Mutant Transformation of Pathogenic *Neisseria* Strains

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Abstract

Neisseria gonorrhoeae is a bacterium that produces ~700,000 new gonococcal diseases in the US each year. While gonorrhea is localized to the urethra in men, in women it can spread to the cervix, uterus and fallopian tubes. Lipooligosaccharide (LOS) is a surface antigen embedded in the outer membrane of all Neisseria strains that causes disease. However, with commensal Neisseria strains disease does not occur. The commensal strains, with the exception of two, all contain a third heptose attached to their core. We hypothesized that if a third heptose could be added to the LOS Beta chains of *Neisseria gonorrhoeae*, then the LOS would become less virulent. The proposed gene sequence that codes for the *HepIII* transferase (enzyme that adds heptose) was located. The DNA was extracted and run in a PCR reaction to amplify the proposed gene sequence, which was then purified. PLES2 plasmid DNA was introduced to competent DH5αMCR cells by heat shock transformation to make multiple copies. The resultant cells were plated onto LB+Ampicillin+Xgal for selectivity and restreaked for growth. As research continues the plasmid will be isolated from the *E. coli* and the purified gene will be inserted into the plasmid. After a few tests, the plasmid with the gene will be inserted into *Neisseria gonorrhoeae* to observe effects.

Introduction

Neisseria gonorrhoeae is a bacterium that causes ~700,000 new gonococcal diseases in the US each year (Center for Disease Control and Prevention [CDC], 2006), costing over one billion dollars annually (Kaiser Family Foundation, 2002). After Chlamydia, Gonorrhea is the second most notifiable sexually transmitted disease in the U.S. (CDC, 1995). If left untreated, it can cause urethritis in men, and lead to pelvic inflammatory disease in women (Fleming & Wasserheit, 1999). Gonococcal infections have also been found to increase susceptibility to and transmission of HIV infections (Rottingen, Cameron & Garnett, 2001). Although the national gonorrhea case rate (120.9 cases per 100,000 populations) has remained fairly stable over the past decade, it increased for the second consecutive year in 2006; particularly in western states. In these areas, rates of Gonorrhea among African Americans, adolescents, young adults and gay males remain high. (CDC, 2006). Due to these increased infection rates, emergence of antimicrobial – resistant *N. gonorrhoeae* strains, and the reproductive and economic implications of gonorrheal infection, the ability to prevent and control gonorrhea has become an important health issue (Eng & Butler, 1997).

Problem Statement

Antimicrobial- resistant *N. gonorrhoeae* strains are becoming a significant obstruction in the control of Gonorrhea. Ciprofloxacin-resistant and quinolone- resistant *N. gonorrhoeae* have become more prevalent in the U.S. (CDC, 2001a; CDC, 2001b), leaving only one group of antimicrobials, cephalosporins, to treat gonorrhea (CDC, 2002). Given the high possibility that *N. gonorrhoeae* strains will become cephalosporin-resistant, new measures to deal with gonorrhea are being sought. One of these new measures is the study of Lipooligosaccharide (LOS) structures. LOS is an important virulence determinant of the *Neisseriaceae* and is composed, in part, by the toxic Lipid A compound that causes disease. Although the genetic basis of LOS production in pathogenic *Neisseria* has been studied extensively, not much research has studied the genetics underlying LOS production in commensal *Neisseria*.
Purpose of Study and Research Question

This study will examine the effects of modifications to the gonococcal virulence determinant, LOS, on the pathogenicity of *Neisseria gonorrhoeae*. The use of data gathered from 16S RNA sequencing of commensal and pathogenic LOS structures, and the analysis of their phylogenetic relationships, has shown that commensal strains with the exception of two, have a third heptose attached to their core. This poses the question:

1. What roles do LOS modifications play in lessening the immune response to LOS' presence?

This study will observe the effects of transforming *N. Gonorrhoeae* with the gene that codes for the enzyme that adds the third heptose, *HepIII*. The *HepIII* gene will be extracted from *N. sicca* 4320.

Significance of Study

The successful transformation and expression of *HepIII* in *N. gonorrhoeae* has much significance. If the immune response is lessened by the addition of a third heptose to *N. gonorrhoeae,* it could provide important information towards a future gonorrheal vaccination.

Analysis and Discussion of the Literature and Presentation of Framework

Neisseria spp.

The genus *Neisseria* is known to consist of 11 similar species of bacteria. The genus was named after Albert Ludwig Sigesmund Neisser, the German Physician and Bacteriologist who first described the organism responsible for gonorrhea. The genus consists of aerobic, gram-negative, generally diplococci bacteria that are also oxidase-positive, catalase – negative, non-motile and non-endospore forming (Stein, 2006). Neisseria have grown over time with humans and are typically commensal inhabitants of the mucous membranes and upper respiratory track of mammals (Knapp, 1988a). Of the species, *N. gonorrhoeae* is the only strict human pathogen; the others show limited virulence, but can cause disease in immunocompromised hosts. Studies have shown the body can be colonized by multiple strains of the *N. sicca* and *N. subflava* group, without any side effects. Even strains of *N. meningitidis* can be found in the upper respiratory tract in dormant states. (Knapp 1988b). The actual pathogenesis of *Neisseria* is caused by a strong inflammatory response of the body to an infection, as opposed to the direct activity of bacterial toxins.

Disease

In males, the most common manifestation of gonococcal infections is urethritis. After a few days (2 to 8), discharge becomes more profuse, purulent and in some cases blood is discharged. Epididymitis is also caused by gonococcal infections, but is seen more in men younger than 35 years of age. When left untreated, it becomes very painful and increases the chances of infertility. In women, the endocervix is the most common site of infection, appearing in 80-90% of all recorded gonococcal infections, followed by the urethra (80%) and the rectum (40%). Symptoms of female gonococcal infection may include vaginal discharge, dysuria, intermenstrual bleeding, dyspareunia and mild lower abdominal pain, but many cases of infection are asymptomatic, and show no symptoms (Wong, Lutwick, Heddurshetti & Cebular, 2009). In congruence with an approaching menstrual period, the female's condition may progress to Pelvic Inflammatory Disease (PID), which affects 10-20% of women with gonorrhea. PID is a secondary disease of gonococcal infection that can also be asymptomatic or symptomatic. Patients with symptoms may experience sharp lower abdominal pain and fever. The long term effects of PID include sterility and the possibility of ectopic pregnancies, the life threatening condition in which the zygote grows outside of the uterus (Hook & Hansfield, 1999). Disseminated gonococcal infections (DGI) result from the spread of gonorrhea into the blood or joints. DGI's generally begin with bacteremia, fever, malaise, and skin lesions which eventually lead to severe septic joint disease (Hansfield, 1984)

Virulence Factors

The ability for *N. gonorrhoeae* to cause disease is due to its many different virulence factors which include, but are not limited to Type IV pili, Opa proteins and Lipooligosaccharides.

Type IV Pili

Pili are filamentous hair-like appendages on the outer membrane of *N. gonorrhoeae*, used for initial attachment of the bacteria to host cells. They are primarily assembled from multiple copies of the structural protein, pilin. The ability for pili to help *N. gonorrhoeae* avoid its hosts' immune response is due to their antigenic variation, a process by which an organism alters the composition of its pili (Hagblom et al., 1985). They are composed of two terminal regions; the N-terminal is the highly conserved region, homologous in the meningococcus and gonococcus, while antigenic variation occurs predominately in the C-terminal region (Deal et al., 1985).

Opa Proteins

Opa Proteins are integral proteins embedded in the outer membrane of *N. gonorrhoeae*. They are synthesized by the bacteria as precursors that contain signaling for inner membrane transport. A gonococcal strain can have up to 11 different *opa* genes, variably expressed by way of a process called phase variation. This process allows the gonococcus to

turn the expression of the Opa proteins on and off, independent of one another (Robertson & Meyer, 1992). Unlike *Neisserial* pili, opa proteins are not required for initial colonization of a host. However, they are associated with cellular binding on a more intimate level. After primary attachment of the bacteria to the cell via pili, Opa proteins have been shown to mediate epithelial binding (de Vries, F.P. et al., 1998).

Lipooligosaccharide

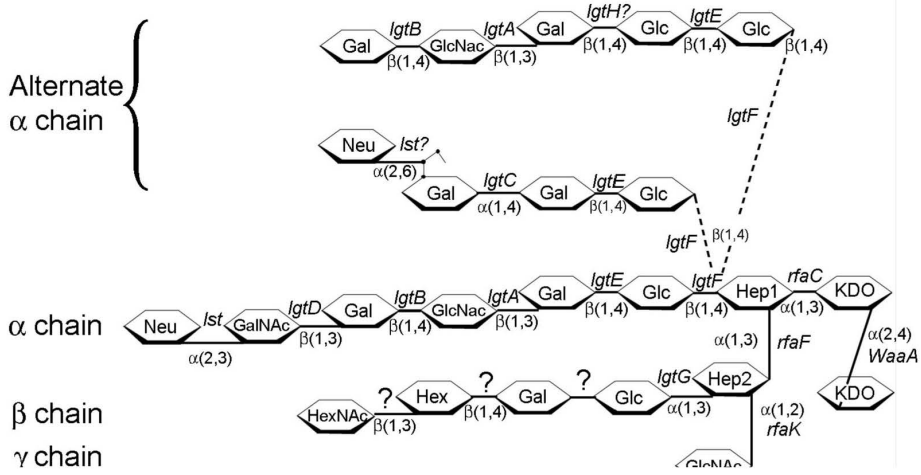
Lipooligosaccharide (LOS) is another important virulence determinant of pathogenic *Neisseria*. It is a glycolipid on the outer membrane involved in immune system evasion (Apicella et al., 1986), host cell invasion and attachment to epithelial cells (Song et al., 2000), mediation of damage to the fallopian tubes (Gregg, et al., 1981) and stimulation for the production of bactericidal antibodies. Not only does LOS allow *Neisseria* to invade the reproductive system in women and the urethra in men, but it also allows the organism to grow in the mouth, throat, eyes and anus (Stein, 2006).

LOS Physiology

Neisserial LOS's have been studied using chemical (Gibson et al., 1993; Griffiss et al., 1987), physical (Yamasaki et al., 1994), biological (Griffiss, 1994), and genetic methods (Petricoin, Danaher & Stein, 1991). They are amphipathic molecules composed of a hydrophilic carbohydrate moiety, also known as the oligosaccharide, and a hydrophobic lipid A moiety. The Lipid A portion is responsible for anchoring the oligosaccharide chains to an inner core embedded in the membrane. The inner core is a conserved portion of the LOS structure made from two KDO and two heptose residues. These residues connect the outer core of the LOS to the lipid A anchor. The genes responsible for LOS synthesis have been found in seven genomic locations (refer to figure 1). The region *kdtA* codes for the enzyme needed to add the two KDO molecules of the inner core to the lipid A moiety (Tzeng et al., 2002); *rfaC* encodes the gene whose product adds a heptose residue to KDO (Zhou, Lee & Apicella, 1994); *lgtF* and *rfaK* initiate the alpha chain and synthesize the gamma chain (Kahler et al., 1996); *rfaF* adds a second heptose to the first heptose residue (Petricoin III & Stein, 1989); *lgtABCDE* code for most of the genes responsible for alpha chain elongation (Gotschlich, 1994); *lgtG*, encodes the gene responsible for beta chain synthesis(Banerjee et al., 1998), and *lst* adds a sialic acid cap to the oligosaccharide chain (Gilbert et al., 1996).

The LOS structures in all *Neisserial* strains possess genes with a high homology to *rfaC* and *rfaF*. In addition, structural data analysis on Neisserial LOS has shown that the inner core of all commensal and pathogenic *Neisserial* strains contain two KDO molecules. Genes adjacent to the *rfaC* and *rfaF* genes, also known as flanking genes, show conservation amongst the meningococcus and gonococcus, but high divergence in commensal strains. Analysis of *Neisserial* 16S RNA sequences show that amongst the commensal strains there is a homolog of heptosyltransferase three, the enzyme that adds a third heptose to the inner core. This suggests that most commensal strains differ from pathogenic strains by the presence of a third heptose.

Figure 1: Pathogenic LOS structure



Gonococcal Vaccines

In recent years, *N. gonorrhoeae* has developed resistances to antibiotics that call for more expensive antibiotics treatments with broader spectrum activity. The treatment of gonorrhoeae with sulfonamides, penicillin's, tetracycline's and crythromycin have all proven to be ineffective with the emergence of relative or absolute multi-resistant strains (Whittington & Knapp, 1988); by 1988 about one third of all gonococcal isolates in the United States had one form of resistance or another (Schwarz et al, 1990). This led to the use of a cephalosporin or flouroquinolone for the treatment of uncomplicated gonorrhea. With the introduction of these broad spectrum antibiotics there has been relative stability in *N. gonorrhoeae*'s pattern of antimicrobial susceptibility However, theoretical concerns arise about the gonococci's potential to form resistance to the broad spectrum treatments. Resistance to flouroquinolones and cephalosporins have been induced in the lab, which caused previously susceptible gonococci to become clinically resistant immediately. This possibility of acquired resistance is making it more and more necessary to develop a vaccine for controlling the disease.

Summary and Implications of Literature

Vaccinations have proven to be a very effective prevention method of disease, but development of a neisserial vaccine has be halted for a number of reasons. One of the problems is not knowing which components of the virulence determinants would elicit the protective immune response needed. In addition, neisserial virulence determinants are able to change surface antigen composition frequently due to antigenic variation, making it harder to find a component of interest. By studying various aspects of neisserial LOS over the years, its importance in pathogenesis and immunobiology has become very well understood. LOS's are a group of complex glycolipids embedded in the outer membrane of Gram- negative bacteria. They possess many different antigenic determinants that have been found to be important in acquired and natural immunity. The understanding of LOS genetic biosynthesis in commensal and pathogenic *Neisseria* has opened doors to the understanding of differences in LOS structure of the two.

Pathogenic Neisserial LOS's have been studied more extensively than commensal Neisserial LOS's have in the past. Recent studies on the commensal *Neisseria* have shown differences in the inner core structures of their LOS that could be the difference between pathogenicity and commensalism in Neisserial strains. There is an addition of a third heptose to the second heptose in commensal Neisserial LOS that is not present in gonorrhoeae strains that are able to cause disease. This finding could prove to be a pivotal discovery towards the development of a vaccine.

Presentation and Discussion of Analytic Framework

This study will observe the effects of a *Hep III* transformation on the pathogenicity of *N. gonorrhoeae*. *Hep III* is the gene that codes for the enzyme responsible for adding the third heptose to the inner core of commensal Neisseria. Upon completion of a successful transformation, the *N. gonorrhoeae* should begin to express the *Hep III* and produce a third heptose on its LOS structure. The hypothesis is that the production of this third heptose will destroy the stereochemistry that allows gonococcal LOS to be pathogenic and interact with host cells. If our hypothesis is correct, it could prove to be a major breakthrough, and a step in the direction of a gonococcal vaccine. To achieve this the gene of interest will be inserted into a plasmid vector. That plasmid vector will then be introduced to the *N. gonorrhoeae* and be taken up by the bacteria. Once it enters the bacteria it should begin to produce heptosyl transferase.

Research Design and Methodology

The genome as pertaining to Neisserial LOS has been almost completely mapped by the Stein Lab, University of Maryland, College Park. Using 16s RNA sequences of Neisserial strains, the coding sequence for the *Hep III* gene has been located and was used to order a primers that would produce our gene of interest via PCR.

Bacterial Strains, Plasmids, Primers and Restriction Enzymes. The bacterial strains, plasmids, primers and restriction enzymes used to conduct this study are all listed in Table 1.

Table 1

Bacterial Strains		
DH5α-MCR *E. coli* cells		New England Biolabs
N. sicca 4320		Dr. Herman Schneider WRAIR
N. gonorrhoeae F62		Dr. Fred Sparlug UNC, Chapel Hill
Plasmid		
pLES 2	Construction and characterization of a new shuttle vector, pLES2, capable of functioning in *Escherichia coli* and *Neisseria gonorrhoeae.*	Dr. Daniel C. Stein et al.
Primers		
NS Hep 3F	5'-CCC GGA TCC AGG AGC AAA CTA TGT CCC TGG CC -3'	This work
NS Hep 3R	5'- AAA GGA TCC GGT TTC AGA CGG CCT TTT CAT ATG GAA -3'	This work

Diluting Primers Upon receipt of the primers the stock tubes were centrifuged. The primers were then diluted with elix water at a 1:10 ratio of nanomoles of primer to microliters of elix water. Then 10 μL was taken from that stock tube and inserted in another tube. It was also diluted at a 1:10 ratio of stock primer to elix water.

Expand Long Template Polymerase Chain Reaction (ELT-PCR) Using diluted primers a mixture of 0.5 μL dNTP, 2.5 μL Buffer 1, 0.5 μL NS Hep 3F, 0.5 μL NS Hep 3R, 21 μL elix water, 0.25 μL DNA polymerase and 1 μL of *N. sicca* 4320 DNA was made; The total was 26 μL. The mixture was run in thermocycler at 95ºC for two minutes, 41 cycles of 95ºC for 30 secs, 63ºC (annealing temp.) for 30 secs and 72ºC for six minutes over night.

Gel Electrophoresis For analysis of the PCR product, 100 mL of 1x TBE buffer was mixed with 0.8- 1.0g agarose and microwaved for 2:15 at 50-60% power. After microwave a spin bar was placed in the mixture and 10 μL of ethidium bromide was added. While cooling down the mixture was placed inside a bucket of water on a hot plate with a magnetic spin function. When the mixture was cooler, but not solid, it was poured in a gel plate and a 20 lane comb was placed inside the plate. After the agarose mixture solidified, the product was ran on the gel electrophoresis for 45 min using lambda digest as the ladder. After gel analysis showed bands around 4 kb, the size of the gene of interest, the DNA was purified using the QIAquick PCR purification system by QIAGEN and stored in the refrigerator.

E. Coli Transformation In order to produce multiple copies of the plasmid, 200 μL of DH5α-MCR cells were obtained from the -80 ºC freezer, making sure not to touch the cells, but to hold the top due to their extreme sensitivity to heat. . They were thawed on ice for 10 minutes until the liquid and solid separated. While thawing the cells, two plastic 10 mL centrifuge tubes were placed on ice so that the cells remained cold once they were transferred. A 5 μL amount of pLES2 plasmid (Stein et al., 1983) was added to one of the cold centrifuge tubes, which was followed by 100 μL of DH5α-MCR competent cells in both tubes. The samples were incubated on ice for 10 minutes and then transferred to a 37 ºC water bath for 2 minutes. After 2 minutes they were immediately transferred back to ice. Then 900 μL LB broth was added bringing the total volume to 1 mL and the tubes are incubated in the rotary shaker for 30 minutes. Upon completion of the 30 minutes, two sets of three volumes (10, 100 and 250 μL) of transformation mix were plated on separate Ampicillin+Xgal plates O/N @ 37ºC. The mixture with the plasmid served as the experimental and the mixture without it was the control.

Alkaline Lysis Plasmid DNA Miniprep Purification Using a sterile toothpick, half of the cells were scraped from the overnight culture plates and suspended in 100 μL of GTE in a microcentrifuge tube; the tube was vortexed. Fresh, ice cold lysis solution was made from 100 μL of 1% SDS solution and 100 μL 0.2 M NaOH. The 200 μL mixture of lysis was added to the vortexed tube and then inverted gently five times to mix it. The mixture was incubated on ice for five minutes, and then 150 μL of cold potassium acetate was added. The tube was inverted several times to mix the contents and then incubated on ice for 10 minutes. The tube was put in the microcentrifuge at 10,000 rpm for 10 minutes at 4°C. The supernatant was transferred to a new tube and 3 μL of RNase was added to it. The mixture was incubated for 10 minutes at room temperature, and then combined with 375 μL of phenol/chloroform/iaa. The solute was vortexed and then spun again in a centrifuge for 5 min. The top layer of the supernatant was removed into a fresh tube making sure not to collect any gunk at the top or bottom of the mix. Ethanol was added in twice the volume of the supernatant extract and incubated on ice for about 30 minutes to allow DNA precipitation. The mixture was spun again at 10,000 rpm at 4°C and the supernatant was discarded carefully, making sure not to get rid of DNA at the bottom of the tube. The tube was allowed to dry on its side until the residual ethanol evaporated, and resuspended in 30-50 μL of elix water. The sample was analyzed on a gel to make sure the plasmid was purified from the cells and the DNA was then stored at 4°C.

Digestion The purified plasmid was digested in 3 separate tubes and the purified PCR product was digested in its own tube. One of the purified plasmid tubes served as a digestion control, the other served as the ligase control, and the last tube was the experimental mixture. In the three plasmid tubes, 8 μL of plasmid DNA was inserted and in the PCR tube the same amount of PCR product was added. All four tubes contained 3 μL of Buffer 4, 3 μL of BSA 10X, 1 μL of Bam H1 enzyme and 15 μL of elix water for a total of 30 μL in each tube. The tubes were then incubated in a 37°C water bath overnight. The next day both samples were purified using the QIAquick PCR purification system by QIAGEN.

Ligation From each of the digestion products 4 μL was taken and combined in one test tube to make 8 μL of DNA total, which was labeled as the experimental. Another tube was labeled ligase control and contained 8 μL of plasmid. The digestion control was not touched. Then 2 μL of T4 DNA ligase buffer, 0.5 μL of T4 DNA ligase and 9.5 μL of Elix water were added to the ligase control and experimental for a total of 20 μL. The mixtures were incubated in 14° C (anti freeze bath) overnight, and in the morning were placed in a 65°C water bath for 10 minutes to deactivate the ligase. After ligation another *E. coli* transformation with all the controls was performed and plated on ampicillin with Xgal plates. The colonies on the experimental plate were white, meaning that the transformation of the E. coli with the plasmid containing the target gene was successful. Another Alkaline Lysis Plasmid DNA Miniprep Purification was conducted.

Transformation of N. Gonorrhoeae For every 1mL of GCP, 10 μL of 4.2% $NaHCO_3$, 1M $MgCl_2$ (filtered), and kelloggs (White &Kellogg, 1965). Enough of the mixture was made for three tubes. Then 5 μL of purified DNA (plasmid + target gene) was added to 1 mL of the media. The mixture was place in a rotary shaker at 37 °C and run for 4 hours. The mix was then plated onto GCK+ampicillin in amounts of 1μL, 10 μL, 100 μL and 250 μL.

Colony PCR A PCR was prepared with all the components except the DNA polymerase. Half of a colony was attained using a yellow pipette tip and pipetted up and down into the PCR reaction. The other half of the colony was streaked onto a new GCK+ampicillin plate. The tube was heated at 95 °C for 5 minutes, then DNA polymerase was added and PCR continued as normal. Once PCR is done, run on a gel and analyze.

Findings, Conclusions and Recommendations for Future Research

Findings

Due to the fact that this research is currently being conducted and has not been completed, there are no findings on the effect of the addition of a third heptose to gonococcal LOS. It is expected that the addition of the third heptose will decrease the pathogenicity of the *N. gonorrhoeae* in vitro. LOS structure is stereospecifically compatible with its function in causing disease in the body, and the addition of a heptose could possibly destroy LOS stereochemistry causing it to lose its functionality. However, whilst conducting the research we were able to establish phylogenetic relationships between commensal and pathogenic strains, which proved that although most *Neisserial* strains have a high homology to *rfaF* and *rfaC*, the genes surrounding them varied between the commensal strains and the gonococcus/ meningococcus. The *HepIII* gene was found in all commensal strains except *N. cinera* and *N. lactamica,* which implies

that commensal strains differ from pathogenic strains by the additions of the third heptose to the Beta chain. Genes for Synthesis of LPS were found in commensal strains, but only *N. sicca* 4320 actually produced LPS. The presence of the glycosyl transferase genes in this strain alone, suggests that horizontal gene transfer occurred; likely from a meningococcus. The KDO residues are the molecules that serve as a base to which transferases add the core and branched oligosaccharides of LOS to. All *Neisserial* strains examined had a gene that encoded for KDO transferase and all *Neisserial* strains studied possessed two KDO residues. Lastly, in pathogenic *Neisseria* the *rfaK* gene codes for an enzyme that adds N-acetylglucosamine (GlcNAc) to the beta chain to form a gamma chain. It has been found amongst test samples that only pathogenic Neisserial strains and those close to it possess the ability to synthesize the GlcNAc on a gamma chain.

Conclusions

It is evident from the analysis of the data collected that genetic acquisition/loss of genetic information as well as recombination has occurred in the Neisseria genus. The Neisseria species that appear to have limited pathogenicity all have the ability to add a third heptose to the LOS core, with the exception of *N. cinerea* and *N. lactamica*. If the transformation is done successfully, the expectation is to see the heptose added to the LOS core. Once added, the stereochemistry of the LOS would be altered, which would cause it to lose its functionality. The potential benefits of this going as expected range from further understanding of the mode of virulence in Neisserial strains to a possible vaccine.

Future Research

Once the effect of the addition of the Heptose to the core residue has been analyzed stereochemically, the next step would be to observe whether the changes affect N. gonorrhoeae's virulence. Transformed *N. gonorrhoeae* would be placed in plates containing human epithelial cells, incubated with the cells and then observed for lysis.

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