    241 ************************************************************

FA1090_nfsb  261 GATGCTTTGGATACCGCCAGTCATTTGGTGGTGTTTTTGGCGAAGAAAAATGCCCGCTTC
M1_nfsB      301 GATGCTTTGGATACCGCCAGTCATTTGGTGGTGTTTTTGGCGAAGAAAAATGCCCGCTTC
consensus    301 ************************************************************

FA1090_nfsb  321 GACAGCCCGTTTATGTTGGAAAGTCTCAAACGGCGCGGCGTTACCGAACCGGATGCCGTA
M1_nfsB      361 GACAGCCCGTTTATGTTGGAAAGTCTCAAACGGCGCGGCGTTACCGAACCGGATGCCGTA
consensus    361 ************************************************************

FA1090_nfsb  381 GAAAAATCTTTGGCGAGGTATCAGGCGTTTCAAGCTGACGACATCAAGATTTTGGACGAT
M1_nfsB      421 GAAAAATCTTTGGCGAGGTATCAGGCGTTTCAAGCTGACGACATCAAGATTTTGGACGAT
consensus    421 ************************************************************

FA1090_nfsb  441 TCGCGCGCCTTGTTTGACTGGTGCTGCCGCCAGACCTATATCGCGTTAGCCAACATGATG
M1_nfsB      481 TCGCGCGCCTTGTTTGACTGGTGCTGCCGCCAGACCTATATCGCGTTAGCCAACATGATG
consensus    481 ************************************************************

                                                Point mutation
                                                       ↓
FA1090_nfsb  501 ACGGGTGCGGCGATGGCGGGTATCGATTCCTGCCCGGTGGAAGGTTTCAACTATGCCGAT
M1_nfsB      541 ACGGGTGCGGCGATGGCGGGTATCGATTCCTGCCCGGTGTAAGGTTTCAACTATGCCGAT
consensus    541 *************************************** ********************

FA1090_nfsb  561 ATGGAACGCGTATTGTCCGGGCAGTTCGGTTTGTTCGATGCGGCAGAATGGGGCGTGTCC
M1_nfsB      601 ATGGAACGCGTATTGTCCGGGCAGTTCGGTTTGTTCGATGCGGCAGAATGGGGCGTGTCC
consensus    601 ************************************************************

FA1090_nfsb  621 GTTGCCGCGACATTCGGCTACCGGGTTCAGGAAATCGTCACGAAAGCGCGCAGGCCCTTG
M1_nfsB      661 GTTGCCGCGACATTCGGCTACCGGGTTCAGGAAATCGTCACGAAAGCGCGCAGGCCCTTG
consensus    661 ************************************************************

                           Stop codon
                              ↓
FA1090_nfsb  681 GAAGAAACCGTTATTTGGGCATAAGGCAGTGCCGTCTGAAAACGCAAGGATTTTCAGACG
M1_nfsB      721 GAAGAAACCGTTATTTGGGCATAAGGCAGTGCCGTCTGAAAACGCAAGGATTTTCAGACG
consensus    721 ************************************************************
```

Fig. 10: The coding region of the *nfsB* gene in FA1090 (wt) and the Poly-G variants were amplified with primers NP1 and NP2. Products were purified and sequenced. Sequences were aligned using ClustalW. Note the inserted poly-guanine tracts of specified length following the ATG in the Poly-G variants and the novel AgeI restriction site (ACCGGT). G5 = strain *nfsB* G5; G6 = strain *nfsB* G6; G7 = strain *nfsB* G7; G8 = strain *nfsB* G8; G9 = strain *nfsB* G9; G10 = strain *nfsB* G10; G12 = strain *nfsB* G12. Consecutive guanines in the poly-guanine track are shown in **BOLD**. Additional nucleotides inserted into the *nfsB* gene, including base pairs added to keep the gene in frame, are shown inside the black box. Start and Stop codons are marked as is the AgeI restriction site.

Figure 10 – *NfsB* Sequence Alignment of FA1090 and Poly-G Variants

```
                                                          START          AGEI site
FA1090     1 GAAATCAATTTGCAATCAAAGTAATAAAAGGATGCACGATG------------ACAGTAT
G5         1 GAAATCAATTTGCAATCAAAGTAATAAAAGGATGCACGATGGGTGGGG---GTACCGGTT
G6         1 GAAATCAATTTGCAATCAAAGTAATAAAAGGATGCACGATGGG-GGG-----TACCGGTT
G7         1 GAAATCAATTTGCAATCAAAGTAATAAAAGGATGCACGATGGGGGGG------ACCGGTT
G8         1 GAAATCAATTTGCAATCAAAGTAATAAAAGGATGCACGATGGGTGGGGGGGGTACCGGTT
G9         1 GAAATCAATTTGCAATCAAAGTAATAAAAGGATGCACGATGGGGGGGG---GTACCGGTT
G10        1 GAAATCAATTTGCAATCAAAGTAA-AAAGGATGCACGATGGGGGGGG---GGACCGGTT
G12        1 GAAATCAATTTGCAATCAAAGTAATAAAAGGATGCACGATGGGGGGGGGGGGTACCGGTT
consensus  1 ************************.***.************.......   ..**.*..*
```

```
FA1090      49 TAAGCAAAGAGCAGGTTCTATCCGCATTTAAAAACCGCAAATCGTGCCGGCATTACGATG
G5          58 TAAGCAAAGAGCAGGTTCTATCCGCATTTAAAAACCGCAAATCGTGCCGGCATTACGATG
G6          55 TAAGCAAAGAGCAGGTTCTATCCGCATTTAAAAACCGCAAATCGTGCCGGCATTACGATG
G7          55 TAAGCAAAGAGCAGGTTCTATCCGCATTTAAAAACCGCAAATCGTGCCGGCATTACGATG
G8          61 TAAGCAAAGAGCAGGTTCTATCCGCATTTAAAAACCGCAAATCGTGCCGGCATTACGATG
G9          58 TAAGCAAAGAGCAGGTTCTATCCGCATTTAAAAACCGCAAATCGTGCCGGCATTACGATG
G10         57 TAAGCAAAGAGCAGGTTCTATCCGCATTTAAAAACCGCAAATCGTGCCGGCATTACGATG
G12         61 TAAGCAAAAAGCAGGTTCTATCCGCATTTAAAAACCGCAAATCGGGCCGGCATTACAATG
consensus   61 ********.***********************************.***********.***

FA1090     109 CGGCACGTAAAATCAGTGCCGAGGATTTTCAGTTTATTTTAGAACTCGGGCGTTTGTCGC
G5         118 CGGCACGTAAAATCAGTGCCGAGGATTTTCAGTTTATTTTAGAACTCGGGCGTTTGTCGC
G6         115 CGGCACGTAAAATCAGTGCCGAGGATTTTCAGTTTATTTTAGAACTCGGGCGTTTGTCGC
G7         115 CGGCACGTAAAATCAGTGCCGAGGATTTTCAGTTTATTTTAGAACTCGGGCGTTTGTCGC
G8         121 CGGCACGTAAAATCAGTGCCGAGGATTTTCAGTTTATTTTAGAACTCGGGCGTTTGTCGC
G9         118 CGGCACGTAAAATCAGTGCCGAGGATTTTCAGTTTATTTTAGAACTCGGGCGTTTGTCGC
G10        117 CGGCACGTAAAATCAGTGCCGAGGATTTTCAGTTTATTTTAGAACTCGGGCGTTTGTCGC
G12        121 CGGCACGTAAAATCAGGGCCAAGGATTTTCAGTTTATTTTAAAACTCGGGCGTTTGTCCC
consensus  121 ****************.***.********************.****************.*

FA1090     169 CCAGTTCGGTCGGTTCGGAGCCTTGGCAGTTTGTTGTGGTTCAAAACCCCGAAATCCGAC
G5         178 CCAGTTCGGTCGGTTCGGAGCCTTGGCAGTTTGTTGTGGTTCAAAACCCCGAAATCCGAC
G6         175 CCAGTTCGGTCGGTTCGGAGCCTTGGCAGTTTGTTGTGGTTCAAAACCCCGAAATCCGAC
G7         175 CCAGTTCGGTCGGTTCGGAGCCTTGGCAGTTTGTTGTGGTTCAAAACCCCGAAATCCGAC
G8         181 CCAGTTCGGTCGGTTCGGAGCCTTGGCAGTTTGTTGTGGTTCAAAACCCCGAAATCCGAC
G9         178 CCAGTTCGGTCGGTTCGGAGCCTTGGCAGTTTGTTGTGGTTCAAAACCCCGAAATCCGAC
G10        177 CCAGTTCGGTCGGTTCGGAGCCTTGGCAGTTTGTTGTGGTTCAAAACCCCGAAATCCGAC
G12        181 CCAGTTCGGCCGGTTCGGACCCTTGGCAGTTTGTTGGGGTTCAAAACCCCAAAATCCGAC
consensus  181 *********.*********.****************.*************.*********

FA1090     229 AGGCAATCAAGCTGTTTTCTTGGGGTATGGCGGATGCTTTGGATACCGCCAGTCATTTGG
G5         238 AGGCAATCAAGCTGTTTTCTTGGGGTATGGCGGATGCTTTGGATACCGCCAGTCATTTGG
G6         235 AGGCAATCAAGCTGTTTTCTTGGGGTATGGCGGATGCTTTGGATACCGCCAGTCATTTGG
G7         235 AGGCAATCAAGCTGTTTTCTTGGGGTATGGCGGATGCTTTGGATACCGCCAGTCATTTGG
G8         241 AGGCAATCAAGCTGTTTTCTTGGGGTATGGCGGATGCTTTGGATACCGCCAGTCATTTGG
G9         238 AGGCAATCAAGCTGTTTTCTTGGGGTATGGCGGATGCTTTGGATACCGCCAGTCATTTGG
G10        237 AGGCAATCAAGCTGTTTTCTTGGGGTATGGCGGATGCTTTGGATACCGCCAGTCATTTGG
G12        241 AGGCAATCAAGCTGTTTTCTTGGGGTATGGCGGATGCTTTGGATACCCCCAGTCATTTGG
consensus  241 ***********************************************.************

FA1090     289 TGGTGTTTTTGGCGAAGAAAAATGCCCGCTTCGACAGCCCGTTTATGTTGGAAAGTCTCA
G5         298 TGGTGTTTTTGGCGAAGAAAAATGCCCGCTTCGACAGCCCGTTTATGTTGGAAAGTCTCA
G6         295 TGGTGTTTTTGGCGAAGAAAAATGCCCGCTTCGACAGCCCGTTTATGTTGGAAAGTCTCA
G7         295 TGGTGTTTTTGGCGAAGAAAAATGCCCGCTTCGACAGCCCGTTTATGTTGGAAAGTCTCA
G8         301 TGGTGTTTTTGGCGAAGAAAAATGCCCGCTTCGACAGCCCGTTTATGTTGGAAAGTCTCA
G9         298 TGGTGTTTTTGGCGAAGAAAAATGCCCGCTTCGACAGCCCGTTTATGTTGGAAAGTCTCA
G10        297 TGGTGTTTTTGGCGAAGAAAAATGCCCGCTTCGACAGCCCGTTTATGTTGGAAAGTCTCA
G12        301 GGGTGTTTTTGGCAAAAAAAAATGCCCGCTTCGACAGCCCGTTTATGTTGGAAAGTCTCA
consensus  301 .************.**.*******************************************
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FA1090     349 AACGGCGCGGCGTTACCGAACCGGATGCCGTAGAAAAATCTTTGGCGAGGTATCAGGCGT
G5         358 AACGGCGCGGCGTTACCGAACCGGATGCCGTAGAAAAATCTTTGGCGAGGTATCAGGCGT
G6         355 AACGGCGCGGCGTTACCGAACCGGATGCCGTAGAAAAATCTTTGGCGAGGTATCAGGCGT
G7         355 AACGGCGCGGCGTTACCGAACCGGATGCCGTAGAAAAATCTTTGGCGAGGTATCAGGCGT
G8         361 AACGGCGCGGCGTTACCGAACCGGATGCCGTAGAAAAATCTTTGGCGAGGTATCAGGCGT
G9         358 AACGGCGCGGCGTTACCGAACCGGATGCCGTAGAAAAATCTTTGGCGAGGTATCAGGCGT
G10        357 AACGGCGCGGCGTTACCGAACCGGATGCCGTAAAAAAATCTTTGGCGAGGTATCAGGCGT
G12        361 AACGGCGCGGCGTTCCCAAACCGGATGCCGTAAAAAAATCTTTGGCGAGGTATCAGGCGT
consensus  361 **************.**.**************.***************************

FA1090     409 TTCAAGCTGACGACATCAAGATTTTGGACGATTCGCGCGCCTTGTTTGACTGGTGCTGCC
G5         418 TTCAAGCTGACGACATCAAGATTTTGGACGATTCGCGCGCCTTGTTTGACTGGTGCTGCC
G6         415 TTCAAGCTGACGACATCAAGATTTTGGACGATTCGCGCGCCTTGTTTGACTGGTGCTGCC
G7         415 TTCAAGCTGACGACATCAAGATTTTGGACGATTCGCGCGCCTTGTTTGACTGGTGCTGCC
G8         421 TTCAAGCTGACGACATCAAGATTTTGGACGATTCGCGCGCCTTGTTTGACTGGTGCTGCC
G9         418 TTCAAGCTGACGACATCAAGATTTTGGACGATTCGCGCGCCTTGTTTGACTGGTGCTGCC
G10        417 TTCAAGCTGACGACATCAAGATTTTGGACGATTCGCGCGCCTTGTTTGACTGGTGCTGCC
G12        421 TTCAAGCTGACGACATCAAGATTTTGGACGATTCGCGCGCCTTGTTTGACTGGTGCTGCC
consensus  421 ************************************************************

FA1090     469 GCCAGACCTATATCGCGTTAGCCAACATGATGACGGGTGCGGCGATGGCGGGTATCGATT
G5         478 GCCAGACCTATATCGCGTTAGCCAACATGATGACGGGTGCGGCGATGGCGGGTATCGATT
G6         475 GCCAGACCTATATCGCGTTAGCCAACATGATGACGGGTGCGGCGATGGCGGGTATCGATT
G7         475 GCCAGACCTATATCGCGTTAGCCAACATGATGACGGGTGCGGCGATGGCGGGTATCGATT
G8         481 GCCAGACCTATATCGCGTTAGCCAACATGATGACGGGTGCGGCGATGGCGGGTATCGATT
G9         478 GCCAGACCTATATCGCGTTAGCCAACATGATGACGGGTGCGGCGATGGCGGGTATCGATT
G10        477 GCCAGACCTATATCGCGTTAGCCAACATGATGACGGGTGCGGCGATGGCGGGTATCGATT
G12        481 GCCAGACCTATATCGCGTTAGCCAACATGATGACGGGTGCGGCGATGGCGGGTATCGATT
consensus  481 ************************************************************

FA1090     529 CCTGCCCGGTGGAAGGTTTCAACTATGCCGATATGGAACGCGTATTGTCCGGGCAGTTCG
G5         538 CCTGCCCGGTGGAAGGTTTCAACTATGCCGATATGGAACGCGTATTGTCCGGGCAGTTCG
G6         535 CCTGCCCGGTGGAAGGTTTCAACTATGCCGATATGGAACGCGTATTGTCCGGGCAGTTCG
G7         535 CCTGCCCGGTGGAAGGTTTCAACTATGCCGATATGGAACGCGTATTGTCCGGGCAGTTCG
G8         541 CCTGCCCGGTGGAAGGTTTCAACTATGCCGATATGGAACGCGTATTGTCCGGGCAGTTCG
G9         538 CCTGCCCGGTGGAAGGTTTCAACTATGCCGATATGGAACGCGTATTGTCCGGGCAGTTCG
G10        537 CCTGCCCGGTGGAAGGTTTCAACTATGCCGATATGGAACGCGTATTGTCCGGGCAGTTCG
G12        541 CCTGCCCGGTGGAAGGTTTCAACTATGCCGATATGGAACGCGTATTGTCCGGGCAGTTCG
consensus  541 ************************************************************

FA1090     589 GTTTGTTCGATGCGGCAGAATGGGGCGTGTCCGTTGCCGCGACATTCGGCTACCGGGTTC
G5         598 GTTTGTTCGATGCGGCAGAATGGGGCGTGTCCGTTGCCGCGACATTCGGCTACCGGGTTC
G6         595 GTTTGTTCGATGCGGCAGAATGGGGCGTGTCCGTTGCCGCGACATTCGGCTACCGGGTTC
G7         595 GTTTGTTCGATGCGGCAGAATGGGGCGTGTCCGTTGCCGCGACATTCGGCTACCGGGTTC
G8         601 GTTTGTTCGATGCGGCAGAATGGGGCGTGTCCGTTGCCGCGACATTCGGCTACCGGGTTC
G9         598 GTTTGTTCGATGCGGCAGAATGGGGCGTGTCCGTTGCCGCGACATTCGGCTACCGGGTTC
G10        597 GTTTGTTCGATGCGGCAGAATGGGGCGTGTCCGTTGCCGCGACATTCGGCTACCGGGTTC
G12        601 GTTTGTTCGATGCGGCAGAATGGGGCGTGTCCGTTGCCGCGACATTCGGCTACCGGGTTC
consensus  601 ************************************************************
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FA1090     649 AGGAAATCGTCACGAAAGCGCGCAGGCCCTTGGAAGAAACCGTTATTTGGGCATAAGGCA
G5         658 AGGAAATCGTCACGAAAGCGCGCAGGCCCTTGGAAGAAACCGTTATTTGGGCATAAGGCA
G6         655 AGGAAATCGTCACGAAAGCGCGCAGGCCCTTGGAAGAAACCGTTATTTGGGCATAAGGCA
G7         655 AGGAAATCGTCACGAAAGCGCGCAGGCCCTTGGAAGAAACCGTTATTTGGGCATAAGGCA
G8         661 AGGAAATCGTCACGAAAGCGCGCAGGCCCTTGGAAGAAACCGTTATTTGGGCATAAGGCA
G9         658 AGGAAATCGTCACGAAAGCGCGCAGGCCCTTGGAAGAAACCGTTATTTGGGCATAAGGCA
G10        657 AGGAAATCGTCACGAAAGCGCGCAGGCCCTTGGAAGAAACCGTTATTTGGGCATAAGGCA
G12        661 AGGAAATCGTCACGAAAGCGCGCAGGCCCTTGGAAGAAACCGTTATTTGGGCATAAGGCA
consensus  661 ************************************************************

FA1090     709 GTGCCGTCTGAAAACGCAAGGATTTTCAGACGGCATTTTTTAATGCTT
G5         718 GTGCCGTCTGAAAACGCAAGGATTTTCAGACGGCATTTTTTAATGCTT
G6         715 GTGCCGTCTGAAAACGCAAGGATTTTCAGACGGCATTTTTTAATGCTT
G7         715 GTGCCGTCTGAAAACGCAAGGATTTTCAGACGGCATTTTTTAATGCTT
G8         721 GTGCCGTCTGAAAACGCAAGGATTTTCAGACGGCATTTTTTAATGCTT
G9         718 GTGCCGTCTGAAAACGCAAGGATTTTCAGACGGCATTTTTTAATGCTT
G10        717 GTGCCGTCTGAAAACGCAAGGATTTTCAGACGGCATTTTTTAATGCTT
G12        721 GTGCCGTCTGAAAACGCAAGGATTTTCAGACGGCATTTTTTAATGCTT
consensus  721 ************************************************
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Nitroreductase Activity of Poly-G Variants

After confirmation that the correct poly-guanine lengths were inserted properly into the nitroreductase gene for each Poly-G variant of FA1090, the nitroreductase activity was analyzed. If the variant does not encode a nitroreductase that has activity in reducing nitrofuran antimicrobials, it will not be useful for a reporter system. The activity of the enzyme was tested by measuring the loss of absorbance at 400 nm using a spectrophotometer. At 400 nm, a loss of absorbance in these studies is the reduction of nitrofurazone in the sample lysates.

The procedure used for the Nitroreductase Activity Assay (NAA) was developed in the lab and then further refined throughout the process of collecting data and suggestions from a paper by Whiteway et al. (1998) [**57**]. Strains of *N. gonorrhoeae* were streaked onto GCK and GCK + Nitrofurantoin (3 μg/mL) to test for nitrofuran sensitivity at the onset of the experiment. Strains were passaged directly from freezer stocks every time to limit mutation of the poly-guanine tract. Then to help minimize differences in cell culture size, the strains were suspended in 4 mL of GCP to a Klett = 100 (roughly 10^9 CFU/mL). 1 mL of the suspended culture was added to 100 mL of GCP supplemented with 1 mL of 4.2% $NaHCO_3$ and 1 mL of Kellogg's. This was incubated overnight in a shaker at 37°C. The next day, the cultures were centrifuged and washed to collect a pinkish-colored pellet. The pellet of bacteria was resuspended in Tris-HCl (pH=7.5) and sonicated to lyse the cells and release the cell contents into solution. This will release the nitroreductase encoded by that strain into the sample solution so it can be tested for activity. The samples were centrifuged further and the supernatant collected with the cell's inner contents including protein suspended in the supernatant. Each

sample was tested one at a time in a quartz cuvette. 800 μl of supernatant (cell lysate) was added to the cuvette with 90 μl of dH_2O. The cuvette was then placed in the spectrophotometer and the sample was blanked at this time to eliminate any background reading or slight differences between cell lysates. Next, nitrofurazone was added to the cuvette to a final concentration of 0.1 mM. To start the reaction, NADPH was added to the cuvette to a final concentration of 0.2 mM. The samples were mixed gently and readings of absorbance taken every 30 seconds for 10 minutes. Originally readings were taken every 20 seconds for 2 minutes, but it was observed that some of these variant strains had a reduced nitroreductase activity which was better analyzed over a ten minute assay. The controls used for each poly-guanine length were samples of FA1090 and M1. These had full activity and no activity, respectively (**Figure 3**). Also, a sample of cell lysate and only nitrofurazone was observed to maintain that NADPH was required and to show that there was no nitrofuran reduction without it. Each Poly-G variant was tested alongside a nitrofuran resistant mutant isolated from that variant strain. This mutant is expected to have little to no activity like the M1 mutant. In the figures for the nitroreductase activity, the circles (●, ○) are the nitrofuran resistant mutants; the triangles (▲, Δ) are nitrofuran sensitive strains. The filled data points (●, ▲) are for the Poly-G variant being tested and the open data points (○, Δ) are controls. All data is representative of experiments done in triplicate.

The variant *nfsB* G5 has nitroreductase activity that reduces the amount of nitrofurazone over the 10 minute sample, but the reduction is not as fast as that observed with FA1090 lysates (**Figure 11**). The absorbance for FA1090 levels off around 100 seconds in most experiments and does not appear to further reduce nitrofurazone after

Figure 11 – Nitroreductase Activity of *nfsB* G5

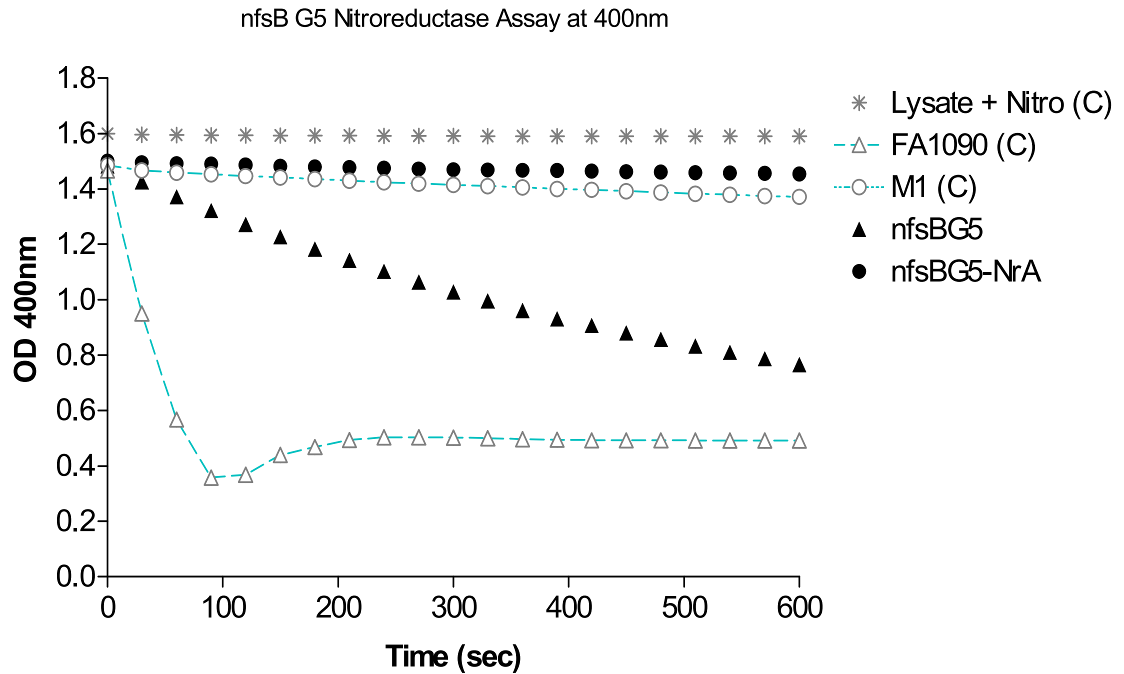


Fig. 11: The Nitroreductase activity of variant strain *nfsB* G5 was tested with the reduction of nitrofurazone at 400nm. *nfsB* G5 (▲, closed triangles) had activity in reducing nitrofurans but not as much as the wild-type FA1090 (Δ, open triangles). A Nit^R mutant of *nfsB* G5, called *nfsB* G5-NrA (●, closed circles) showed no activity just like M1 (○, open circles). No activity is observed without the co-factor NADPH (*, stars). The data is from experiments done in triplicate, errors bars calculated from Standard Error of the Means (SEM).

that, which could represent the complete conversion of the nitrofurazone or the assay mix runs out of NADPH. The reduction by FA1090 is faster than the Poly-G variants but *nfsB* G5 possesses distinguishable activity from both M1 and its Nit^R-counterpart. Like *nfsB* G5, *nfsB* G6 has activity distinguishable from M1 and its Nit^R-mutant but the activity falls somewhere between the mutants and the sensitive control (**Figure 12**). Variant *nfsB* G7 possessed nitroreductase activity that mimicked the wild-type FA1090 (**Figure 13**). The activity was the best of all the variants and was closest to the wild-type strain before adding poly-guanine tracts, while the mutant of *nfsB* G7 did not possess any activity. Variant *nfsB* G8 possessed reduced nitroreductase activity but was still discernable from both M1 and the *nfsB* G8 mutant (**Figure 14**). *nfsB* G8 reduces nitrofurazone but at a much slower rate than *nfsB* G7, *nfsB* G5, and *nfsB* G6. Poly-G variant *nfsB* G9 possessed activity relatively similar to *nfsB* G5 and *nfsB* G6, while reducing the nitrofurans slower than the wild-type FA1090 (**Figure 15**). Variants with poly-guanine tracts of lengths 5, 6, and 9 may eventually reach an absorbance level as low as FA1090 given more time. It appears that these variants are slower in reducing nitrofurans than the wild-type FA0190, but still possess activity. Variants *nfsB* G10 (**Figure 16**) and *nfsB* G12 (**Figure 17**) both have very little nitroreductase activity and the drop in absorbance observed is barely distinguishable from the Nit^R mutants. Both of these variants appear to have lost most of their activity by the insertion of these longer poly-guanine tracts or the long tracts creates a higher frequency of nitrofuran resistant mutants and the activity observed is diluted.

Figure 12 – Nitroreductase Activity of *nfsB* G6

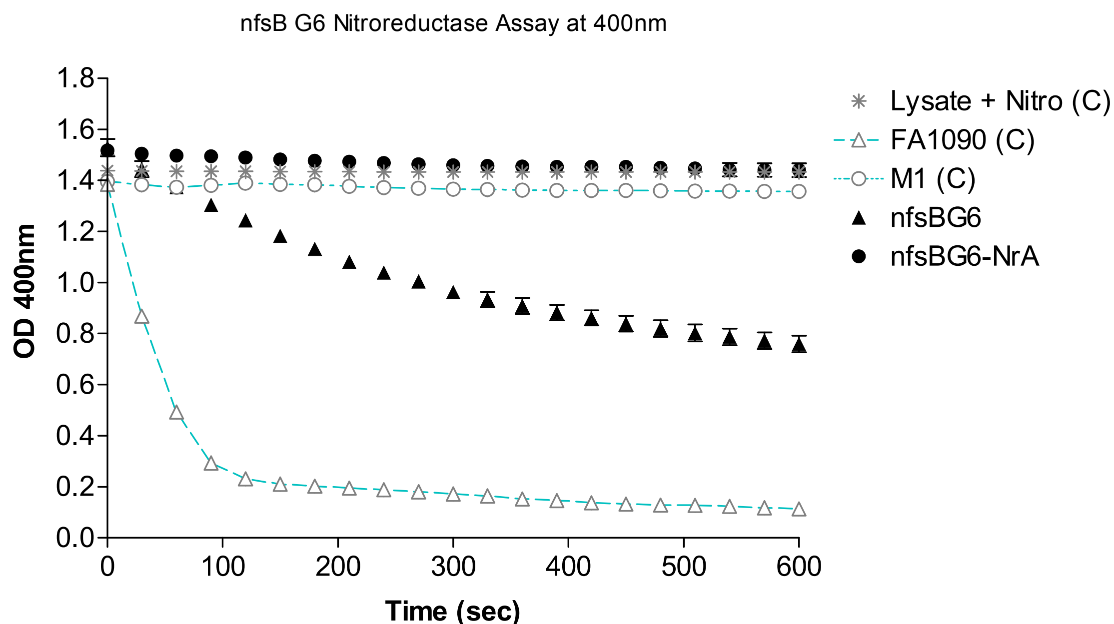


Fig. 12: The Nitroreductase activity of variant strain *nfsB* G6 was tested with the reduction of nitrofurazone at 400nm. *nfsB* G6 (▲, closed triangles) had activity in reducing nitrofurans but not as much as the wild-type FA1090 (Δ, open triangles). A Nit^{R} mutant of *nfsB* G6, called *nfsB* G6-NrA (●, closed circles) showed no activity just like M1 (○, open circles). No activity is observed without the co-factor NADPH (*, stars). The data is from experiments done in triplicate, errors bars calculated from Standard Error of the Means (SEM).

Figure 13 – Nitroreductase Activity of *nfsB* G7

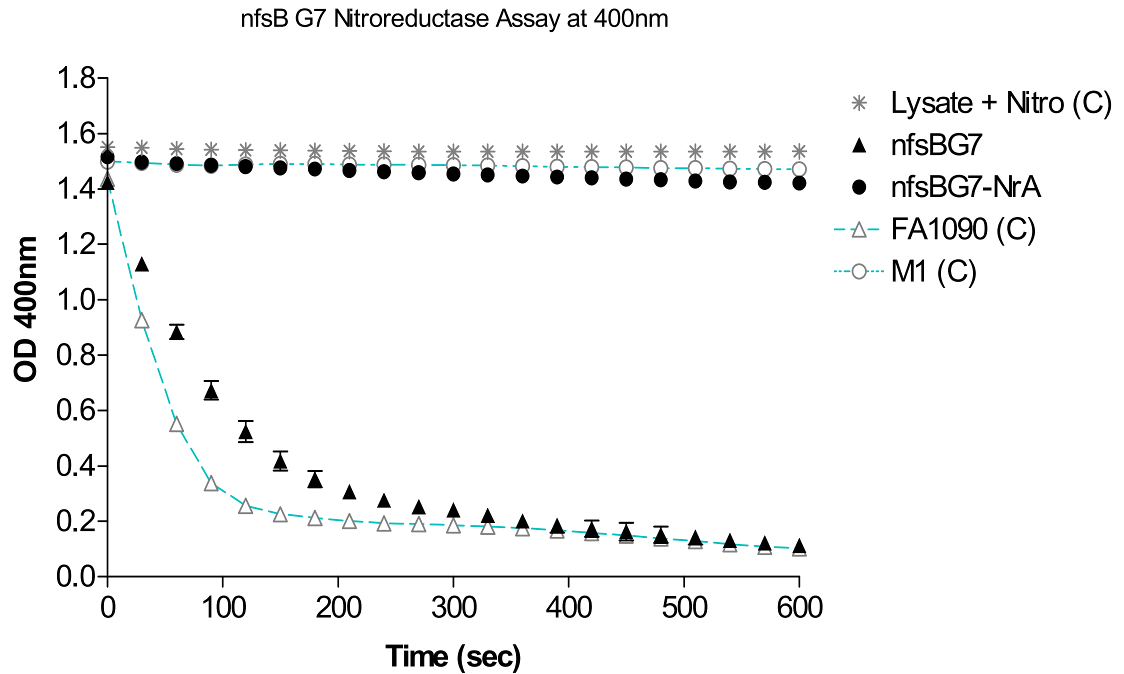


Fig. 13: The Nitroreductase activity of variant strain *nfsB* G7 was tested with the reduction of nitrofurazone at 400nm. *nfsB* G7 (▲, closed triangles) had activity in reducing nitrofurans close to the wild-type FA1090 (Δ, open triangles). A Nit^R mutant of *nfsB* G7, called *nfsB* G7-NrA (●, closed circles) showed no activity just like M1 (○, open circles). No activity is observed without the co-factor NADPH (*, stars). The data is from experiments done in triplicate, errors bars calculated from Standard Error of the Means (SEM).

Figure 14 – Nitroreductase Activity of *nfsB* G8

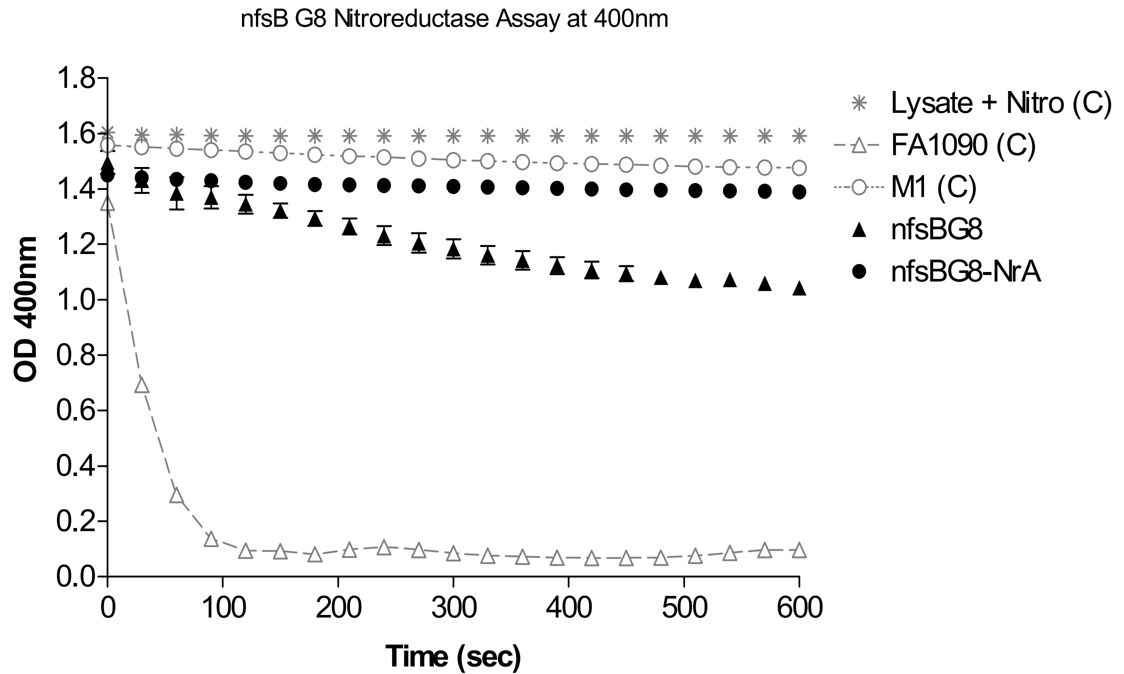


Fig. 14: The Nitroreductase activity of variant strain *nfsB* G8 was tested with the reduction of nitrofurazone at 400nm. *nfsB* G8 (▲, closed triangles) had activity in reducing nitrofurans but small activity when compared to the wild-type FA1090 (Δ, open triangles). A Nit^R mutant of *nfsB* G8, called *nfsB* G8-NrA (●, closed circles) showed no activity just like M1 (○, open circles). No activity is observed without the co-factor NADPH (*, stars). The data is from experiments done in triplicate, errors bars calculated from Standard Error of the Means (SEM).

Figure 15 – Nitroreductase Activity of *nfsB* G9

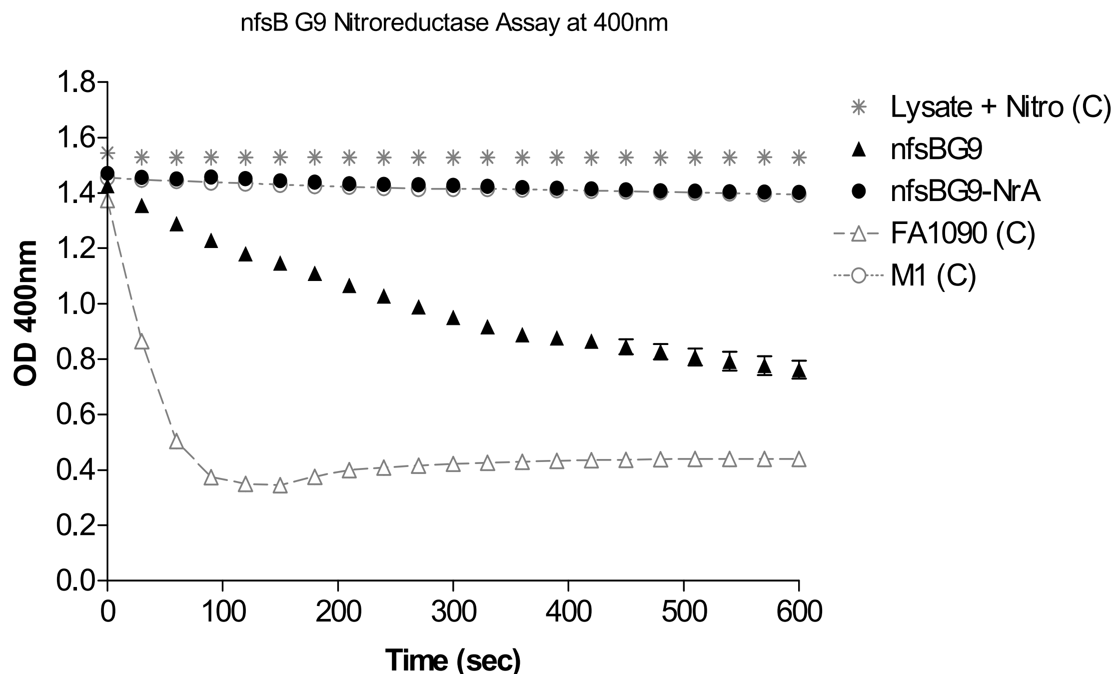


Fig. 15: The Nitroreductase activity of variant strain *nfsB* G9 was tested with the reduction of nitrofurazone at 400nm. *nfsB* G9 (▲, closed triangles) had activity in reducing nitrofurans but not as much as the wild-type FA1090 (Δ, open triangles). A Nit^R mutant of *nfsB* G9, called *nfsB* G9-NrA (●, closed circles) showed no activity just like M1 (○, open circles). No activity is observed without the co-factor NADPH (*, stars). The data is from experiments done in triplicate, errors bars calculated from Standard Error of the Means (SEM).

Figure 16 – Nitroreductase Activity of *nfsB* G10

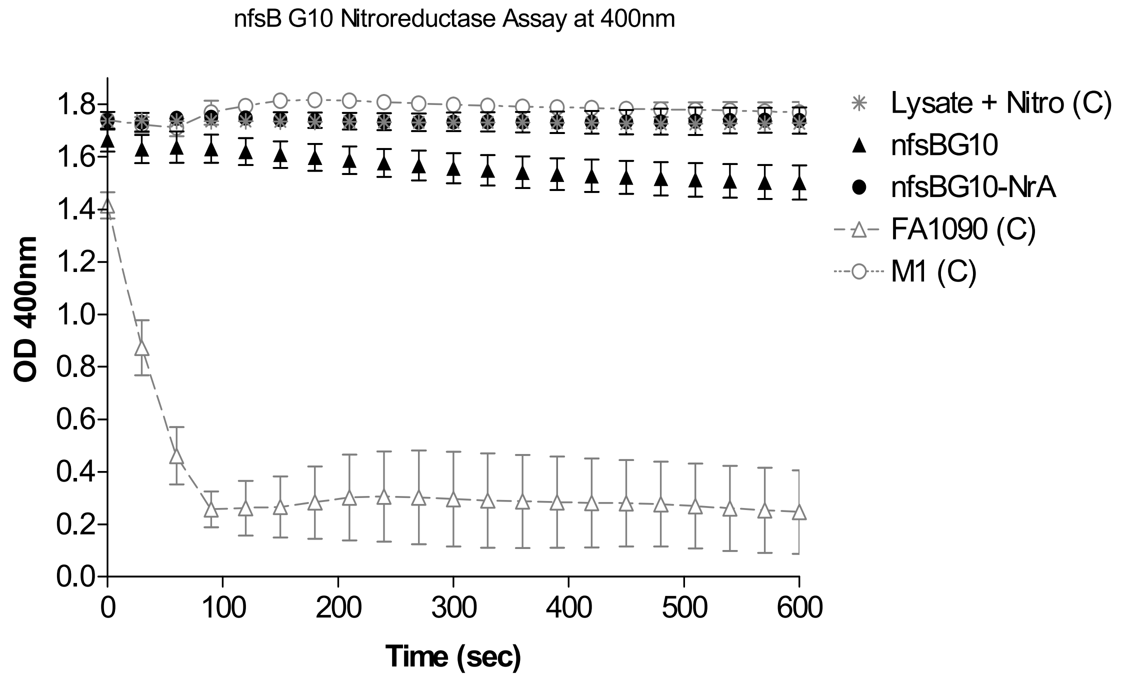


Fig. 16: The Nitroreductase activity of variant strain *nfsB* G10 was tested with the reduction of nitrofurazone at 400nm. *nfsB* G10 (▲, closed triangles) had activity in reducing nitrofurans but small activity when compared to the wild-type FA1090 (Δ, open triangles). A Nit^R mutant of *nfsB* G10, called *nfsB* G10-NrA (●, closed circles) showed no activity just like M1 (○, open circles). No activity is observed without the co-factor NADPH (*, stars). The data is from experiments done in triplicate, errors bars calculated from Standard Error of the Means (SEM).

Figure 17 – Nitroreductase Activity of *nfsB* G12

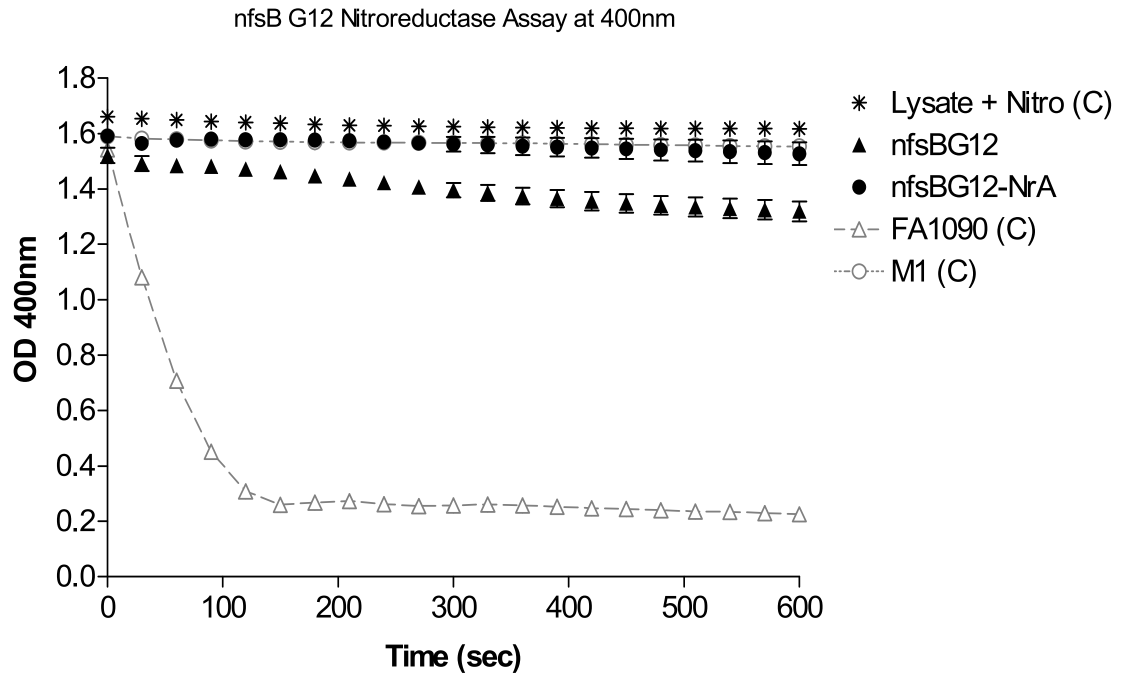


Fig. 17: The Nitroreductase activity of variant strain *nfsB* G12 was tested with the reduction of nitrofurazone at 400nm. *nfsB* G12 (▲, closed triangles) had activity in reducing nitrofurans but small activity when compared to the wild-type FA1090 (Δ, open triangles). A Nit^R mutant of *nfsB* G12, called *nfsB* G12-NrA (●, closed circles) showed no activity just like M1 (○, open circles). No activity is observed without the co-factor NADPH (*, stars). The data is from experiments done in triplicate, errors bars calculated from Standard Error of the Means (SEM).

Nitroreductase activity of *nfsB* G7 resembles FA1090, while *nfsB* G5, *nfsB* G6, and *nfsB* G9 are less than *nfsB* G7. *nfsB* G8 activity is less than those variants, while more than both *nfsB* G10 and *nfsB* G12.

The differences observed in nitroreductase activities among the poly-G variants may have been due to the amount of protein found in each sample, which can be impossible to control for cells lysed by sonication given currently available experimental tools. In the future, antibodies may be available for NfsB and the protein and be properly purified and quantified. Furthermore, it is possible that in the process of releasing proteins by sonication, some of the protein may be irreversibly denatured. If a sample contained more bacteria, the cell lysate would contain more protein and more expressed nitroreductase. The Poly-G variants were tested together striving to keep the conditions equivalent. Bio-Rad protein assay was used to determine the protein concentration in the cell lysates, and then the lysates were normalized for concentration of protein. This assay will determine general amounts of protein to help show that variation observed in previous NAA are not due to large differences in sample size or conditions, such as a change in CFU/mL. This assay will not tell us anything about the specific expression level of nitroreductase in each variant, but can rule out differences in sample size or environmental conditions. The normalized cell lysates were tested using NAA protocol and activity observed (**Figure 18**). The activities of the variants were similar to those observed in the individual assays.

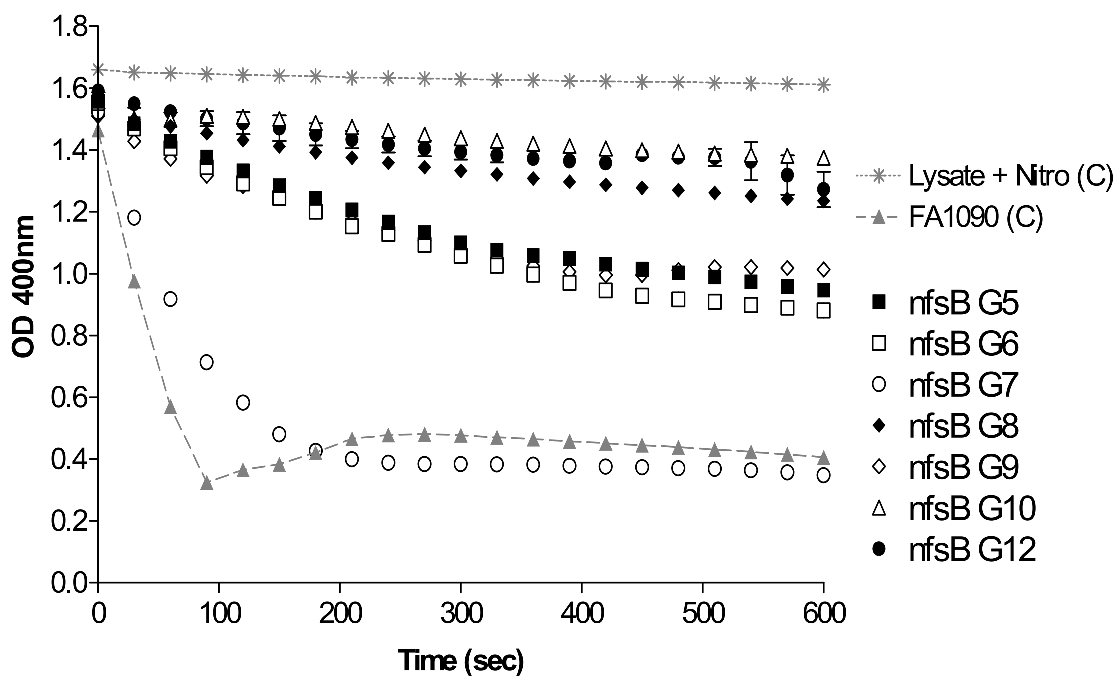
Spontaneous Mutation Frequency of Poly-G Variants

It was important to know the spontaneous mutation frequency of the nitroreductase to a nitrofuran resistant state after addition of poly-guanine tracts. It was

Fig. 18: The nitroreductase activities of all the Poly-G variants were tested simultaneously while the protein concentration was normalized to the wild-type FA1090. The experiment hoped to rule out environmental factors and difference in bacterial concentration. Protein concentration was determined using BSA protein concentration assay. The table shows mean protein concentrations and the fold differential from the wild-type detected in each sample. The samples were normalized to FA1090 protein concentration, so all samples would have the same concentration of protein present. Activities are shown by a decrease in absorbance at 400 nm, meaning nitrofurans are being reduced. Data is shown in the graph for decrease in absorbance over time at 400 nm.

Figure 18 – Nitroreductase Activity of Poly-G Variants with Protein Normalization

Strain	Total Protein (mg/mL)	Fold differential from FA1090
FA1090	2.57	**1.0**
nfsB G5	3.17	**1.2**
nfsB G6	3.45	**1.3**
nfsB G7	4.29	**1.7**
nfsB G8	3.78	**1.5**
nfsB G9	4.98	**1.9**
nfsB G10	3.07	**1.2**
nfsB G12	6.01	**2.3**



hypothesized that more mutants would be observed as the length of guanines increased due to slipped-strand mispairing during replication. The function of the reporter system relies on knowing the spontaneous mutation frequency to our selectable agent before work can begin. The mutation frequency was determined by exposing the strains to nitrofurantoin and measuring the survival of mutants that are now Nit^R. Unmutated bacteria will die as a result of reducing nitrofurantoin. A suspension of bacteria (~10^9 CFU/mL or Klett = 100) was serially diluted and plated on selective and non-selective GCK plates. The most concentrated serial dilution samples were plated onto GCK + nitrofurantoin (3 μg/mL). The least concentrated dilutions were plated onto GCK. After 48 hours of incubation, colonies were counted. Colonies on the plates with nitrofurantoin were used to calculate mutants/mL and colonies on GCK were used to calculate original CFU/mL of the sample. It was possible to determine mutation frequency using these concentrations (**Figure 19**).

It was thought that the largest increase in mutation frequency would be seen where poly-guanine lengths reached 10, this length seems to be associated with efficient LOS phase variation. However, it appears that the rate increases beginning with lengths around 5, 6, and 7. The minimum length of poly-guanine tracts that is important for increased slipped-strand mispairing is shorter than first hypothesized. The mutation frequency increases 5 to 10 fold from FA1090 (~1.28×10^{-7}) to *nfsB* G5 (~6.24×10^{-7}) to *nfsB* G6 (~1.22×10^{-5}) and to *nfsB* G7 (~5.18×10^{-4}). The increase in mutation frequency levels off in poly-guanine lengths over 8 between 10^{-4} and 10^{-5}. The mutation frequencies of *nfsB* G8, *nfsB* G9, *nfsB* G10, and *nfsB* G12 observed were ~4.36×10^{-5}, ~6.32×10^{-5}, ~2.27×10^{-4}, and ~4.85×10^{-4}, respectively. Spontaneous mutation frequencies

Figure 19 – Spontaneous Mutation Frequency of Poly-G Variants

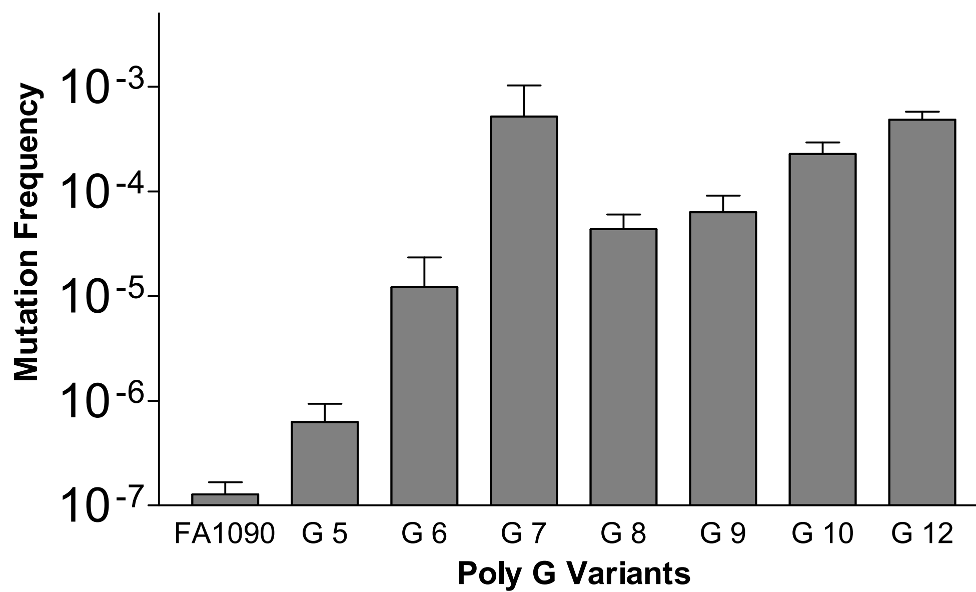


Fig. 19: The spontaneous mutation frequencies of the strains were tested as the frequency of mutation from a Nit^S phenotype to a Nit^R phenotype in the presence of 3 μg/mL nitrofurantoin. The longer the length of poly-guanines in the nitroreductase the more likely the strain is to obtain a mutation conferring resistance. Each data set is from at least 3 experiments. The actual values are listed in **Table 7.** Error bars were calculated using Standard Error of the Means.

Table 7 – Mutation Frequencies for Poly-Guanine Strains

N. gonorrhoeae strain	Spontaneous Mutation Frequency
FA1090	$1.28x10^{-7} \pm 3.94x10^{-8}$
nfsB G5	$6.24x10^{-7} \pm 3.14x10^{-7}$
nfsB G6	$1.22x10^{-5} \pm 1.11x10^{-5}$
nfsB G7	$5.18x10^{-4} \pm 5.08x10^{-4}$
nfsB G8	$4.36x10^{-5} \pm 1.65x10^{-5}$
nfsB G9	$6.32x10^{-5} \pm 2.81x10^{-5}$
nfsB G10	$2.27x10^{-4} \pm 6.65x10^{-5}$
nfsB G12	$4.85x10^{-4} \pm 9.06x10^{-5}$

were calculated from data including at least three separate experiments for every poly-G strain. **Table 7** contains the frequencies and **Figure 19** is a bar-graph representation of the increase in mutation frequency due to poly-guanine tract length increase. The data suggest there is a relationship between poly-guanine tract length and increased mutation frequency. Lengths as short as 5 consecutive guanines can cause increased mutation.

Detection of Frame-shifting by Denaturing Gradient Gel Electrophoresis

The next step in the project was to determine the genetic basis for the phenotype switch that is observed in the Poly-G strains. Many Nit^R mutants were obtained and isolated from calculating spontaneous mutation frequency. It was hypothesized that many contain mutations in the poly-guanine tract with an addition or deletion of a nucleotide that would cause a frame-shift. This could prevent expression of a functional nitroreductase. Other mutant phenotypes may be due to mutations in different areas of the *nfsB* gene. A method was sought that could be used to easily detect frame-shifting insertions or deletions prompting a Nit^R phenotype. The general approach was to amplify the *nfsB* region from the Nit^R mutants and their parent cells and compare amplicon fragment sizes. The expectation was that fragments from Nit^R mutants would be 1 to 2 base pairs larger or smaller due to extra guanine insertion or deletion viewed by electrophoresis.

To accomplish the best resolution between single base pair differences, samples would be run on poly-acrylamide gels and a GC-clamp would be attached [**71**]. The GC-clamp would be added using select PCR primers. An area approximately 150 bp would be amplified that includes the poly-guanine tract. The primers were specifically designed to add a 40-mer of guanines and cytosines to one end of the PCR product. The GC-clamp

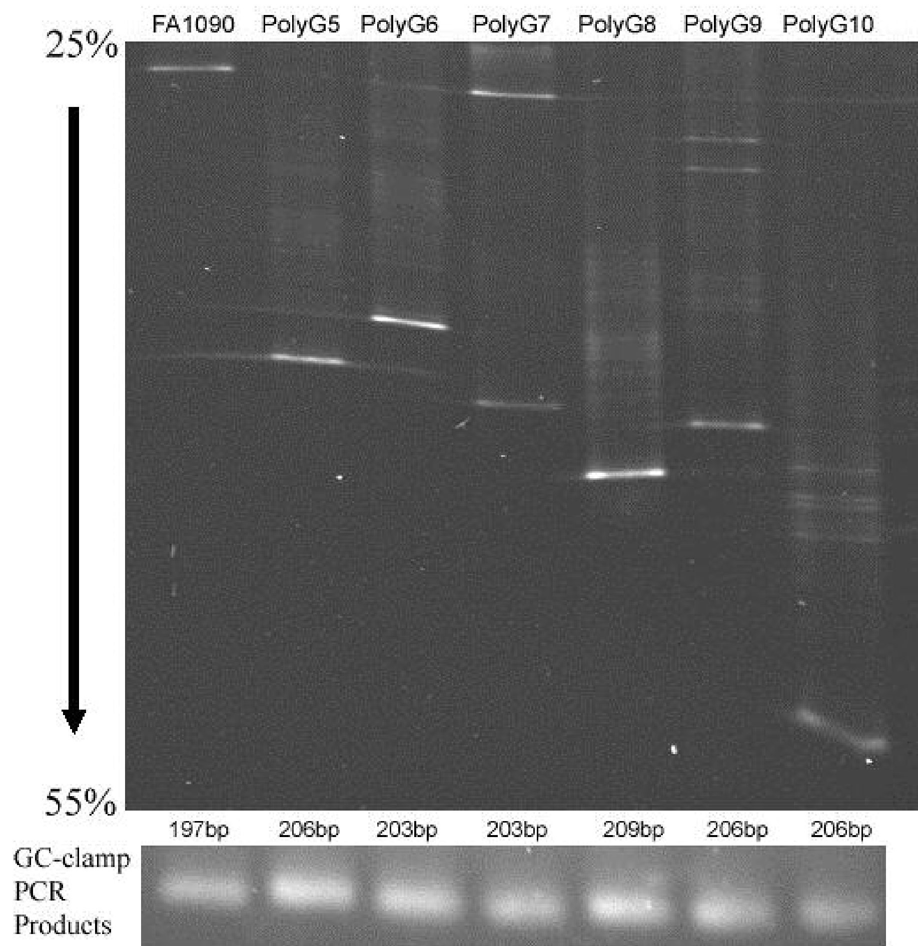
is added by primer nfsBG-DGGE-F (gc) (**Table 3**) to the 5' end in respect to the *nfsB* gene. The other primer, nfsBG-DGGE-R (**Table 3**), positions the poly-guanine tract near the 3' end of the PCR product in respect to the gene. This places the GC-clamp and the poly-guanine tract at opposite ends of the small ~200 bp fragment. A procedure known as denaturing gradient gel electrophoresis (DGGE) combines the use of the GC-clamp with a linear gradient of urea and formamide created when pouring the poly-acrylamide gel. Urea and formamide when combined with high temperature (60°C) form a linear denaturing gradient that increases from top to bottom in the gel. In most cases the top of the gel, near the loading chambers for samples is 0% denaturant (no urea or formamide), and the bottom of the gel is 100% denaturant. 100% denaturant solution contains 42% urea and 40% formamide. DNA samples run in this gradient will denature from double-stranded to single-stranded will traversing down the gel. The amount of denaturant that is required to denature a given piece of DNA is dependant on the nucleotide make-up of a DNA sample. For example, higher concentration of denaturants is needed for DNA with a higher T_m value. Long stretches of guanines and/or cytosines have a higher T_m value and will take a higher concentration of urea and formamide to denature. Therefore, a DNA sample will denature at a certain point in the gel when it reaches the minimal concentration of denaturant required in the linear gradient. The GC-clamp was designed so that it will not be denatured. The other nucleotides in the PCR products used here denature but the GC-clamp remains double-stranded, so the DNA acts like a long single-stranded piece of DNA in the gel. Because it acts like a long-single-stranded piece of DNA it will run slower through the gel. Samples with a poly-guanine tract will not denature as quickly in the gel. In these samples, both ends of the PCR product will

remain double-stranded longer and therefore travel farther in the gel. The more guanines present in the tract, the harder to denature, so the farther a fragment will run in the gel before behaving as a long single-stranded DNA. For example, DNA with a tract of 10 guanines will run farther in the gel than DNA with a tract of 5 guanines. Both of these fragments would run farther than DNA without a poly-guanine tract (**Figure 20**).

The Poly G variants and FA1090 DNA were amplified using the specified primers and the products were run on a DGGE gel. The sizes of the fragments vary due to added guanines and other nucleotides added to keep some guanine tracts in frame. The sequences are shown in **Figure 10** and the sizes are as follows: FA1090 – 197 bp, *nfsB* G5 – 206 bp, *nfsB* G6 – 203 bp, *nfsB* G7 – 203 bp, *nfsB* G8 – 209 bp, *nfsB* G9 – 206 bp, *nfsB* G10 – 206 bp, *nfsB* G12 – 209 bp. The poly-acrylamide gel was poured to create a gradient ranging from 0% denaturant solution at the top of the gel to 100% denaturant solution (42% urea, 40% formamide) at the bottom of the gel. After a minimum of 2 hours of polymerization, a comb was inserted and a 0.7% agarose stacking gel was poured. After an hour of solidification, the comb was removed and lanes washed with 1xTBE. Samples were loaded into the lanes of the gel while the gel was submerged in 1xTBE in a buffer chamber. The TBE was kept at a constant 60 degrees Celsius. The gel was run at 70 volts for 15 hours. The gel was stained with ethidium bromide and subsequently washed with dH_2O, then viewed under UV light. **Figure 20** illustrates a section of that gel containing FA1090 and Poly G variant samples. The percentage of gradient viewed in the gel was calculated based on a linear gradient from 0 to 100% throughout the gel. Here the denaturant solution is ~25% at the top and ~55% at the

Fig. 20: A small region of the *nfsB* gene was amplified using PCR with primers that added a GC-clamp. Those PCR products are shown in the bottom panel taken from a 1% agarose gel. The exact base pair size of each PCR product is labeled above each lane. The DNA samples were separated using denaturing gradient gel electrophoresis. The gradient was poured to achieve a 0% to 100% denaturant gradient. The top panel represents a small portion of that gel and the percentage of denaturant was calculated based on a linear gradient. The addition of guanines in the tract increases the T_m value; therefore the product travels further before it encounters a concentration of denaturant that creates single-stranded DNA. This can be observed in the change from FA1090 (no G-tract) to G6 then G7 then G8 and G10. Poly G9 may have secondary structure that causes complications in denaturation, therefore observations for these samples is not as expected. Poly G5 has additional base pairs to keep the gene in frame and these extra base pairs are also guanines. This may account for the unexpected finding observed with Poly G5.

Figure 20 – DGGE of Poly G Variants



bottom. The noticeable shifts in the bands can be contrasted to the PCR products viewed in a 1% agarose gel shown in the bottom panel where the DNA is aligned. FA1090 DNA traveled the least in the gel because it contained no poly-guanine tract to impede denaturation. A contrast of the distance traveled in the gel is observed in G6, G7, and G8 DNA corresponding to the extra guanine impeding denaturation. *nfsB* G10 DNA traveled the farthest in the gel as expected with a long guanine tract. Light extra bands can be observed in some lanes that are most likely intermediates of denaturation. DNA of *nfsB* G5 did not denature or travel as expected. However, the sequence of *nfsB* G5 contains extra guanines added to keep the gene in frame. These are separated from the 5 guanine tract by a thymine. Therefore, G5 DNA is larger in size (206bp) and contains 3 extra guanines that will hinder denaturation more than expected with the 5 guanine tract alone. Thus *nfsB* G5 travels further than the 6 guanine tract and what is observed is most reliable. The DNA of *nfsB* G9 is observed traveling slower in the gel than G8 but slightly faster than G7. This DNA may contain secondary structure that further complicates the denaturing process. Also, *nfsB* G8 may travel farther because like G5, it contains extra guanines before a thymine to maintain the open reading frame. With DGGE and these samples, it looks as if the main target for study may be in lengths of 6, 7, and 8.

It was hoped that the genetic basis for mutation in *nfsB* G7 mutants could be detected as single nucleotide deletions or insertions in the guanine tracts using *nfsB* G6, *nfsB* G7, and *nfsB* G8 as controls for DGGE. However, surprising results were observed for numerous *nfsB* G7 Nit^R mutants tested with DGGE. None of the mutants tested contained frame-shifting mutations in the guanine tract (data not shown). Every DNA sample from the mutants contained the tract of 7 guanines because they traveled the same

distance through the gel as the *nfsB* G7 control DNA. Even though the hope was to observe frame-shifting mutations in *nfsB* G7 Nit^R mutants, the genetic basis for the mutant phenotype of the samples studied was not due to addition or deletion of guanines within the tract as expected.

Analysis of *nfsB* G8 Nit^R mutants used DGGE and the gels were analyzed for a frame-shifting mutation. **Figure 21** illustrates one gel containing frame-shifting mutations resulting in altered migration/mobility in the gel. DNAs from control samples of *nfsB* G7, *nfsB* G8, *nfsB* G9, and *nfsB* G10 were used as controls to define the expectations of denaturation in the gel. Mutants isolated were picked from plates grown on GCK and freezer stocks were made. These mutant isolates were arbitrarily named using G8 then the date found (month then day) and a number to identify, such as G8 1112-1. G8 mutants numbered 1112-1 to 1112-8 and 420-10 to 420-11 are viewed in this figure and the numbers above the lane signify the final number in the name designation. This gel was poured with a gradient that ranged from 25% to 75% denaturant solution and the portion of the gel viewed contains roughly 30% to 45% denaturing gradient calculated based on a linear gradient. Mutants G8 1112-1 and G8 1112-6 traveled in the gel similar to the G8 control so must still contain 8 guanines in the poly-guanine tract and much like the *nfsB* G7 mutants the mutations lies outside the area of detection. Mutant G8 1112-7 and mutant G8 420-11 contained a deletion of a guanine since they shifted to travel in the gel like *nfsB* G7. The other mutants in this gel G8 1112-2 thru -5 and G8 420-8 thru -10 contained a shift that is unidentifiable. The DNA traveled farther than *nfsB* G8 DNA but not as far as *nfsB* G10 DNA. These may be a true representation of a

Figure 21 – DGGE Detection of *nfsB* G8 Mutants

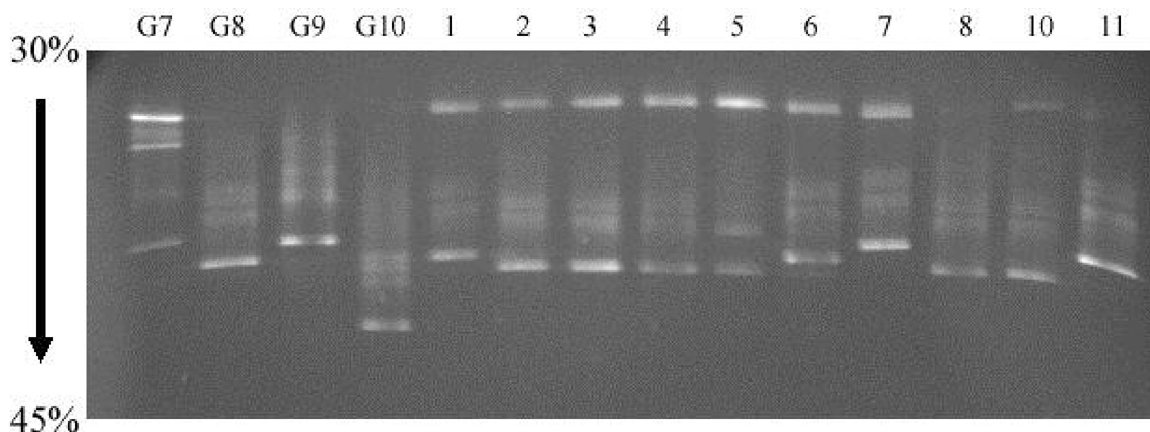


Fig. 21: Denaturing gradient gel electrophoresis (DGGE) was used to detect single nucleotide additions or deletions due to frame-shifting mutations. This will determine whether frame-shifting mutations are the genetic basis for the Nit^R phenotype acquired in these *nfsB* G8 mutants. In this case a gradient was poured to achieve a 25% to 75% of denaturant gradient throughout the gel. The figure shows a portion of that gel with the percentages of denaturant calculated based on a linear gradient. G7, G8, G9, and G10 serve as controls for unmutated Poly-G strains with select lengths of guanines in the tract. Lanes 1-11 are *nfsB* G8 nitrofuran resistant mutants arbitrarily numbered from the point of isolation. Mutants #1 and #6 contain 8 guanines and the genetic basis for their mutant phenotype must not be due to frame-shifting. Mutants #7 and #11 have shifted in the gel and resemble a guanine tract of length 7; therefore these mutations were caused by the deletion of a base. Mutants #2-5 and #8, #10 are shifted slightly from both G8 and mutants #1 and #6 and appear to be true poly-G9 tract shifts.

poly-guanine tract with 9 guanines without the secondary structure. This means that these mutants were created by the addition of a single guanine in the tract. Many of these samples of *nfsB* G8 were mutated from a Nit^S phenotype to a Nit^R phenotype as a consequence of frame-shifting. In the small sampling of *nfsB* G8 mutants including the ones shown here and others not shown (40 samples), about two-thirds (66.67%) of the mutants possessed an additional guanine (G9) while about one-sixth (16.67%) possessed a deletion (G7) and the other one-sixth (16.67%) the numbers of guanines in the tract were unchanged (G8). It appears that by using DGGE, frame-shifting mutations in the poly-guanine tract of the reporter system can be detected by observing band-shifts because of the addition or deletion of a guanine.

DNA sequencing of the *nfsB* region amplified by the GC-clamp primers for the Nit^R mutants verified the results in the DGGE gel. The DNA sequence of the ~200 bp product was analyzed to determine the number of guanines in the tract, but this only analyzed the small region near the 5' end of the gene and does not give any information about the rest of the coding region of the gene. Mutants G8 1112-1 and -6 have 8 guanines, while Mutant G8 1112-7 and Mutant G8 420-11 have 7 guanines. Mutant G8 1112-2 thru -5, Mutant G8 1112-8, and Mutant G8 420-10 underwent addition mutations and possess 9 guanines. The mutants included contain the wide variety of mutations that cause a Nit^R phenotype, either addition or deletion of a nucleotide or a mutation found elsewhere in the *nfsB* gene.

Discussion

Overview

Studies of the connection between DNA repair and phase variation have been hampered by the low spontaneous mutation frequencies to most agents that could be used to select for mutants in DNA repair pathways. Also, there is no method of selection that has proved useful for measuring all types of phase variation prevalent in the gonococcus. A working knowledge of phase variation has also been hampered by the difficulty in observing variation in genes. Many examples of phase variation are known in *N. gonorrhoeae* but the exact way that phase variation occurs is not clear. The goal of this work was to create a reporter system that could allow for easy observable measurement by using a positive selection. The selection process for growth versus non-growth in the presence of nitrofuran antimicrobials allowed for easy detection of gene expression. A nitroreductase gene, *nfsB*, which was not phase variable, was modified by the insertion of the poly-guanine tract within its coding sequence. Strains were created in this project that varied in the length of poly-guanine tract, and these strains were used to determine the effect of poly-G tract length on phase variability.

The results of the spontaneous mutation frequency plating experiments and the subsequent genetic analysis showed that nitrofurantoin resistance is a potential target for analyzing mutation in the gonococcus that is relevant to phase variation because the gene had a low inherent spontaneous mutation frequency. Mutations occurred at a higher rate in the strains containing poly-guanine tracts and the frequency of mutation increased as the poly-guanine tract length increased within the range of 5 to 7 guanines. The simplest explanation was that a long tract of similar nucleotides is prone to slipped-strand

mispairing during DNA replication, and DNA repair pathways in the gonococcus cannot repair these mistakes as efficiently. However, these defects in repair of these homopolymeric tracts allowed for the phase variability of genes that can be advantageous at times for the bacteria to increase pathogenicity and evade host immunity.

This mutation frequency data indicate that *N. gonorrhoeae* encodes one nitroreductase (*nfsB*) because a mutation in this gene resulted in resistance to nitrofurans and loss of nitroreductase activity. Biochemical data were obtained that supports this conclusion (**Figure 3**). Comparison of the nitroreductase activity of cell lysates of wild-type FA1090 and a Nit^R mutant M1 showed that a mutation in *nfsB* alone will result in the loss of activity. Cell lysate controls that did not contain the co-factor NADPH show that without the important co-factor present there is no nitroreductase activity. Together this indicates that NfsB was the only oxygen-insensitive nitroreductase found in the gonococcal genome and any reduction of nitrofurans observed was solely from NfsB.

Sequence alignment analysis of numerous NfsB and NfsB-like proteins from various bacteria (**Figure 2**) indicated that the gonococcal NfsB contained stretches of amino acids that are conserved and functionally important in well characterized nitroreductases. Most notably is the region of conserved amino acids near the N-terminus, which has been hypothesized to be the FMN-binding site [**57**]. Sequence alignment also led to the hypothesis that the first amino acids are not important to function because there was little conservation observed in the extreme N-terminus (**Figure 2**). This suggested that the nitroreductase gene could be manipulated by inserting a poly-guanine tract at the very beginning of the coding region.

Poly-guanine tracts of lengths 5 to 10 and 12 were added to *nfsB* in FA1090 to create 7 variant strains. These new strains allowed us to monitor the phase variability of a poly-guanine tract because of its inclusion in a gene encoding a protein whose expression can be positively selected.

Nitroreductases have been used as reporter systems for chemical mutagenesis studies in *Salmonella*. A series of plasmids was constructed containing the *E. coli* genes *nfsA* and *nfsB*. These plasmids were used to create *Salmonella typhimurium* tester strains that were used to study the mutagenicity of various nitro-substituted compounds [**73**]. Biochemical activity of the nitroreductases was determined using absorbance levels at a wavelength of 400nm much like in this project. This method could be used to characterize biochemical activity of various nitroreductases to many of these nitro-aromatic compounds. Each of these new strains created in this project was subjected to biochemical analysis. All the strains possessed a level of nitrofuran reductase activity. However, some required increased incubation times to visualize the activity, suggesting that the alterations at the amino-terminus effected enzymatic function, protein expression levels, or protein stability. All strains appeared to have enough activity to impart nitrofuran sensitivity on the gonococcus. Even though it was hypothesized that tampering with the amino acid sequence at the N-terminus would be harmless, these activities observed may be due to the glycines added to the N-terminus of the nitroreductase by the poly-guanine tract. There also could be a difference in expression level of nitroreductase among the strains. Also, because the lysate samples are not purified, other proteins may be expending the NADPH. The modified nitroreductases may reduce nitrofuran and NADPH slower, and these contaminating proteins are

exhausting the NADPH before the nitroreductase can use the co-factor. Therefore, an abundance of NADPH may need to be added to our assay mixes to obtain truer measures of nitroreductase activity. However, *nfsB* G7 possessed an activity similar to FA1090 (**Figure 13**) and was a useable strain for the reporter system.

The reporter system works because mutations in *nfsB* were obtained when wild-type nitrofuran sensitive gonococci were plated on nitrofuran-containing media and the resulting mutants lack nitroreductase activity. This creates a positive selection where our mutants of interest grow in the presence of the antimicrobial at the MIC of 3 μg/mL.

The spontaneous mutation frequency was assayed for every poly-guanine variant strain (**Figure 19**). It was hypothesized that the minimal length of guanines to observe an increase in mutation frequency would be 10, because surface proteins like LOS undergo phase variation due to poly-guanine tracts lengths of 10 or higher [**31, 32**]. However, the observations here show a minimum length of guanine tract to be smaller, around 6 or 7 guanines. Strain *nfsB* G5 had a mutation frequency roughly 10-fold higher than wild-type, then strain *nfsB* G6 was 10-fold higher than that, and finally strain *nfsB* G7 possessed a mutation frequency 10-fold higher still. The mutation frequencies of poly-guanine tracts of increased lengths plateau around 10^{-4}. The minimal length important for phase variability appeared to be shorter than expected. Strains *nfsB* G7 and *nfsB* G8 could be important in future work on phase variation using these reporter system strains.

Observations of the mutation frequency of the varying poly-G tract lengths reveal that *nfsB* G7 has the highest mutation frequency. The mutation frequency observed may be higher than any of the other poly-G strains due to the activity of the nitroreductase.

The strain with 7 guanines was the only strain to have activity similar to wild-type activity and higher activity may place more pressure for mutation to a Nit^R phenotype.

When performing studies using this reporter system, it would be important to note which mutants gained the Nit^R phenotype due to frame-shifting mutations. Denaturing gradient gel electrophoresis (DGGE) proved to be a reliable method to detect frame-shifting mutants fairly quickly. DGGE was able to detect insertion or deletions of guanines in the poly-guanine tract when a frame-shift mutation is responsible for the observed change in phenotype. Results in **Figure 21** showed that detection of nucleotide insertions or deletions in the poly-guanine tract of strain *nfsB* G8 was possible. Using *nfsB* G8 as the reporter strain, it would be possible to easily detect mutants, arising from plating assays with nitrofuran antimicrobials, which contain frame-shift mutations due to the high resolution gel when using DGGE.

Future Work

Now that a reporter system for poly-guanine tract analysis has been created, it can be used to study phase variation and the effect of the various DNA repair pathways on this mutation. This system could have implications in determining what proteins and genes act upon homopolymeric nucleotide tracts. These genes/proteins could be important or necessary for phase variation. And because of proposed mechanisms of action of these proteins, it may provide further insight into DNA repair pathways in *N. gonorrhoeae*, plus elucidate connections between DNA repair and phase variation.

Previously described was a reporter system based on the HpuA/B hemoglobin receptors [**43**]. Tn-mutagenesis defined genes/proteins important for phase variation of the hemoglobin receptor *in N. meningitidis*. This reporter system functioned like the one

created in this project because it was based on phase-variable expression of the hemoglobin receptor that was essential for growth of the bacteria. Much like these others, genetic studies using this reporter system for an output of manipulation can lead to better understanding of a pathway or mechanism. The focus of this system will be on further defining how gonococci use phase variation and a better understanding of how it is accomplished.

Knock-out mutants of proposed genes or proteins can be made and the mutation frequency of a poly-guanine strain can be assayed through plating experiments with nitrofurantoin. An observed change in mutation frequency would show that this gene/protein affects homopolymeric nucleotide tracts. If a strain such as *nfsB* G8 is to be used in such studies, the mutation frequency is already known (**Figure 19**; **Table 7**). DGGE can also be used to determine if frame-shifting mutations have occurred. Random transposon mutagenesis of the gonococcal genome as described by Kleckner et al. (1991), [**74**] can lead to discovery of genes important to phase variation through homopolymeric nucleotide tracts using the nitroreductase/poly-guanine tract reporter system from this project. These may be genes previously known to be involved in phase variation or DNA repair or genes that had not been previously connected. These genes that appear to have a connection to phase variation can be further analyzed and a more defined picture of phase variation can be determined.

A potential problem that may arise in using this reporter system requires extra vigilance when performing plating assays. The reporter system relies on the Nit^S phenotype of the strains. And with the added poly-guanine tracts in the nitroreductase, mutation frequency is increased. Therefore, these strains can acquire a Nit^R phenotype at

a considerably higher rate than is possible for strains harboring wild-type *nfsB*. When performing assays, it is important to assure that the strains are Nit^S or the mutation frequency outputs of the assay will be unreliable. This is especially imperative with the longer guanine tracts such as 10 or 12, because it can be easy to streak the strains onto GCK and pick a colony that has undergone a frame-shift mutation.

This reporter system may have limitations associated with using a poly-guanine tract. While many suspected phase variation genes do use poly-guanine tracts, others also use poly-cytosine tracts and pentameric repeats. This nitroreductase reporter system focuses on poly-guanine tracts, but any genes/proteins found to alter mutation frequency of poly-guanine tracts most likely will have the same mode of action on any type of homopolymeric tract. Mutation and phase variability of poly-guanine tracts should be equal to variability of poly-cytosine tracts, because of the dual nature of DNA replication. While one strand of the DNA would be replicating using a poly-guanine tract, the other strand is replicating using a poly-cytosine tract. Plus any knowledge gained could be useful for understanding any type of phase variation. In future work, the *nfsB* coding region could be modified to contain poly-pentameric runs to replace the poly-guanine tracts. These strains could be used to study pentameric repeats using a positive selection.

Phase variation in genes responsible for the expression of various cell surface antigens is an important aspect of the pathogenesis of *N. gonorrhoeae.* Phase variation may be important for the bacteria to attach to host cells and then make a switch to become invasive. Expression of Opa proteins is essential for attachment [**12, 13**] and the variable expression of type IV pili is important for initial attachment and a major antigenic determinant [**17**]. Frame-shifting in homopolymeric nucleotide tracts is a

common and important mechanism to accomplish phase variation which can lead to varying protein expression patterns. Surface proteins that are encoded by genes containing homopolymeric nucleotide tracts and undergo phase variation include LOS [**31**, **32**], pilC [**22**], and iron-utilization receptors FetA [**34**] and HpuAB and HmbR [**35 – 37**]. Opa-coding genes also undergo phase variation due to a similar mechanism by altering the number of pentameric repeats at the start of the genes [**14**]. The propensity for frame-shifting mutations in homopolymeric nucleotide tracts supports the theory that pathogenic gonococci use frame-shift mutations to accomplish antigenic variation. This creates more precedent for the use of a poly-guanine tract dependent reporter system for the future studies of the connection of phase variation and DNA repair. The power of the reporter system will be to allow for deeper and clearer understanding of how phase variation occurs and how the bacterium effectively uses phase variability.

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