ABSTRACT

Title of Document: ROLE OF GONOCOCCAL SURFACE GLYCOCONJUGATES, THEIR DIVERSITY AND THEIR ROLE IN BACTERIA-BACTERIA INTERACTION AND BACTERIA-HOST INTERACTION.

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Doctor of Philosophy, 2015

Directed By: Dr. Daniel Stein, Professor, Cell Biology and Molecular Genetics

Bacterial interactions with each other and with host cells play a critical role in the pathogenesis of gonorrhea. Bacterial aggregation was observed to be mediated by the interaction between lipooligosaccharide (LOS) and the opacity-associated protein. In this study, I identified a gene encoding a beta-hexosaminidase (NagZ) in *Neisseria gonorrhoeae* that modulates gonococcal aggregation and biofilm formation. In comparison to the parental strain, a strain with the *nagZ* gene deleted produced a biofilm with increased mass. Scanning electron microscopy and confocal laser microscopy were able to visualize differences in the biofilms formed by the two strains. Biofilms formed by a strain deficient in *nagZ* were disrupted by addition of

exogenously added purified NagZ. This is the first study to demonstrate that an enzyme thought to be restricted to peptidoglycan recycling is able to moonlight as biofilm modulator. NagZ could play an important role in promoting bacterial escape from a biofilm, along with previously characterized agents such as Nuc thermonuclease. Using strains defective in surface protein glycosylation, I demonstrate that the increase in biofilm formation seen in *nagZ* mutant is dependent on PglC-mediated surface protein glycosylation. This is the first study demonstrating the role played by surface glycoconjugates in gonococcal biofilm formation. I used bioinformatic analysis to study the diversity of the other major glycoconjugate on gonococcal surface, LOS. I identified significant differences in the LOS core structure between commensals and pathogens within the *Neisseriaceae*. I generated preliminary data suggesting that *N. gonorrhoeae* activate inflammasomes in epithelial cells, resulting in production of IL-18. While activation of inflammasomes does not affect production of other cytokines such as IL-8, IL-8 levels were reduced by using MyD88-inhibitors. Gonococcal-induced inflammasome activation in epithelial cells and production of inflammasome-dependent cytokines were further confirmed in human cervix explants.

ROLE OF GONOCOCCAL SURFACE GLYCOCONJUGATES, THEIR DIVERSITY AND THEIR ROLE IN BACTERIA-BACTERIA INTERACTION AND BACTERIA-HOST INTERACTION.

By

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List of Abbreviations

AIM2	Absent in melanoma 2
AP-1	Activator Protein 1
ASC	Apoptosis-associated speck-like protein containing CARD
BLAST	Basic Local Alignment Search Tool
BODIPY	Boron-dipyrromethene
C3R	Complement component 3 receptor
CAMP	Cationic antimicrobial peptide
CAZY	Carbohydrate-active enzymes database
CCL20	Chemokine (C-C motif) ligand 20
CDC	Centers for Disease Control and Prevention
CEACAM	Carcinoembryonic antigen-related cell adhesion molecules
CMP	Cytidine-5'-monophosphate
CRISPR	Clustered regularly interspaced short palindromic repeats
DAMP	Danger-associated molecular pattern
DATDH	Diacetamido-2,4,6-trideoxyhexose
DGI	Disseminated gonococcal infection
E2	Estradiol
EAEC	Enteroaggregative *E. coli*
eDNA	Extracellular deoxyribonucleic acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
EPEC	Enteropathogenic *E. coli*

EPS	Extracellular polymeric substance
FIR	Fluorescence Intensity Ratio
FLICA	Fluorescent Inhibitor of Caspase 1 Assay
FMK	Fluoromethylketone
FRT	Female reproductive tract
Gal	Galactose
GalNAc	N-acetylgalactosamine
GC	*Neisseria gonorrhoeae*
GGI	Gonococcal Genomic Island
GH	Glycosylhydrolase
Glc	Glucose
GlcNAc	N-acetylglucosamine
GlcNAC	N-acetylglucosamine
GMP	Guanosine monophosphate
HBD	Human β - defensins
HD5	Human α - defensin 5
Hep	Heptose
HIV	Human Immunodeficiency Virus
HMGB1	High mobility group protein B1
I:C	Polyinosinic acid:polycytidylic acid
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin

IPTG	Isopropyl β-D-1-thiolgalactopyranoside
IRAK	Interleukin-1 receptor-associated kinase
JNK	c-Jun N-terminal kinase
KDO	2-keto-3-deoxyoctulosonic acid
LAMP1	Lysosomal-associated membrane protein 1
LBP	LPS-Binding Protein
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
MAL	Myelin and lymphocyte protein
MALT	Mucosa-associated lymphoid tissue
MAPK	Mitogen-activated protein kinase
MD2	Myeloid differentiation protein 2
MFC	Microbial Fuel Cells
MFI	Mean Fluorescence Intensity
MIC	Minimum Inhibitory Concentration
MIP-α	Macrophage inflammatory protein α
MMP	Matrix Metalloproteinases
MSCRAMM	Microbial Surface Components Recognizing Adhesive Matrix
MurNAc	N-acetylmuramic acid
MyD88	Myeloid differentiation primary response 88
NANA	N-acetyl neuraminic Acid (also known as sialic acid)
NAP	Nucleoid-Associated Proteins
NBD	Nucleotide-Binding Domain

NCBI	National Center for Biotechnology Information
NF-κB	Nuclear factor kappa-light-chain-enhancer of activator B cells
NGO	*Neisseria gonorrhoeae*
NLRC4	NLR family CARD domain-containing protein 4
NLRP3	NOD-like receptor family, pyrin domain containing 3
NOD	Nuclear oligomerization domain
OMV	Outer Membrane Vesicles
ORF	Open Reading Frame
PAGE	Polyacrylamide gel electrophoresis
PEA	Phosphoethanolamine
PI	Propidium Iodide
PID	Pelvic inflammatory disease
PNAG	Poly N-Acetyl Glucosamine
PNP	p-Nitrophenol
PRR	Pattern Recognition Receptors
PYD	Pyrin domain
RIP	Receptor-interacting protein kinase
RLR	RIG-like receptor
SDS	Sodium Dodecyl Sulfate
SEAP	Secreted Alkaline Phosphatase
SEM	Scanning electron microscopy
SLP	Secreted Leukocyte Protease Inhibitor
SP-A	Surface Protein A

SREC	Scavenger receptor expressed by endothelial cell-1
ST	Sequence Type
STAT-3	Signal transducer and activator of transcription 3
STD	Sexually Transmitted Diseases
STI	Sexually Transmitted Infection
TEER	TransEpithelial Electrical Resistance
TGF-β	Transforming Growth Factor β
Th	T-helper cell
TIR	Toll-Interleukin Receptor
TLR	Toll-Like Receptor
TNF-α	Tumor Necrosis Factor α
TRAM	TRIF-Related Adaptor Molecule
TRIF	TIR-domain-containing adapter-inducing interferon-beta
UPEC	Uropathogenic *E. coli*
WT	Wildtype

Chapter 1: Introduction

1.1 Gonorrhea, its impact and significance:

Gonorrhea, one of the oldest known diseases (1), is a sexually-transmitted infection caused by the human obligate gram-negative diplococci, *Neisseria gonorrhoeae* (gonococci, or GC). First described by Albert Neisser as the causative agent of gonorrhea (2), interest in studying the pathogen increased in the early twentieth century. The discovery of penicillin was a major step in fighting the disease, especially during the Second World War. While prevalence of disease peaked during 1970s, possibly due to a combination of factors such as increase in sexually active population, and preference of non-barrier contraceptives over barrier methods, the incidence has since then continually dropped. Recently, there has been a small uptick in disease. According to the Centers for Disease Control and Prevention (CDC), there were 334,826 cases of gonorrhea in the US in 2012, marking a 4.1% increase from 2011 (3). However, CDC also estimates that more than 800,000 people get infected with gonorrhea, with less than half of them being diagnosed and treated. There has also been a consistent and continual increase in the prevalence of cephalosporin-resistant gonorrhea, over the last two decades (4).

Gonorrhea is currently being treated with a two-drug regimen (consisting of injectable ceftriaxone along with one of the two oral drugs, doxycycline or azithromycin), following increased resistance against even third-generation cephalosporins (Figure 1.1) (3, 5). In the last decade, strains with reduced susceptibility to cephalosporins are being reported more frequently from Japan,

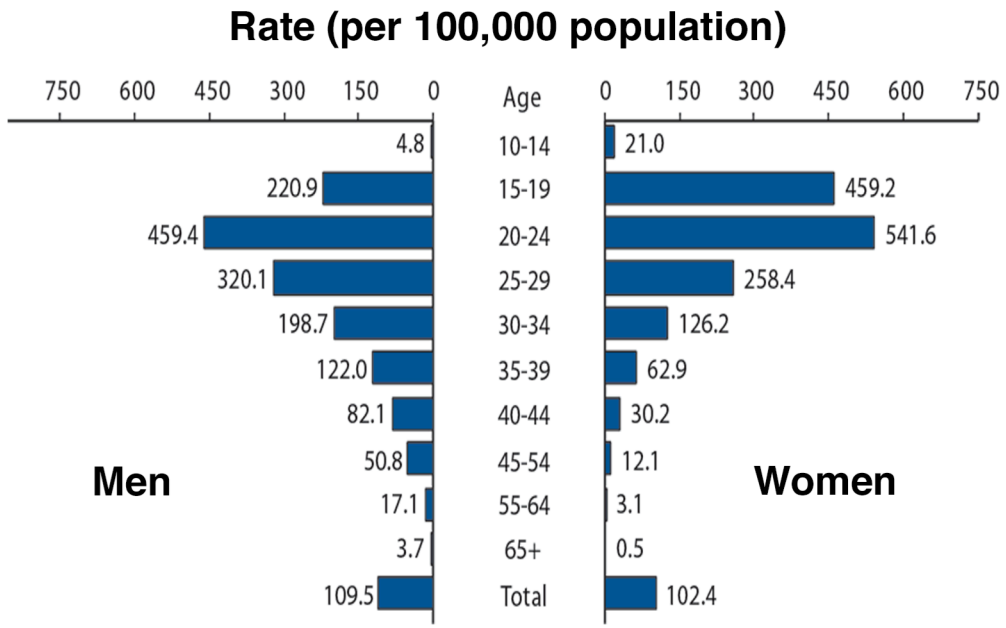

Figure 1.1 Incidence of gonorrhea. The incidence of gonorrhea in men and women, by age based on CDC data. The rate of incidence per 100,000 population is shown age group and sex. (Adapted from CDC Report on STD Surveillance 2013)

Europe, Canada and the US (5). The CDC considers the threat of ceftriaxone treatment failure to be "imminent", forcing it to increase surveillance and launch a response plan. *N. gonorrhoeae* primarily causes urethritis in men, and cervicitis in women. However, it has also been demonstrated to cause oral and rectal infections. In neonates, GC can cause conjunctivitis and dermatitis by contact transmission. Patients infected with GC have an increased risk of acquiring human immunodeficiency virus (HIV) (6). In addition, untreated women could go on to develop ascending infection of their genital tract resulting in pelvic inflammatory disease (PID), or disseminated gonococcal infection (DGI), which is associated with dermatitis and arthritis (7, 8). PID can also lead to other complications such tubal scarring resulting in infertility and predisposing women to ectopic pregnancy, while neonatal conjunctivitis can cause corneal scarring and blindness (9–11). Renewed interest in gonococcal research has occurred in light of these observations and the emerging threat of multidrug-resistant strains.

1.2 *N. gonorrhoeae* virulence determinants

The major surface virulence factors are porin, pili proteins, opacity-associated proteins, lipooligosaccharide (LOS), reduction modifiable protein, H.8 protein, IgA protease, aerobically-induced proteins and iron-binding proteins. Figure 1.2 represents the various surface virulence determinants in *Neisseria* spp., and their corresponding ligands on the host tissue.

Porins are the most abundant outer membrane protein in *Neisseria* spp. They form pores on the bacterial membrane allowing preferential exchange of anions in

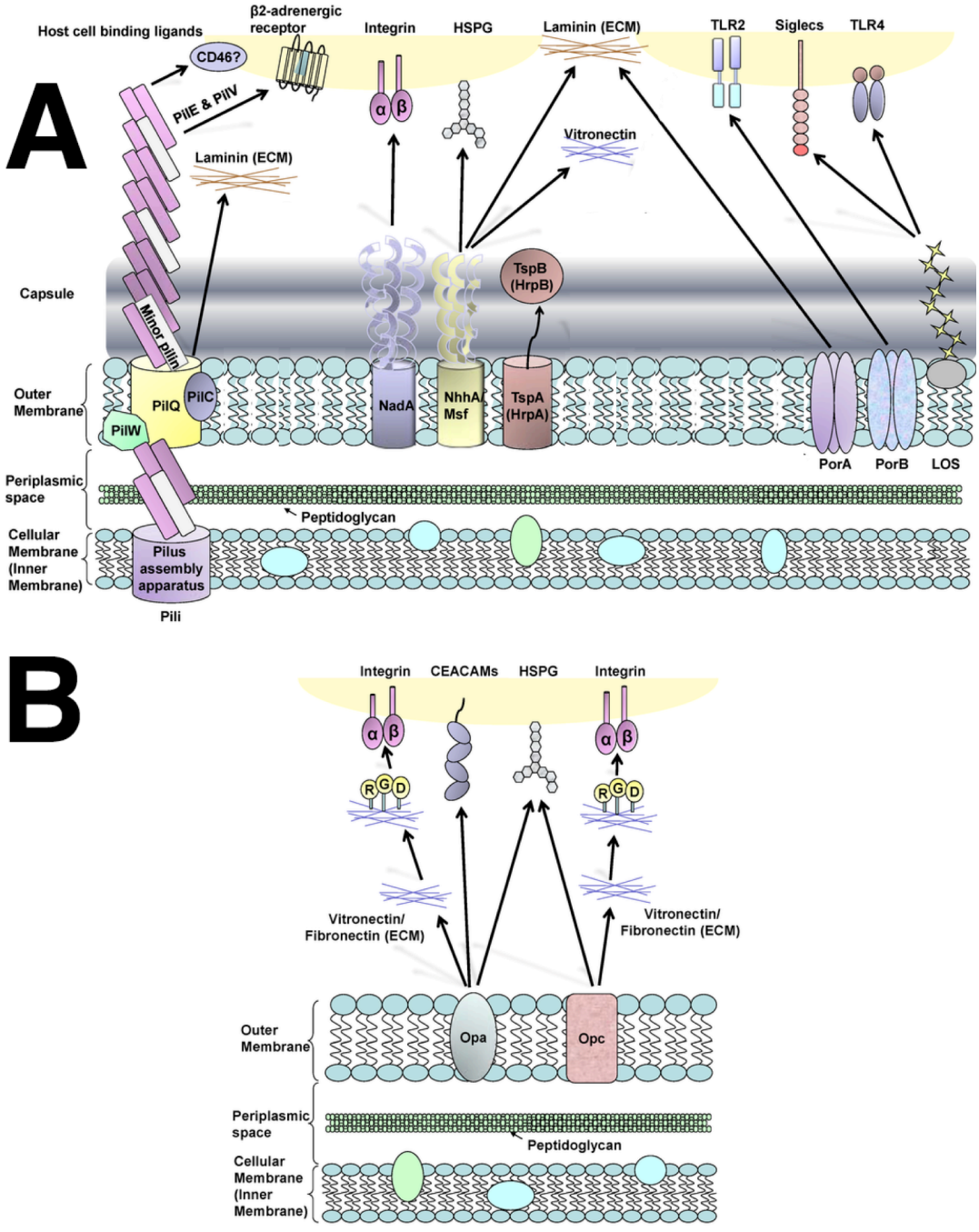

Figure 1.2 Virulence determinants of *Neisseria*. (A) Schematic representation of various surface virulence determinants of pathogenic *Neisseria* spp. and their corresponding ligands on host tissue. (B) Schematic representation of the various ligands (in host tissue) for Opa protein and Opc protein. Figure adapted from (123).

gonococci. Porins exhibit allelic variation, but there is very little variation of structure within a given strain. While meningococci has two paralogs of the *por* gene designated as *porA* and *porB,* gonococci have only one gene which is orthologous to the meningococcal *porB*. Previously referred as Protein 1 (P1), gonococci porin has been shown to elicit a strong immune reaction during infection. Porins are also involved in gonococcal invasion of cervical epithelial cells through C3R integrin and the scavenger receptor SREC (12).

Porin was observed to translocate into host cell membranes, resulting in impairment of phagocytosis, actin polymerization, lysosome maturation and secretion of microbicidal peptides (13–15). PorB1b has been reported to induce calcium influx from outside the cells, resulting in apoptosis induction (16). Since an increase in intracellular calcium results in activation of NLRP3-mediated inflammasome, and there was inhibition of porin-induced apoptosis by treating with YVAD (a caspase 1 inhibitor), it is possible that porins can also result in inflammasome activation (12, 17). Porins also function as immunomodulators by binding and activation TLR2 (18). Porin 1A can impair complement-mediated killing by binding to complement factor H (19). These features, and porin's ability to induce a T-cell dependent response make porin an ideal candidate for inclusion in vaccines, since the other major antigen, LOS, is T-cell independent (20, 21).

LOS is a glycolipid similar to LPS in other gram-negative bacteria but lacking the repeating oligosaccharide outer core (22–24). LOS molecules have a lipid component, commonly called lipid A, and a carbohydrate component, divided into inner and outer cores. The lipid A component binds to TLR4 and is responsible for

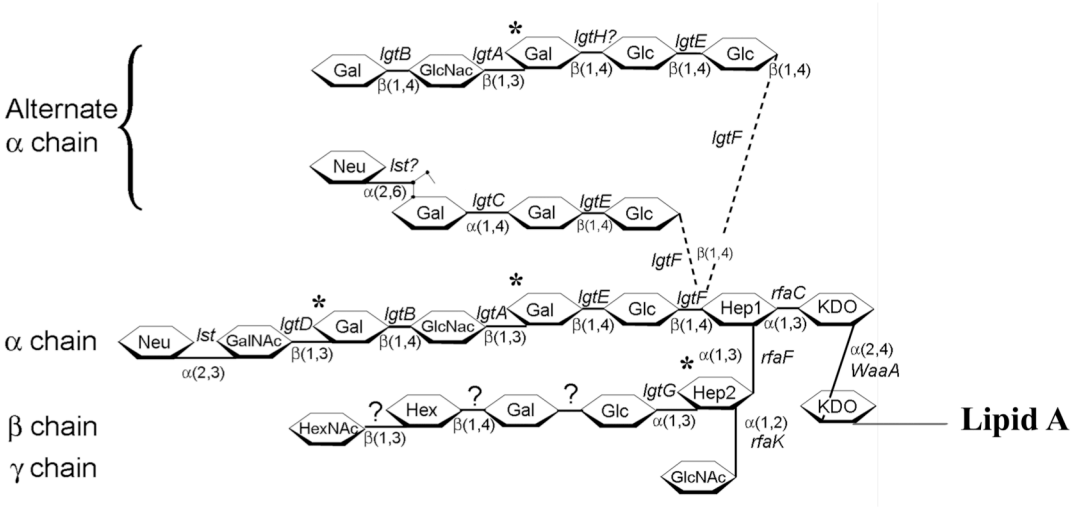

Figure 1.3 Schematic representation of neisserial LOS. The various enzymes involved in LOS biosynthesis are represented, in addition to the sugar moieties added by them. Phase variable enzymes are LgtD, LgtA and LgtG. In addition, the α-chain can also sialylated by Lst using host-derived CMP-NANA as donor. Figure modified from (22).

the characteristic “cytokine storm” seen in endotoxic shock (25, 26). In *Neisseria*, the outer core consists of three carbohydrate chains: α, β and γ. Some of the glycosyltransferases involved in outer core synthesis have been observed to be phase-variable, resulting in antigenic variation of outer core structure (27, 28). The schematic in figure 1.3 shows the various enzymes involved in biosynthesis of LOS molecule, and phase-variable enzymes are denoted by an asterisk. In addition, the LOS can be decorated with host-derived sialic acid, which prevents recognition by the immune system. Sialylation of LOS reduces phagocytosis by neutrophils, complement-mediated bacterial killing, and binding of IgM and IgA antibodies (29, 30). LOS is essential for gonococcal survival (31).

Neisserial LOS, like LPS, has been shown to activate TLR4, with the help of CD14 and MD2 (24). Before LOS interacts with TLR4-MD2 heterodimer, it has to be extracted from the outer membrane by human proteins such as LBP or soluble CD14 (32–34). The stability of LOS on the outer membrane can affect the immunostimulatory potential of the molecule by controlling availability of LOS. The immunostimulatory ability of LOS can be further be modified by decoration of lipid A with a phosphoethanolamine moiety. John CM *et al* observed that PEA increases the inflammatory potential of LOS, and that lack of a PEA in lipid A due to the absence of the *lptA* gene explains the reduced inflammation caused by *Neisseria* commensals (35). In addition, the lipid A PEA group was also found to modulate neisserial resistance against cationic antimicrobial peptides (36). Our lab had earlier characterized the *lpt3* gene and showed by biochemical analysis that the Lpt3 portioncan add a PEA group to the second heptose in the inner core of LOS (37).

Lpt6 has been shown to add PEA to the same heptose. While the biological role PEA decoration of lipid A has been studied in great detail, the importance of PEA in LOS inner core region is not completely understood.

LOS has been shown to interact with Opa proteins, suggesting that Opa acts as a lectin (38, 39). Opa proteins impart some degree of opaqueness and shine in gonococcal colonies grown on solid translucent media. Interestingly, there is no relationship between colony opacity and expression of Opa proteins in meningococci (40). Opa proteins, also known as heat-modifiable protein or PII, are transmembrane proteins with eight β-sheets and four outer loops (40). Opa proteins are phase-variable due to changes in the repeated pentameric sequence (CTCTT) within their coding sequence (41, 42). The frequency of phase variation has been estimated to be between 10^{-3} and 10^{-4} per generation (43). All eleven copies of the *opa* gene in gonococci have been found to have the pentameric repeats. Opa proteins have been found to play critical and diverse roles in the pathogenesis of gonorrhea. Opa can bind to HSPG receptors, independent of pili, and mediate invasion (44). They have also been shown to bind to CD66 receptors (a subtype of carcinoembryonic antigen-related cell adhesion molecule (CEACAM)). CEACAM family members are expressed in genital epithelial cells and neutrophils, suggesting a critical role for GC-CEACAM interaction (45). Transgenic mice expressing human CEACAM1 and CEACAM3 have been developed to study GC interaction with host immune cells (46). GC binding to CEACAM3 in neutrophils induces opsonin-independent phagocytosis, and this has been shown to trigger a powerful cascade of Syk-dependent signaling resulting in an NF-κB-dependent pro-inflammatory response (47). While a lot is

known about the role of the complete Opa protein, it is also possible that frame-shifting of the *opa* gene could result in an alternate protein being expressed with novel functions (40). The highly phase-variable nature of the protein also makes it an unlikely vaccine candidate in spite of a significant role in the pathogenesis of gonorrhea (21).

Pathogenic *Neisseria* secrete IgA protease has been shown to cleave LAMP1 protein and cause lysosomal disruption in infected cells (40). Loss of the IgA protease is associated with defects in intracellular survival and transcytosis of polarized T84 epithelial cell monolayers (48). *Neisseria* also express type IV pili, which plays an important role in invasion of epithelial cells. GC exhibit phase variation of pili as well as antigenic variation of the *pilE* gene which encodes the pilin subunit (40).

1.3 Biofilm: Architecture, Organization & Significance

The idea of bacteria living as part of a larger community has been well-established for almost a century, based on studies of *Rhizobium* and other soil bacteria (49, 50). Bacterial acquisition of multicellular behavior has been shown to have happened multiple times independently, conferring evolutionary advantage through various mechanisms (49). One of the classical examples of transient bacterial multicellularity is biofilm formation (Figure 1.4) (51). Biofilms represent the oldest form of organization of life on earth (51). Stromatolites, dating back to 3.4 billion years, represent fossilized remnants of cyanobacterial biofilm (52). Biofilms are believed to contain more than 98% of all bacteria in the world, and more than 50% of earth's biomass (53). Biofilms represent an organizational unit of microorganism, encased in a matrix, that operates as a cooperative consortium.

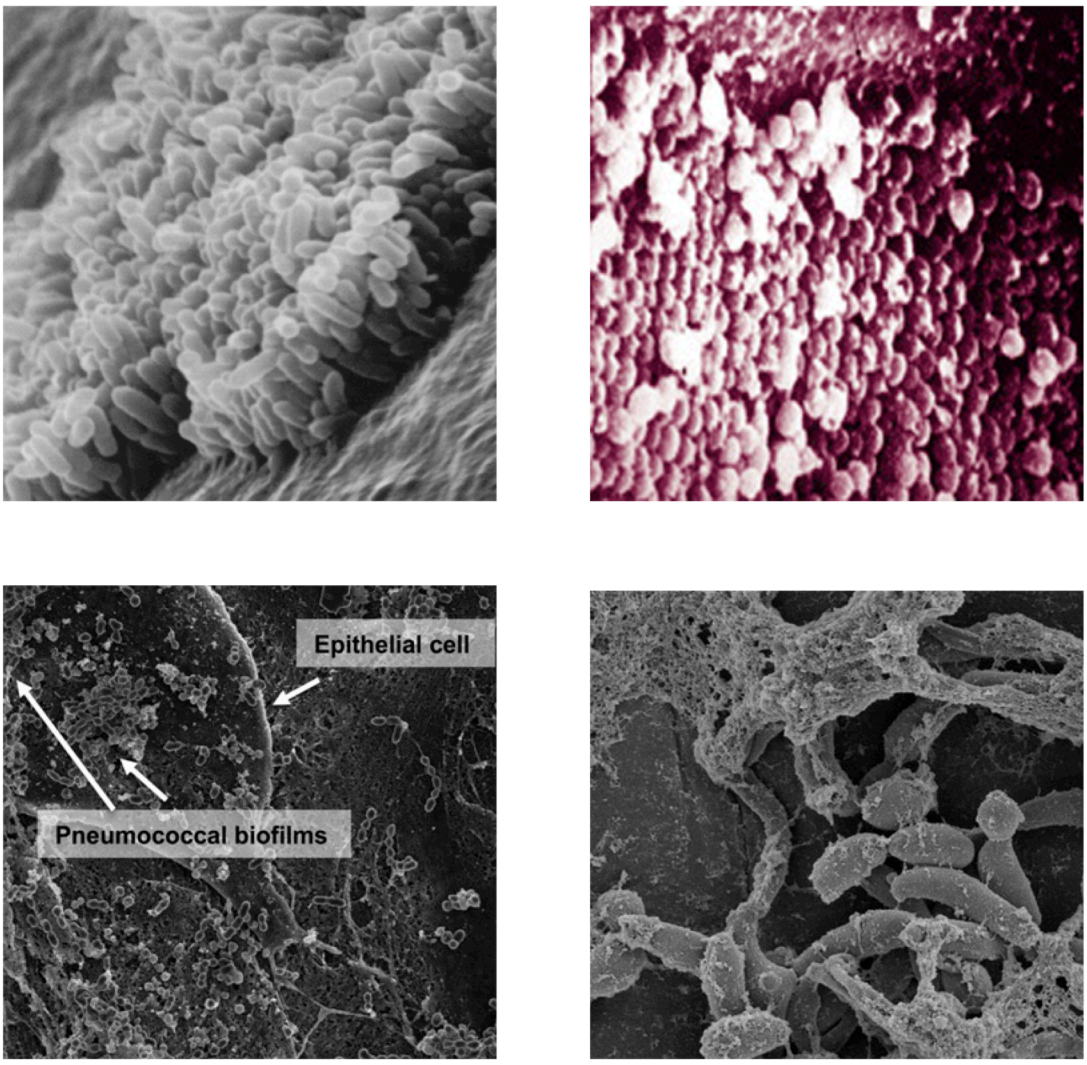

Figure 1.4 Examples of biofilm formed in various environments. Clockwise from top left are SEM images of a *Proteus mirabilis* biofilm formed on a urinary catheter, a *S. aureus* biofilm in a cardiac pacemaker, a fungal biofilm formed on contact lens surface, and a pneumococcal biofilm on top of epithelial cell surface. (Modified from (53, 235))

In a majority of biofilms, the matrix contributes over 90% of the dry weight. The matrix is composed of extracellular materials that act as a scaffold for the biofilm, and also contributes for attachment to the surface and for cohesion within the biofilm. The extracellular matrix can be composed of proteins, exopolysaccharides and extracellular DNA (eDNA) (54–56). The biofilm matrix i also protects the bacteria from antibiotics, toxins and other competing bacteria (55, 56). However, it is paramount to understand that naturally occurring biofilms are seldom formed by a single bacterial species (53). Studies on multispecies biofilms have not only revealed the level of complexity involved in bacterial interaction, but have also highlighted the limits of current technology in studying interaction between different species (57).

The architecture of a biofilm determines the metabolic status in a given biofilm by virtue of changes in concentration, cohesion, charge, sorption capacity of the various components in the matrix, and three-dimensional properties of the matrix such as pores, distribution of pores and water channels (53). Biofilms exhibit various three-dimensional architecture. They can be smooth and flat, rough, fluffy, mushroom-shaped, or filamentous (56). Some bacteria, such as *P. aeruginosa* and *B. subtilis*, form pellicles at the air-liquid interface under static aerobic conditions (58–60). Most biofilms also have variability in pore distribution (61–63). These pores, described as "water channels" play a critical role in transporting nutrients and removing waste products from various microenvironment in the biofilm (53). They possibly also provide conduits for soluble factors that play a role in bacteria-bacteria communication. The organization of biofilms also creates a gradient of nutrition and oxygen availability from the top to bottom of the biofilm. This difference in

oxygenation has been shown to result in anaerobic respiration in biofilms especially in the bottom layers in *P. aeruginosa* and *N. gonorrhoeae* (64–66). Studies using microelectrodes to measure oxygen levels within the biofilm have demonstrated that there are variations in oxygen level on a micrometer scale. Multispecies biofilms have been observed in chronic wounds, marine sediments, urinary catheter, dental plaques and industrial sludge. Multispecies biofilms are a good way to study competitive as well cooperative interactions (67). *P. aeruginosa* was reported to kill *Candida* in biofilms (67). Similarly, *Streptococcus mutans* is killed by hydrogen peroxide generated by *S. oligofermentans* in multispecies oral biofilms. In addition, these biofilms facilitate horizontal gene transfer as observed in dental plaques. Cooperative interactions between bacteria in biofilms have been exploited to improve efficiency of microbial fuel cells (MFCs) (68, 69).

Bacterial biofilms go through various stages over time (Figure 1.5). The role of biofilms in disease is well-established for oral infections and colonization of catheters and other devices. Bacterial biofilms have also been implicated in the pathogenesis of infected renal stones, bacterial endocarditis, infections in cystic fibrosis patients, chronic prostatits, biliary tract infections, otitis media, osteomyelitis, chronic tonsillitis, and necrotizing fasciitis (67). Bacterial biofilms have been implicated in providing a favorable environment for antibiotic resistance, because of the presence of suboptimal concentrations of antibiotics and a lower metabolic state that further impedes the bactericidal activity of antibiotics (70). In addition, biofilms have also been observed to promote horizontal transfer of antibiotic resistance. Biofilms are believed to cost billions of dollars to the US economy every year in energy losses,

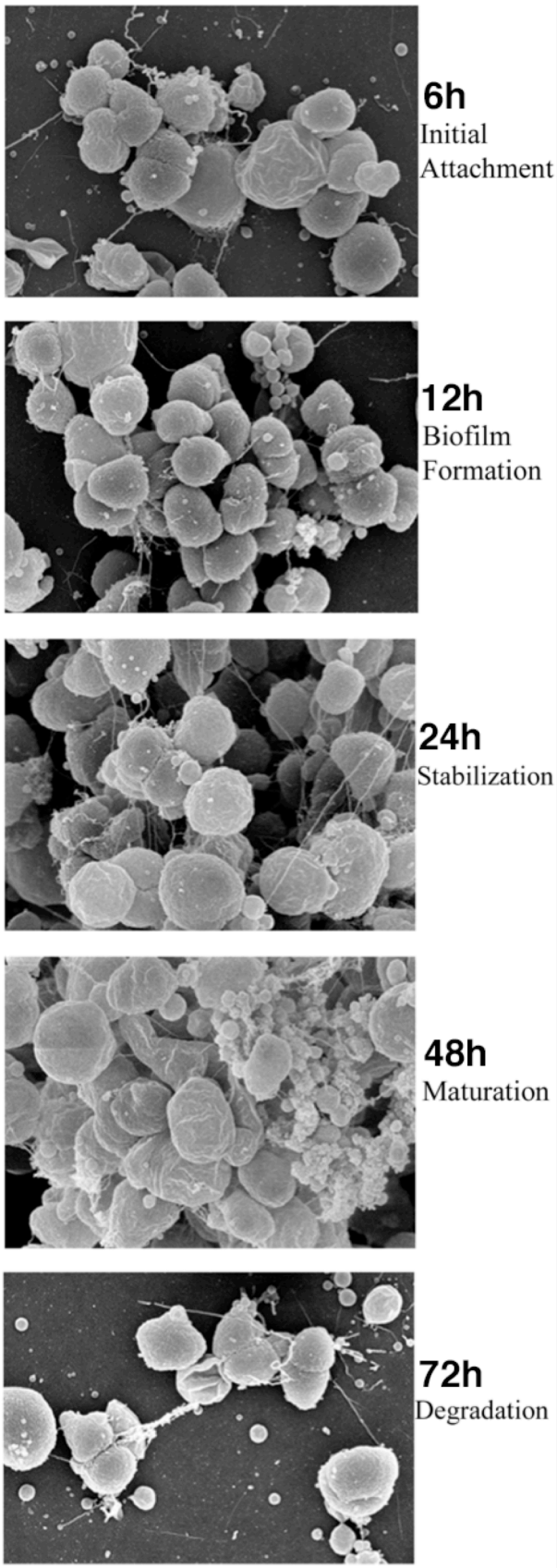
6h
Initial
Attachment
12h
Biofilm
Formation
24h
Stabilization
48h
Maturation
72h
Degradation

Figure 1.5 Structural evolution of biofilm. Free-floating or planktonic bacteria come in contact with each other and with a solid surface, forming microcolonies as they continue to aggregate (Initial attachment). As the biofilm is being formed, EPS production starts and the biofilm begins to stabilize (Biofilm formation). Production of EPS allows the biofilm to develop into a mature, complex, three-dimensional structure (stabilization). As the overall complexity and bacterial density increases, biofilms start maturing and continuously grow at the outermost layer while the innermost layer remains quiescent and anchors the biofilm to a surface. Over time, the biofilm degrades or begins to propagate by detachment of individual or small clumps of cells. This degradation and bacterial dispersal is usually mediated by enzymes and aided further by shear stress. Shown are SEM images of gonococcal biofilm taken at time points of 6h, 12h, 24h, 48h and 72h after seeding.

industrial equipment damage, industrial product contamination and hospital associated infections. However they also have potential applications in the food industry, bioenergy development, and astrobiology (53, 67, 70).

1.4 Biofilm matrix structure and function

Bacterial biofilm matrix is composed of proteins, exopolysaccharides, bacterial outer membrane, and eDNA (56, 71). The majority of those are sourced from the biofilm forming bacteria. The matrix offers a three-dimensional scaffold for the biofilm and keeps the bacteria in close proximity, facilitating close interaction and retaining released cellular debris (56). Hence, the matrix functions as a chamber that facilitates recycling of bacterial debris. A list of various functions served by various components of biofilm matrix is given in Table 1.1.

Polysaccharides are a major component of the biofilm matrix (55, 56). Advances in fluorescence microscopy such as Raman microscopy in combination with fluorescent lectin stains have furthered our understanding of the structure of these polysaccharides in EPS. The vast majority of exopolysaccharides are heteropolysaccharides composed of neutral and charged sugar residues, such as alginate (in *P. aeruginosa*), colanic acid (in *E. coli*), and PNAG (poly N-acetylglucosamine in *S. aureus*). Some bacteria can produce more than one kind of exopolysaccharide (56). These heteropolysaccharides can be composed of different monomers, and also at different ratios. *P. aeruginosa* has been shown to produce three different kinds of exopolysaccharide - alginate, Pel, and Psl (54, 72). Homopolysaccharides are also found in EPS produced by bacteria such as *Gluconacetobacter xylinus* and *Agrobacterium tumefaciens* which form

Function	Relevance for biofilms	EPS components involved
Adhesion	Allows the initial steps in the colonization of abiotic and biotic surfaces by planktonic cells, and the long-term attachment of whole biofilms to surfaces	Polysaccharides, proteins, DNA and amphiphilic molecules
Aggregation of bacterial cells	Enables bridging between cells, the temporary immobilization of bacterial populations, the development of high cell densities and cell–cell recognition	Polysaccharides, proteins and DNA
Cohesion of biofilms	Forms a hydrated polymer network (the biofilm matrix), mediating the mechanical stability of biofilms (often in conjunction with multivalent cations) and, through the EPS structure (capsule, slime or sheath), determining biofilm architecture, as well as allowing cell–cell communication	Neutral and charged polysaccharides, proteins (such as amyloids and lectins), and DNA
Retention of water	Maintains a highly hydrated microenvironment around biofilm organisms, leading to their tolerance of dessication in water-deficient environments	Hydrophilic polysaccharides and, possibly, proteins
Protective barrier	Confers resistance to nonspecific and specific host defences during infection, and confers tolerance to various antimicrobial agents (for example, disinfectants and antibiotics), as well as protecting cyanobacterial nitrogenase from the harmful effects of oxygen and protecting against some grazing protoza	Polysaccharides and proteins
Sorption of organic compounds	Allows the accumulation of nutrients from the environment and the sorption of xenobiotics (thus contributing to environmental detoxification)	Charged or hydrophobic polysaccharides and proteins
Sorption of inorganic ions	Promotes polysaccharide gel formation, ion exchange, mineral formation and the accumulation of toxic metal ions (thus contributing to environmental detoxification)	Charged polysaccharides and proteins, including inorganic substituents such as phosphate and sulphate
Enzymatic activity	Enables the digestion of exogenous macromolecules for nutrient acquisition and the degradation of structural EPS, allowing the release of cells from biofilms	Proteins
Nutrient source	Provides a source of carbon-, nitrogen- and phosphorus-containing compounds for utilization by the biofilm community	Potentially all EPS components
Exchange of genetic information	Faciliates horizontal gene transfer between biofilm cells	DNA
Electron donor or acceptor	Permits redox activity in the biofilm matrix	Proteins (for example, those forming pili and nanowires) and, possibly, humic substances
Export of cell components	Releases cellular material as a result of metabolic turnover	Membrane vesicles containing nucleic acids, enzymes, lipopolysaccharides and phospholipids
Sink for excess energy	Stores excess carbon under unbalanced carbon to nitrogen ratios	Polysaccharides
Binding of enzymes	Results in the accumulation, retention and stabilization of enzymes through their interaction with polysaccharides	Polysaccharides and enzymes

Table 1.1 Components of biofilm matrix. List of various EPS components and their function in biofilm. (From (56))

cellulose (56). EPS formed by different strains of the same species can have differences in their EPS components. Observations showing lack of biofilm formation by mutations that compromise EPS biogenesis demonstrate the vital role played by EPS in biofilm formation.

Biofilms possess an abundance of extracellular DNA (eDNA), facilitating genetic exchange (73). The source of eDNA could either be from lysis of a bacterial subpopulation (aided in part by autolysins) or by active secretion of DNA: some strains of *N. gonorrhoeae* that possess the gonococcal genomic island (GGI) secrete DNA by a Type IV secretion system (74, 75). In addition, eDNA can also function as a structural component, as evidenced by destruction of biofilms by treatment with DNase. eDNA has been shown to be a major structural component in biofilms formed by *S. aureus, B. cereus, P. aeruginosa, N. meningitidis* and *N. gonorrhoeae* (76–79). Studies on gonococcal biofilm have shown the different roles played by ssDNA versus dsDNA. While ssDNA was found to play a vital role in initiating biofilm formation, unlike dsDNA it was not incorporated in the mature biofilm (71). Targeted destruction of ssDNA only prevents biofilm formation but has no effect on preformed biofilms (71). The structural role of eDNA has also been observed to show significant differences depending on the organism studied (55, 56). For example, eDNA forms a grid-like structure in *P. aeruginosa*, it has been shown to form a network of fine strands and thicker 'rope' like structures in *H. influenzae* biofilm (81, 82).

1.5 Other components of matrix, and mechanical properties of matrix:

The other major components of the biofilm matrix are proteins (enzymes and structural proteins), lipids, surfactants and water. Enzymes in the matrix can either be

secreted or released by bacterial lysis. These enzymes have also been shown to have very diverse function, such as rearrangement of EPS into a more refractory polymer in marine stromalites, and promoting dispersal by degrading the EPS as observed in *Actinobacillus actinomycetemcomitans* (56).

Proteins can form structural components of a biofilm (83, 84). Extracellular proteins such as curli play a quintessential role in biofilm formation by *E. coli* and *Salmonella* spp (85). Bacterial structures such as pili and flagella also provide proteinaceous structural components to biofilm (86).

Lipids are also observed in biofilms formed by *Rhodococcus* spp., *Thiobacillus ferrooxidans, Serratica marcescens* and *N. gonorrhoeae. Rhodococcus* spp. biofilm has an EPS with hydrophobic properties that allow it to adhere to teflon and colonize waxy leaf surfaces (56). The lipids in gonococcal biofilms are derived from outer membrane vesicles released by the bacteria (87). Outer membrane-like filaments seen in gonococcal biofilm have also been observed *in vivo* in cervical biopsy samples (87, 88). The hydrophobic nature of some components in the matrix be caused by biosurfactants. These surface active molecules are believed to act during the initiation phase by promoting bacterial aggregation (56). The last and the most abundant component in biofilm matrix is water. The EPS matrix, being hygroscopic, provides a hydrated environment by retaining water and buffering against environmental fluctuations. However, the matrix seems to be able to sequester particles and ions from the water phase and function as a molecular sieve (55).

1.6 Biofilm formation, maturation, and dispersal:

Biofilms begin when individual bacteria aggregate to form microcolonies. This initial aggregation is aided in part by bacterial motility (89–91). For example, *P. aeruginosa* strains defective in flagellar motility and type IV pili were not able to form microcolonies (86). Pilus-negative Group A *Streptococci* (GAS) are defective in biofilm formation (92). Pilus-negative gonococcal strains formed thinner biofilm compared to pilus-positive strains (Unpublished observations). Sherlock *et al* also showed that intercellular TibA-TibA interaction contributes towards bacterial autoaggregation in *E. coli* (93). Other factors associated with increased bacterial aggregation are fimbriae, AIDA, curli fibers, Ag43 (all in *E. coli*), Bap (*S. aureus*), flagellin (*C. jejuni*), Opa and AutA (*N. meningitidis*), ShdA and MisL (*Salmonella enterica*) (39, 94–98). It is interesting to note that a vast majority of these bacterial adhesins are autotransporters. The role of interbacterial interaction in biofilm is clearly demonstrated by Veiga *et al* when they expressed a chimeric protein with domains from Jun and Fos in *E. coli* (99). Fos and Jun form heterodimers through strong interaction between their coiled coils (99). In addition to promoting interbacterial aggregation, bacterial adhesins such as curli fibers and Ag43 also facilitate attachment of bacteria to biotic and abiotic surfaces. Curli fibers also promote binding of bacteria to host extracellular components such as laminin, fibronectin, and plasminogen (85). Similarly in *P. aeruginosa* and UPEC, components of CUP (Chaperone Usher Pathway) fimbriae promote bacterial surface adherence (100). Other factors shown to be involved in bacterial attachment to surface are SagA, Acm (*E. faecium*), Ace, Esp (*E. faecalis*), Opa (*N. meningitidis* and

N. gonorrhoeae), MSCRAMM family of proteins, Embp, protein A, FnbpA, FnbpB, and SasG (*S. aureus* and *S. epidermidis*) (101–105) .

The initial aggregation and surface contact between bacteria and a surface can lead to changes in gene expression, up regulating genes encoding proteins involved in matrix formation (106). High levels of 3,5-cyclic diguanylic acid (cyclic-di-GMP) promote expression of adhesins such as CdrA in *P. aeruginosa*, which then binds to Psl, and stabilizes the biofilm (107). Cyclic-di-GMP has also been found to affect production of alginate, Pel and Psl (108). There is evidence that cell-to-cell communication plays a key role in regulating matrix production and biofilm strengthening. The quorum sensing system, called Agr (accessory gene regulator) in *S. aureus,* upregulates biofilm-degrading enzymes such as proteases, and downregulate expression of adhesins (109). Quorum sensing systems have also been implicated in biofilm maturation by increasing eDNA amounts in the matrix. Although eDNA increases in quantity over time because of bacterial lysis, mature *P. aeruginosa* biofilms were less sensitive to DNase digestion than younger biofilms, suggesting a more significant contribution from other components of EPS rather than DNA in improving structural stability (56). Contrastingly, experiments in our lab using gonococci did not show any difference in DNase sensitivity vis-a-vis age of the biofilm. Gonococcal biofilms grown for various time periods were all equally sensitive to DNase treatment and were almost completely dispersed (Unpublished observation). The complex interaction between the various components of the matrix, including exopolysaccharide, adhesins and eDNA complemented by increasing availability of intracellular components such as nucleoid-associated proteins (NAPs,

in *S. aureus*), form a biofilm scaffold to support the multiplying bacteria during this phase. The mature biofilm thus serves as a safe haven for the resident bacteria, offering protection against host immune cells and other toxic molecules.

As the bacterial population increases, the biofilm stability begins to be compromised due to a plethora of reasons, not limited to loss of structural stability, susceptibility to shear stress, lack of nutrient percolation, accumulation of toxic metabolites, and release of biofilm degrading enzymes (110). Levels of cyclic-di-GMP are inversely correlated with bacterial dispersal from a biofilm (111). This dispersal appears to be coordinated and highly regulated, as a subpopulation of cells undergo lysis and release nutrients which act as cues for dispersal of another subpopulation (111). The dispersal cells undergo changes in gene regulation by down regulating genes involved in biofilm formation such as *pilA*, and upregulate other genes such those encoding flagella and proteins involved in chemotaxis (110). Recently, incorporation of D-amino acids in peptidoglycan trigger dispersal in *Bacillus* spp., *S. aureus*, and *P. aeruginosa* (112).

In general, there are three modes of dispersal namely erosion, sloughing and seeding (111). Erosion refers to continuous bacterial release over the entire lifecycle of the biofilm at a low level, whereas sloughing refers to sporadic bursts of detachment of huge parts of the biofilm. Seeding dispersal is characterized by central hollowing where small clusters of bacteria leave the matrix resulting in a hollowing. These are believed to help in the formation of new pores which can facilitate access of nutrients to remote parts of the biofilm. These mechanisms can either be either active or passive, where active dispersal is initiated by the organisms themselves

EPS-degrading enzymes	Thermonuclease (*S. aureus*)
Phage-mediated	Pf4 (*P.aeruginosa*), PlyC (*S. pyogenes*)
Surfactant-mediated	Rhamnolipids (*P.aeruginosa*)
Cell division mediated	*E. coli*
Modulation of pili	EAEC
Induction of motility	*E. coli, P. aeruginosa*
Release of amyloid fibers	*B. subtilis*
Nutrient limitation	*Shewanella oneidensis*, *P. aeruginosa, Acinetobacter* sp.

Table 1.2 Mechanisms of biofilm dispersal. List showing the various mechanisms by which bacteria mediate biofilm dispersal. (EAEC is Enteroaggregative *E. coli*) (Modified from (110, 236, 237))

whereas passive dispersion is mediated by external agents such shear stress, human intervention (eg. brushing of teeth) and predator grazing (eg. phagocytosis) (111). Table 1.2 lists the various mechanisms behind biofilm dispersal.

EPS-lysing enzymes are significant contributors for bacterial dispersal. Table 1.3 shows a list of various EPS-degrading enzymes in different bacteria. These enzymes can be classified into DNases, proteases, and glycolichydrolases. While EPS-degrading enzymes breakdown the covalent links, surfactant molecules produced by bacteria can breakdown biofilm by destroying non-covalent linkages. Bacterial dispersal agents are also aided partly by host-derived factors such as higher body temperature, norepinerphine, extracytopalsmic ATP, increased nutrients that promote biofilm destruction (110). Another class of agents that promote biofilm dispersal are bacteriophages. The bacteriophage Pf4 has been shown to promote dispersal of *P. aeruginosa* str. PAO1 biofilm (113). Although some bacteriophages have been known to encode an EPS-degrading enzyme, the mechanism behind phage-induced biofilm dispersal is not clearly understood. It is hypothesized that during the later stages of biofilm development, the switch from lysogenic to superinfective phage form drives cell death, which is believed to dump intracellular enzymes that can aid bacterial dispersal. In support of this hypothesis, deletion of CRISPR elements can reduce bacterial swarming and increase biofilm formation (111).

1.7 Gonococcal biofilm formation:

Neisserial biofilm studies, initiated by Igor Stojilijkovic showed biofilm formation by *N. meningitidis* using a static method to grow biofilms on glass

Alginate lyase	Alginate	*P. aeruginosa*
Aureolysin	Unknown	*S. aureus*
Chitinase	Chitin	*Pseudoaltermonas* sp. S91
Disaggregatase	Polymer of GalNAc, galacturonate, and glucuronate	*Methanosarcina mazei*
Dispersin B	PNAG	*A. actinomycetemcomitans*
Endo-β-1,4-mannanase	Unknown	*Xanthomonas campestris*
Exopolysaccharide lyase	Unknown	*P. fluorescens*
Hemagglutinin protease	Bacterial receptors on human intestinal cells	*V. cholerae*
Hyaluronidase	Hyaluronan	*S. internedius*
LapG protease	LapA exopolysaccharide-binding protein	*P. putilda*
Sp1 protease	Unknown	*S. aureus*
Surface-protein-releasing enzyme	Antigen P1	*S. mutans*
Thermonuclease	eDNA	*S. aureus*
Alphamylase	Unknown	Bacillus amyloliquefaciens
Glucoamylase	Unknown	Aspergillus niger

Table 1.3 List of EPS-degrading enzymes. Table shows some of the characterized biofilm-degrading enzymes, their targets and corresponding organisms. (Modified from (110, 238))

coverslips (114–116). Apicella *et al* extended the studies to *N. gonorrhoeae*, showing that GC can form biofilm in a continuous flow chamber (88). GC were observed to form microcolonies, which eventually developed into biofilm on the surface of urethral and cervical epithelial cells. The biofilms had a tridimensional structure characterized by a matrix interspersed with water channels to supply nutrients to the deeper regions of the biofilm. Interestingly, using lectin-binding they also demonstrated the role of outer membrane vesicles (OMVs) in biofilm formation (88). Since *N. gonorrhoeae* does not make any EPS, it is believed that the OMVs play an important role by giving structural support to the biofilm matrix. OMVs have also been shown to contain large amounts of DNA (117).

The role of DNA in GC biofilm matrix was established when it was demonstrated that *nuc* knockouts formed thicker biofilms compared to the wildtype strains (118). The *nuc* gene encodes a nuclease, which was found to be active on both single-stranded as well as double-stranded DNA. Further studies on incorporation of DNA in GC biofilms revealed that it was predominantly dsDNA that was incorporated in mature biofilms, although biofilm formation was reduced by treating with Exonuclease I, which specifically destroys ssDNA (80). This suggests different roles for ssDNA and dsDNA in biofilm initiation and maturation. A similar role for dsDNA was suggested by studies in meningococci, where it was found that eDNA released by cell lysis contributed to shear force resistance (76).

The importance of studying GC biofilms is highlighted by observation of biofilm formation in patients diagnosed with gonococcal cervicitis (87). TEM analysis of cervical biopsy specimens suggested that biofilms form on epithelial cell

surfaces *in vivo.* The significance of GC biofilm formation should also be seen in light of observations in *N. meningitidis* where it was found that 30 percent of carriage isolates formed biofilm in comparison to only 12.5 percent of invasive strains (114). Our lab observed similar trend when gonococcal isolates from cervix were compared with isolates from gonococcal isolates from the blood of the same patients; the blood isolates form less biofilm when compared to the cervical isolates (Nadia Kadry, unpublished observation). This observation correlates with studies on *N. meningitidis* where Lapann *et al* observed that clonal complexes that are unable form biofilm, such as ST-11 and ST-8, tend have a poor colonization ability but higher transmission rates (76).

1.8 Gonococcal protein glycosylation:

Gonococcal pili be heavily glycosylated post-translationally by addition of either an O-linked trisaccharide or O-linked disaccharide depending on the expression of *pglE* gene (119). Phase variation of the glycosylation pattern adds another level of heterogeneity to the outer membrane of *Neisseria* spp. This was demonstrated in a study by Virji *et al* where they observed that differences in strength of adherence of meningococci to epithelial cells was affected by both the primary sequence of pilin subunits as well as glycosylation status of pilin subunits (120). Adherence rates were higher with *N*-linked glycosylation of pilin subunits. While the various proteins involved in pilin glycosylation have been characterized, bacterial glycosylation in general has not been fully characterized (119, 121).

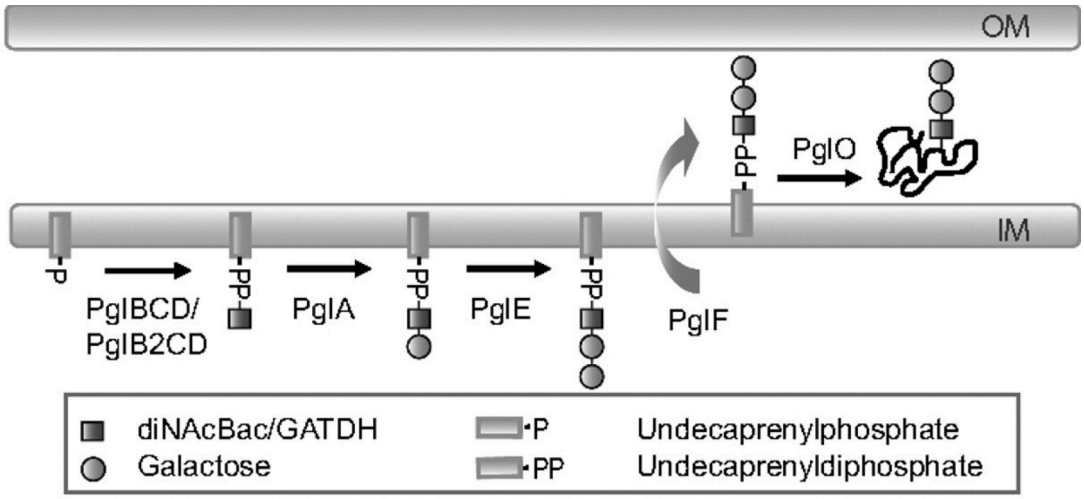

Figure 1.6 Protein glycosylation system in *Neisseria* spp. Schematic representation of the various enzymes involved in glycosylation of surface proteins in *N. gonorrhoeae* and *N. meningitidis,* showing sequential action of various enzymes which add the sugar moieties on to undecaprenyl-lipid carrier and then transfer the glycosyl moiety to proteins in the peripasmic space (239).

Systematic peptide-based assays have revealed the extent of protein glycosylation in prokaryotes. The gonococcal protein glycosylation system has been well characterized although the heterogeneity of surface glycans is still being uncovered. The promiscuity of bacterial glycosylation was demonstrated by Vik *et al* when they observed that the pilin glycosylation system was able to glycosylate other non-pilus associated proteins such as AniA, PotF and DsbA (121). These proteins were glycosylated at their serine residues, similar to the *pilE* glycosylation at Ser-63 site. Allelic variations in *pglH*, which encodes a protein involved in gonococcal protein glycosylation downstream of PglC, were observed to result in addition of N-acetylglucosamine and peptidoglycan fragments to surface proteins (Figure 1.6)(122). The role of bacterial glycosylation in promoting bacterial aggregation and biofilm formation has been extensively shown by studies on serine-rich adhesins (123). Streptococcal glucosyltransferases Gtf1 and Gtf2 be involved in GlcNAc decoration of Fap1 protein and the Fap-1-like serine-rich proteins, which play important role in bacterial aggregation (124). This GlcNAc decoration has been shown to promote biofilm formation in *S. parasanguinis* (124). Similar observations have been made in pneumococci and *A. baumannii* (125, 126).

1.9 Innate and adaptive immunity:

The innate immune system is the first line of defense against pathogens in humans and mammals. Innate immunity differs from the other arm of human immunity, adaptive immunity, in that it is present from birth, and does not confer long-term immunity (127). Studying the interaction between the different arms of immune system and gonococci will help us understand the mechanism behind

asymptomatic gonorrhea. Understanding how gonococci would suppress immune response would help us develop better vaccines and diagnostic tests for better diagnosis of asymptomatic disease.

Innate immunity protects against pathogens by employing rapid, non-specific and highly conserved mechanisms while the adaptive immune system prepares to mount a more specific and robust response, which might take days to weeks to develop. Innate immunity involves macrophages, dendritic cells, neutrophils, mast cells, basophils, eosinophils, natural killer cells, and γδ T cells (128). At a molecular level, proteins of the complement system and pattern-recognition receptors (PRRs) are major components of innate immunity. Adaptive immunity, unlike innate immunity, creates a specific and targeted immune response with immunological memory (127). There are two major adaptive immune responses - antibody production and a cell-mediated response. While antibody production is mediated by B-cells and cell-mediated response is orchestrated by T-cells, there is significant interaction between the two responses (127).

There are three major groups of T-cells - CD8 T cells, CD4 T cells and γδ T cells. CD8 cells are cytotoxic cells involved in directly killing pathogens or dysfunctional host cells by using perforin and granzyme. There are numerous subgroups of Th cells such as Th1, Th2, Th9, Th17, Th22 and Treg cells (129). Th cells play a crucial role in modulating adaptive immune response. While Th1 cells, which secrete IFN-γ, activate bactericidal macrophages, cytotoxic CD8 cells and induce B-cells to make opsonizing antibodies, Th2 cells are characterized by a predominant humoral response. Th17 cells are a subclass of T helper cells, abundant

at mucosal surface, which produce IL-17, IL-17F and IL-22 (130). They differentiate in response to STAT-3 activating cytokines IL-6, IL-21 and IL-23, along with IL-1β and TGF-β. By mediating neutrophilia and secretion of antimicrobial peptides by epithelial cells (131), Th17 cells play a vital role in fighting mucosal infection (130). All the diverse and discrete units of the immune system generally communicate through chemicals referred to as cytokines.

Cytokines are an assortment of soluble polypeptides and glycoproteins, similar to hormones but are not endocrine-derived (132). Cytokines generally act in a local environment, in autocrine and paracrine fashion. Cytokines play a crucial role in maintaining homeostasis as well as responding to any disturbance in homeostasis (133–135). The balance between levels of proinflammatory cytokines such as IL-1, IL-6, IL-8, TNF-α and anti-inflammatory cytokines such as IL-10 and TGF-β is important in preventing a disproportionate immune response (136). Epithelial cells, which form the first line of defense against the external environment, can secrete cytokines following stimulation by other cytokines or by pathogens (137).

Epithelial cells in bronchial airway secrete IL-10 constitutively which prevents an excessive immune response against pathogens (138). Alteration in epithelium-derived levels of IL-6 has been observed in patients suffering from asthma (139). The immune regulatory role of airway epithelium and gut epithelium has been well-characterized (140). Cytokines derived from epithelia in the female genital tract have also been studied, although their role in infection is not completely understood. Studies in mice and rat have shown that epithelial cells in the FRT are able to produce TNF-α, TGF-β and MIPα/CCL20. MIPα/CCL20 levels were increased in uterine

epithelial cells exposed to *E. coli* whereas *L. rhamnosus* had no effect, suggesting that epithelial cells are able to differentiate between commensals and pathogens (141).

From an anatomical perspective, innate immune barriers include skin, mucosal layers in the gastrointestinal tract, respiratory tract and urogenital tract, and conjunctival layer in eyes. Among these, one of the busiest portals of bacterial entry is the mucosal layer, which shows the importance of mucosal immunity in human health. The mucosal immune system consists of epithelial cells (and extra epithelial secretions) and mucosa associated immune cells, which can either be unorganized or organized into specialized lymphoid tissue referred as mucosa-associated lymphoid tissue (MALT). The epithelial layer observed in endocervix and ectocervix, the two regions first infected by *N. gonorrhoeae,* are fundamentally different (1, 141). While the epithelial layer in the endocervix forms a tight polarized monolayer of columnar epithelial cell characterized by tight and adherens junctions, the ectocervix is lined by multiple layers of loosely adherent squamous epithelial cells (141).

Epithelial cells form a physical barrier against bacterial invasion, aided in part by tight junction formation between neighboring cells (142). The integrity of tight junctions can be measured using an ohmmeter that measures the electrical resistance across the epithelial monolayer (143). Tight junction barrier function is also regulated by cytokine production and growth factors. Previous work in our lab has established how gonococci was able to disrupt tight junction in polarized epithelial cells (144). The physical barrier is further reinforced by mucosal secretion which also contains microbicides such as human β-defensins (HBDs), human α-defensin-5 (HD5), lysozyme, lactoferrin, secreted leukocyte protease inhibitor (SLPI), surfactants such

as surfactant protein A (SP-A), and cathelicidins, of which only LL-37 is found in FRT (145). GC resistance against cationic antimicrobial peptides (CAMPs) is mediated by the decoration of LOS with phosphoethanolamine (PEA), and by the action efflux pumps such as NorM (146). Mucus secretions mainly consist of mucins and are also rich in antibodies. Soluble mucins are believed to prevent bacterial pathogens from invading epithelial cells by binding and trapping the bacteria (147).

In addition to the challenging task of identifying potential pathogens from beneficial commensals, the mucosal layer in female reproductive tract (FRT) has "to balance protection with procreation" (148). This balance is aided in part by the hormonal regulation of immune cells. The immune cells as well as epithelial cells in FRT respond to cyclical changes in levels of estrogen and progesterone. Estrogen (E2) has been shown to reduce LPS-induced IL-6 production and transepithelial electrical resistance (TEER) in epithelial cells (141, 148). E2-driven keratinization of vaginal epithelial cells prevents GC infection of those cells. As a result vaginal gonorrhea is limited to prepubescent girls (149).

Epithelial cells also express pattern-recognition receptors such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs) (128). TLRs are evolutionarily conserved transmembrane proteins that recognize either exogenous PAMPs or endogenous danger-associated molecular patterns (DAMPs). Ten human TLRs and their corresponding ligands have been characterized, as listed in Table 4. TLRs function as dimers. All TLRs except TLR2, which forms heterodimers with either TLR1 or TLR6, function as homodimers (150, 151). The receptors form a dimer following stimulation by ligand. TLR signaling is mediated by two distinct

TLR	Immune Cell Expression	PAMPs	DAMPs	Signal Adaptor	Production
TLR1+ TLR2	Cell surface Mo, MΦ, DC, B	Triacylated lipoproteins (Pam3CSK4) Peptidoglycans, Lipopolysaccharides	(TLR2 DAMPs listed below)	TIRAP, MyD88, Mal	IC
TLR2+ TLR6	Cell surface Mo, MΦ, MC, B	Diacylated lipoproteins (FSL-1)	Heat Shock Proteins (HSP 60, 70, Gp96) High mobility group proteins (HMGB1) Proteoglycans (Versican, Hyaluronic Acid fragments)	TIRAP, MyD88, Mal	IC
TLR3	Endosomes B, T, NK, DC	dsRNA (poly (I:C)) tRNA, siRNA	mRNA tRNA	TRIF	IC, type1 IFN
TLR4	Cell surface/ endosomes Mo, MΦ, DC, MC, IE	Lipopolysaccharides (LPS) Paclitaxel	Heat Shock Proteins (HSP22, 60, 70,72, Gp96) High mobility group proteins (HMGB1) Proteoglycans (Versican, Heparin sulfate, Hyaluronic Acid fragments) Fibronectin, Tenascin-C	TRAM, TRIF TIRAP, MyD88 Mal	IC, type1 IFN
TLR5	Cell surface Mo, MΦ, DC, IE	Flagellin		MyD88	IC
TLR7	Endosomes Mo, MΦ, DC. B	ssRNA Imidazoquinolines (R848) Guanosine analogs (Loxoribine)	ssRNA	MyD88	IC, type1 IFN
TLR8	Endosomes Mo, MΦ, DC, MC	ssRNA, Imidazoquinolines (R848)	ssRNA	MyD88	IC, type1 IFN
TLR9	Endosomes Mo, MΦ, DC, B,T	CpG DNA CpG ODNs	Chromatin IgG complex	MyD88	IC, type1 IFN
TLR10	Endosomes Mo, MΦ, DC	profilin-like proteins		MyD88	IC

Mo: monocytes, MΦ: marcophages, DC: dendritic cells, MC: Mast cells, B: B cells, T: T cells, IE: Intestinal epithelium, IC: Inflammatory cytokines

Table 1.4 TLRs and their distribution in human cells. Table lists their subcellular location, endogenous and exogenous ligands, signal adaptor molecules and resulting response. (Adapted from www.invivogen.com)

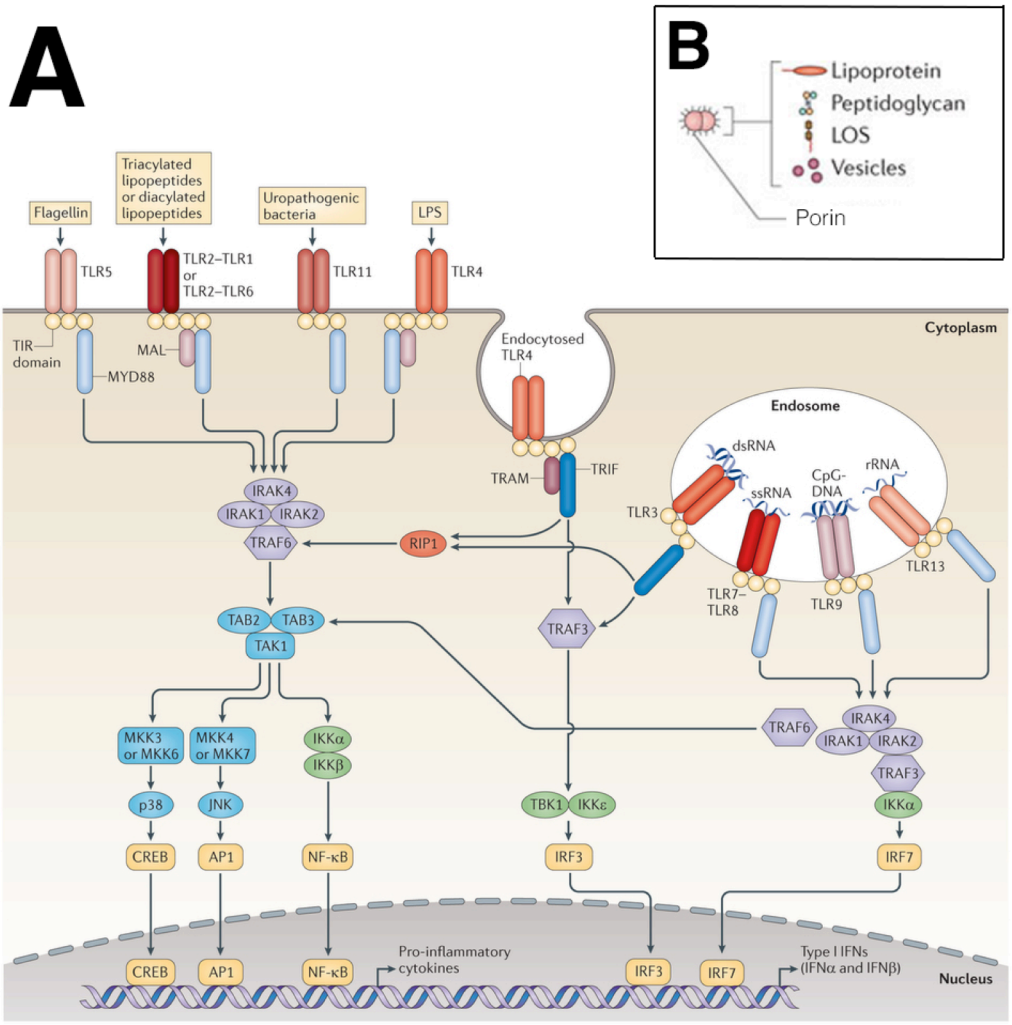

Figure 1.7 TLR activation and signaling. (A) Activation of TLRs result in downstream recruitment of adaptor molecules such as MyD88 and MAL. TLR signaling is mediated by two distinct pathways, MyD88-dependent and MyD88-independent. Following recruitment and activation of the adaptor molecules, transcription factors such as NF- κB and AP-1 are activated, resulting in transcription of genes encoding inflammatory cytokines such as IL-8 and IFN. (B) Schematic representation of various ligands on GC surface that activate TLRs on host cells. (Adapted from (240, 241))

pathways, namely a myeloid differentiation primary-response protein 88 (MyD88)-dependent pathway resulting in expression of inflammatory cytokines and a MyD88-independent pathway resulting in expression of type I interferons. Following ligand-induced dimerization, the TLRs bind to MyD88 and MyD88-adaptor-like protein (MAL) or to TIR domain-containing adaptor protein inducing IFNβ (TRIF) and TRIF-related adaptor molecule (TRAM) (151, 152). Following binding of signaling adaptor molecules IRAKs and TRAFs, there is activation of MAPKs, JKNs, p38 and activation of transcription factors, NF-κB and, AP-1, CREB and IRFs (Figure 1.7). TLR activation results in production of pro-inflammatory cytokines.

Cells in all regions of FRT express TLRs 1-6, as shown by immunohistochemical techniques, although vaginal and ectocervical epithelial cells do not to express TLR4 (153). The absence of TLR4 in lower female reproductive tract could be a result of the need to tolerate useful commensal bacteria such as *Lactobacillus* spp. and diphtheroids (141, 154, 155). Gonorrhea in females primarily affects the endocervical epithelial cells, but could ascend into the endometrial epithelial cells in the uterus, resulting in pelvic inflammatory disease (PID) (1). The endocervical epithelial layer is composed of a monolayer of polarized epithelial cells. Primary endocervical epithelial cells express TLRs 1-3, and TLR6, while primary endometrial epithelial cells expressed TLRs 1-9 (156). Primary endometrial epithelial cells only respond to the TLR3 agonist poly (I:C), suggesting that additional signaling factors such as soluble CD14 might be needed to stimulate primary endometrial epithelial cells in solitary culture (157). HEC 1-B cells (human endometrial carcinoma cell line responds to TLR3 and TLR5 ligands (158).

The other classes of pattern recognition receptors are NODs, RIG-like receptors (RLRs), C-type lectin receptors (CLRs) and NOD-like receptors (NLRs). While NOD1 recognizes diaminopimelate-containing peptidoglycan, this is limited to gram-negative bacteria. NOD2 is activated by muramyl dipeptide, which is produced by both gram-negative and gram-positive bacteria (128). Although TLRs and NODs both activate NF-κB, they use different adaptor proteins. Unlike TLRs which are transmembrane proteins and detect extracellular or membrane-bound ligands, NODs, RLRs and NLRs are cytosolic and are activated by cytosolic ligands (159). More than 23 NLRs have been identified in humans. NLRs, on activation by danger-associated molecular patterns or by bacterial products such as flagellin, form an inflammasome complex resulting in caspase 1 activation, inflammatory cytokine production and cell death.

1.10 Inflammasomes and their role in bacterial infection

An inflammasome is a high molecular weight protein complex formed in the cytosol, which serves as a platform to recruit proinflammatory caspases such as caspase 1, caspase 4 and caspase 5 in humans, and caspase 11 and caspase 12 in mice, although only recruitment of caspase 1 has been well-established (159, 160). While the canonical inflammasome activation pathway involves NLR-medicated recruitment and assembly of ASC and caspase-1, caspase-1 activation has also been shown to be established by a novel mechanism involving caspase-11 (160). The non-canonical inflammasome activation pathway involves sensing cytosolic LPS, and activation of a caspase-11 containing inflammasome mediated by NLRP3 and ASC (160).

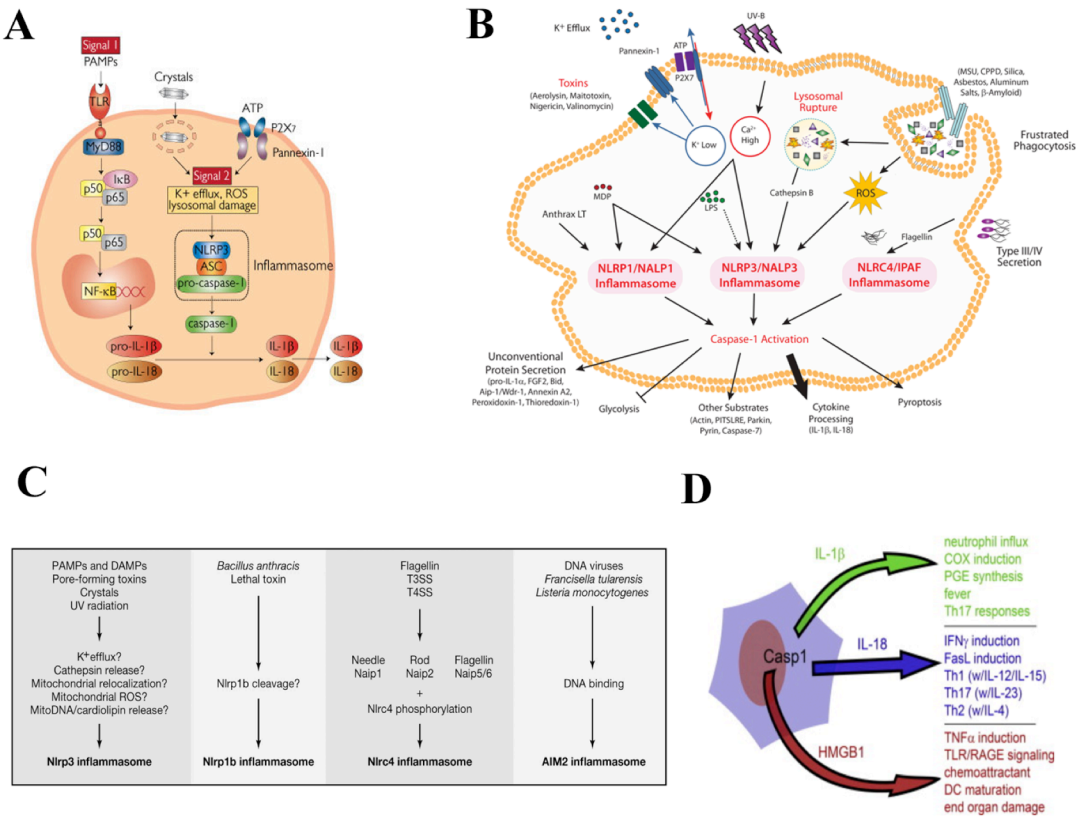

Figure 1.8 Inflammasome activation and its effects. Schematic showing the various steps involved in (A) inflammasome assembly and caspase 1 activation, (B and C) various stimuli that play activate the different inflammasomes, (D) biological function of caspase 1-dependent cytokines. (Modified from www.invivogen.com, (242) and (160))

Inflammasomes are activated in response to very diverse stimuli, ranging from intracellular ion changes to presence of crystals such as urate. Bacterial toxins such as lethal toxin (LT) from *B. anthracis* and bacterial type III and type IV secretion systems activate inflammasomes (161). In general, inflammasome activation has been shown to require two signals: Signal 1 works through the NF-κB pathway to increase expression of NLRs, pro-caspase, pro-IL-1β and pro-IL-18, and signal 2 activates the NLRs and triggers the assembly of inflammasome (160). The NLRs are auto inhibited in the absence of a ligand. In the presence of a suitable stimuli or ligand, the autoinhibition is released and NLR molecules then oligomerize through NBD and PYD domains (Figure 1.7) (160).

Inflammasome activation, irrespective of the stimuli or the mechanism, results in production of IL-1β, IL-18, and secretion of alarmins such as HMGB1 (160). Although IL-1β production is primarily inflammasome-mediated, there are other mechanisms of inflammasome-independent IL-1β production including caspase-8, ripotosome-(involving RIP1), and TNF receptor (TNFR)-mediated mechanisms (159). Proteinases such as matrix metalloproteinases (MMPs), cathepsin G, elastase, streptococcal exotoxin B have been shown *in vitro* to cleave pro-IL-1β into its active form (162). In addition to inducing cytokine production, inflammasome activation also protects against microbial infection by another mechanism called pyroptosis (163). Pyroptosis is a programmed cell death mechanism, unlike necrosis, but highly proinflammatory, unlike apoptosis. Pyroptosis is characterized by cytokine release by microvesicle shedding, cytokine secretion through caspase 1-dependent pores, chromosomal DNA destruction by endonuclease, cell swelling and lysis (159, 163).

Pores, roughly with a diameter of 1.1 to 2.4 nm, are formed on the plasma-membrane, which allows water influx and osmotic lysis (163).

The downstream effects of inflammasome activation include IL-1β-mediated neutrophil influx, production of prostoglandins and leukotrienes, a strong Th17 response and induction of fever. IL-1β, unlike IL-1α, is not expressed in all cells and limited mainly to myeloid cells (164). In addition, IL-1β needs to be cleaved into its active form before it can be secreted. The receptors for both IL-1β and IL-18, contain a Toll-IL1 related (TIR) domain and can signal through the adaptor protein MyD88 to result in NF-κB activation (164). IL-1β has been shown to activate a Th17 response by blocking IFNγ. In addition, IL-1β has also been shown to inhibit TGFβ- induced Foxp3 expression, both of which result in an augmented Th17 response. Although Th17 response has been found to be protective in STIs such as vaginal candidiasis, it had no protective function in a murine model of gonorrhea (165). In contrast, inhibition of Th17 response resulted in development of a protective immunity in the mice model for gonococcal infection (166). The other product of inflammasome is IL-18. Unlike IL-1β, IL-18 has been shown to promote skewing of immune response towards Th1 response, along with IL-12 or IL-15 (167). In some cases, IL-18 also promotes a Th17 response along with IL-23. Occasionally IL-18 has also been shown to perform a protective role by suppressing T-cell mediated response in mice model of *H. pylori* infection, preventing T-cell mediated tissue damage (168). Not all inflammasome activation is protective and useful. Under some conditions, IL-1β and IL-18 are either not beneficial or harmful (Table 1.5).

Beneficial	Baneful
IL-1β	
S. aureus	CAPS
Francisella tularensis	Gout
Influenza A virus	Rheumatoid arthritis
Herpes simplex 1 virus	Type 2 diabetes
Rhinovirus	Heart disease
Leishmania	EAE
Toxoplasma	*Pseudomonas*
	Burkholderia
	Leishmania
	Trypanosoma
Beneficial	Baneful
IL-18	
Listeria	CAPS
Shigella	Arthritis
NAFLD	Heart disease
Metabolic syndrome	EAE
Francisella tularensis	IBD
Burkholderia	*Pseudomonas*
Vaccinia	HIV
Influenza A virus	*Leishmania*
Leishmania	*Toxoplasma*
Toxoplasma	
Plasmodium	
Trypanosoma	

Table 1.5 Biological function of IL-1β and IL-18. Diseases in which IL-1β and IL-18 play beneficial or baneful role. (From (242))

1.11 Immune response against GC

Study of host immune response against *N. gonorrhoeae* is important to understand the absence of protective immunity and to understand subclinical nature of gonococcal infection. Studies in human volunteers have been limited due to practical and ethical considerations. Even these studies, however, were limited to male volunteers. Ramsey *et al* showed elevated levels of IL-8, IL-6, TNF-α and IL-1β in urine of male volunteers 48 hours after infection (169). However, only IL-1β levels correlated with onset of symptoms and an increase in leukocyte in urine sediment, whereas the other three cytokines increased as early as 2 hours post-inoculation. The correlation of IL-1β with onset of clinical symptoms implies that the source of IL-1β are migrating inflammatory cells. Production of IL-8 and IL-6 by endocervical epithelial cells has also been demonstrated. This is believed to be independent of TLR4 because endocervical epithelial cells lacked both TLR4 as well as MD2 (156).

N. meningitidis can activate TLR2 and TLR4 in THP1 cells (human monocyte cell line) using LOS (170). While this activity is not affected by sialylation of LOS or the presence of a capsule, other LOS variations affect cytokine levels in cell culture models (35, 170, 171). GC activation of TLR2 was observed to enhance infection of HIV-1 infection of T lymphocytes (172). The neisserial outer membrane proteins, porin, H.8 protein and Lip activate TLR2 and induce expression of inflammatory cytokines (24). Gonococci release peptidoglycan fragments outside, which are TLR2 agonists (24). Using CD4+ T cells, Mallet *et al* showed that gonococci-derived hexose monophosphate acted as a PAMP and induced cytokine production (173). While gonococci have a number of PAMPs that can potentially induce

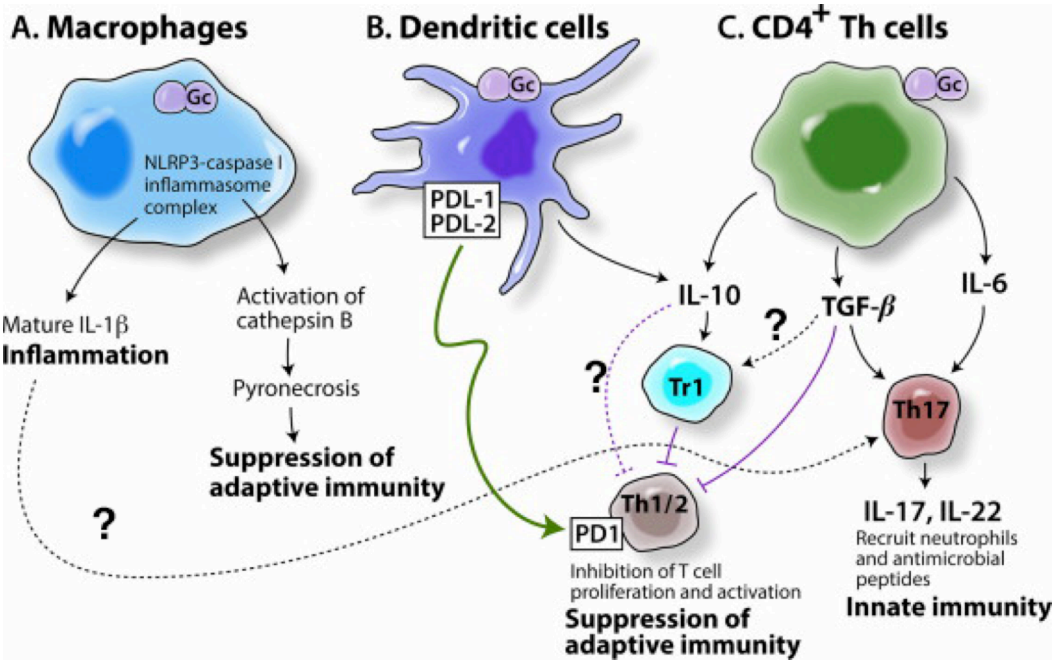

Figure 1.9 GC modulation of innate and adaptive immune response. (A) GC can activate inflammasome in macrophages, resulting in IL-1b production and pyronecrosis of APCs. (B) GC interaction with DCs results in IL-10 production and PDL-1 and PDL-2 mediated apoptosis of PD1-expressing Th cells. (C) GC interaction with CD4 cells induces production of IL-10, TGF-b and IL-6. IL-10 and TGF-b inhibit Th1 and Th2 activation. TGF-β and IL-6 induce Th17 cell response, leading to the recruitment or induction of innate defenses such as PMNs and anti-microbial peptides. (Adapted from (21)).

proinflammatory cytokine production the mechanism underlying asymptomatic infections are not yet completely understood (21).

Recently, Duncan *et al* showed that GC can activate cathepsin B in THP1 cells which in turn activated NLRP3 inflammasome resulting in subsequent production of IL-1β (174). In monocytes, porin has been shown to induce calcium influx and cause apoptosis, which was inhibited by using a caspase 1-specific inhibitor zYVAD-FMK (16). Since calcium influx has been reported to cause NLRP3 inflammasome formation, it is possible that porin can also induce inflammasome-mediated IL-1β release. While the role of IL-1β in gonorrhea is yet to be studied in detail, it has been shown as a very potent pro-inflammatory cytokine involved in pathogenesis of other autoimmune diseases such as rheumatoid arthritis, gout, and inflammatory bowel disease (159). It is suggested that GC-induced pyroptosis could lead to death of APCs and tissue monocytes, resulting in problems associated with development of protective immunity (Figure 1.9) (21, 174).

Gonococci interaction with CD4+ T cells has been shown to result in production of anti-inflammatory cytokines IL-10 and TGF-β (175). TGF-β has been shown to be responsible for mediating the switch from Th1/Th2 response to Th17 response in mice experiments, resulting in a strong innate immune response but weak adaptive immunity (166). Blocking TGF-β resulted in a stronger adaptive immune response. While these experiments explain the lack of adaptive response, the strong innate response seen in lab experiments using cell lines contradicts the feeble innate response seen in gonorrhea among female patients. In this study, I strove to understand innate immune response using cervical tissues obtained from anonymous

volunteer patients undergoing hysterectomy. Previous organ culture experiments with *N. gonorrhoeae* were limited to fallopian tube (FT) explants (176). Cytokine profile of GC incubation with FT explants showed secretion of TNF-α, IL-6, IL-1β. The FT model was useful to identify the correlation between TNFα levels and sloughing of ciliated epithelial cells, which could result in scarring and infertility associated with PID. However, fallopian tube infections are late events in the natural history of gonorrhea and are not representative of the vast majority of gonococcal infections.

1.10 Rationale

Gonorrhea is the second-most prevalent bacterial STI after Chlamydia infection (177). There are an estimated 820,000 infections per year in the US, with about 30% of them being resistant or showing reduced susceptibility to at least one antibiotic. The disease has the highest incidence in the sexually active age groups of 20 and 30 years (177). The general focus of this dissertation is to identify the diversity surface glycoconjugates in the *Neisseriaceae*, and study the role of glycoconjugates in gonococcal interaction with each other. Modulation of surface virulent determinants is one of the most common ways bacteria can manipulate the outcome of host-pathogen interaction. Our lab has focused on gonococcal LOS, its biosynthesis, and its role in immune response. Gonococci have a very elaborate surface protein glycosylation mechanism originally identified as pilin glycosylation system. The promiscuity of this system leads to numerous other surface proteins being glycosylated, leading to extensive surface glycoconjugate diversity. I hypothesized that modulation of surface protein glycosylation would be able to affect biofilm formation by affecting bacteria-bacteria interaction.

In the first part of this study, I look at other neisserial species and use bioinformatics tools to identify LOS biosynthetic genes in commensal *Neisseriaceae* strains. Using this approach has helped identify the differences in LOS between neisserial commensals and pathogens (22). Neisserial LOS is a major virulence determinant that activates TLR4 on host cell surface. LOS also a plays an important role in bacteria-bacteria interaction, by binding to Opa protein. This interaction can be characterized by measuring biofilm formation by the gonococci. While gonococcal biofilm formation has been demonstrated on biopsy samples from patients with gonorrhea, the mechanisms behind biofilm formation, maturation, bacterial escape and eventual degradation have not been identified. Biofilm formation has been observed to help bacteria evade immune system by attenuating host proinflammatory responses, leukocyte-mediated killing and complement activation. Understanding biofilm formation by gonococci would help us understand the role of biofilm in asymptomatic infections. In order to study this, I have developed techniques to form and visualize gonococcal biofilm on abiotic and biotic surfaces. I have used human cervical tissue samples to look at biofilm formation in a biologically relevant system and used scanning electron microscope to visualize and quantify biofilm formation In this study, I have identified a novel function for an intracellular enzyme previously thought to be only involved in peptidoglycan recovery pathway.

In the final part of this study, I demonstrate gonococcal activation of inflammasome in epithelial cells as well as cervical explant tissue. Inflammasome activation by gonococci has been shown in human monocytic cell lines. Pyroptosis, caused by inflammasome activation, is purported to reduce immune response by

destroying immune cells. The focus of the study is on the role of surface glycosides on *N. gonorrhoeae* and to understand their role in bacteria-bacteria interaction as well as bacteria-host interaction.

1.11 Specific Aims and Approaches

1.11.1 Aim 1: To characterize the diversity of LOS structure among the various strains *Neisseriaceae.*

While the LOS biosynthetic genes in neisserial pathogens, *N. meningitidis* and *N. gonorrhoeae*, have been well characterized, little is known about the LOS biosynthetic genes in commensal strains and their distribution among the various commensals. Using an *in silico* approach I will identify the different biosynthetic genes and their distribution among neisserial strains.

1.11.2 Aim 2: To understand how gonococci modulate glycosylation of their surface proteins to impact bacteria-bacteria interaction and biofilm formation.

Bacteria-bacteria interaction plays a major role in biofilm formation and bacterial escape from biofilm. I show the role of surface glycosylation and its modulation by NagZ in neisserial biofilm formation. Using confocal microscopy and scanning electron microscopy to visualize biofilm, I aim to understand the structure of gonococcal biofilm and its evolution over time.

1.11.3 Aim 3: To understand how gonococci interaction with host cells affects inflammasome-mediated immune response.

Bacterial interaction with epithelial cells resulting in an inflammatory response is the hallmark of acute disease. Using a fluorescence assay to detect activated caspase 1, I show that gonococci can activate inflammasome in epithelial cells. I also used a human cervical explant model to study interaction of *N. gonorrhoeae* with host tissue and study cytokine production in response to gonococci.

Chapter 2: Lipooligosaccharide diversity in Neisseriaceae and their analysis using genomic data

2.1 Introduction

Members of the genus exhibit diverse LOS phenotypes. While *N. meningitidis* is an opportunistic pathogen, *N. gonorrhoeae* is an obligate pathogen (40). However, meningococcal infections are associated with a higher rate of morbidity and mortality in comparison to gonococcal infections (178). Colonization with *N. lactamica* has been found to induce cross-reacting antibodies that protect against meningococcal meningitis, while none of the other commensals elicit any clinically observable immune response (179). This suggests a spectrum of virulence among *Neisseria* spp, with *N. meningitidis* on one end and all the other commensals at the other end with *N. gonorrhoeae* in between the two. As seen in figure 2.1, phylogenetic analysis showed that most of the commensals cluster away from the pathogens except *N. lactamica*, *N. cinerea* and *N. polysaccharea*, (180). The phylogenetic clusters also correlate with the observed spectrum of virulence within *Neisseriaceae.* The reasons for these varied host-pathogen interaction are still not completely understood.

Pathogenic *Neisseria* spp. elicit a stronger innate immune response than any of the commensal *Neisseria* spp (35). The major virulence determinants in *Neisseria* are capsule (present only in *N. meningitidis*), colony opacity-associated proteins (Opa and Opc), lipooligosaccharide (LOS), Type IV pili, H.8. protein and porin (40). The

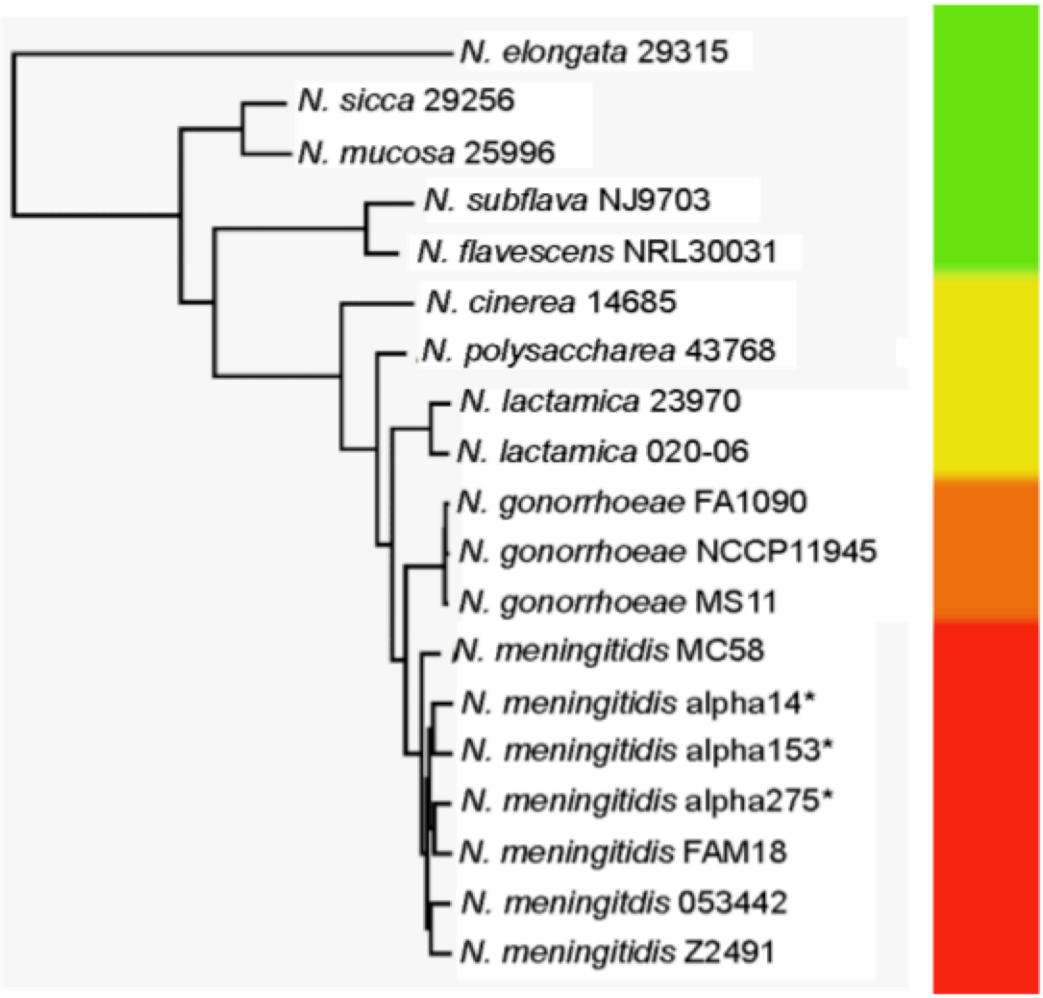

Figure 2.1. Phylogenetic analysis of the various members of *Neisseriaceae.* Maximum likelihood tree based on the concatenated sequence of 896 core neisserial genes shared by all members. The carrier meningococci strains are marked by an asterisk. The phylogenetic relationship is consistent with observed virulence of each of these organisms, which is represented on the right with decreasing degrees of virulence from bottom to top, with red being associated with significant disease, and green indicating the absence of disease. Figure is modified from (180).

majority of neisserial commensal strains lack Opa proteins, porin homologs, and functional pili (180). LOS is a naturally occurring variant of lipopolysaccharide (LPS). LOS, unlike LPS, lacks the O-repeating carbohydrate polymer, and instead typically has a shorter oligosaccharide chain in its place. Both molecules however have a core polysaccharide unit covalently connected to the lipid A moiety. Lipid A typically has six fatty acid tails attached to two glucosamine residues in various combinations in different organisms (22, 27). On a given bacterium it has been estimated that it contains approximately 2 million lipid A residues (181).

Pathogen-Associated Molecular Patterns (PAMPs), such as LPS are recognized by Pattern Recognition Receptors (PRRs), such as TLR4. Although LOS lacks the O-carbohydrate, it has still been shown to activate TLR4 (182). Neisserial LOS, by causing widespread activation of TLR4 and hence 'cytokine storm', causes the endotoxic shock seen in fulminant meningococcal shock (183). The biologically active portion of both LOS and LPS has been established as the lipid A portion (184). Lipid A activates TLR4 by forming a heterodimer of TLR4 and MD-2 (185, 186). Myeloid Differentiation factor-2 (MD-2) is a co-receptor for LPS/LOS (187). MD-2 is essential for expression of TLR4 on the cell surface as well as down- stream signaling following activation of TLR4 (186). Structural studies of this LPS-TLR4-MD-2 complex have shown that LPS bridges TLR4 and MD-2 by having five of the six fatty acid tails buried in hydrophobic pocket in MD-2 and the other fatty acid forms a hydrophobic interaction with the conserved phenylalanine in TLR4 (188).

The first step in LOS activation of TLR4 is extraction of individual LOS molecules from the bacterial outer membrane. This process is aided by human LPS-

binding protein (LBP), an acute phase protein, secreted by hepatocytes (34). LBP-LOS complex is readily detected by both soluble and membrane-bound CD14 (a GPI-linked protein on the surface of myeloid cells), which further relays LOS to host cell membrane-bound receptors. While LBP is not essential for LOS binding to CD14 (but it significantly enhances LOS binding to CD14), CD14 is essential for LOS' proinflammatory activity (189). LBP enzymatically transfers LOS to CD14, both membrane-bound and soluble. Transfer of LOS to TLR4 by CD14 leads to the formation of TLR4-MD2-LOS complex, that then dimerizes, leading to downstream signaling resulting in a myriad of effects (190). Two different pathways, namely MyD88-dependent and MyD88-independent, mediate the downstream activation. Distinct cytokine profiles have been observed for each one of these pathways. While the MyD88-dependent pathway results in secretion of TNFα, IL-1β, MCP-1 and MIP-3α, the MyD88-independent pathway releases IFNβ, EDRF, IFNγ inducible pro- tein 10 (IP-10) and Type-I IFN (190). Bacterial LOS/LPS can activate both pathways. LOS-activated TLR4-MD-2 complex turns on the downstream cascade, resulting in nuclear translocation of transcription factors such as NF-κB, which promote expression of proinflammatory cytokines. Variations in the levels of cytokine, induced by TLR4 activation, have been observed between different types of LOS molecules (191).

Neisserial pathogens induce a wide array of cytokines by TLR2- and TLR4-mediated pathways. Absence of a TLR4 response was found to be associated with increased bacterial load and defective neutrophil-mediated killing in mice models of gonorrhea (192). While it is tempting to believe that non-engagement with TLR4

would lead to a favorable environment for gonococci, LOS is essential for viability of non-capsulated *Neisseria*. Gonococci have evolved to engage TLR4 and still survive the innate immune response. Neisserial engagement of TLR4 was found to induce a specific subset of cytokines leading to a Th17 response, which precludes development of immune memory, induces neutrophil infiltration, and secretion of cationic antimicrobial peptides (165). Experiments using anti-TGFβ to divert immune response from Th17 to Th1/Th2 response resulted in faster clearing of primary infection, and protection against secondary infection (166). While the mechanism by which gonococci guides TLR4 activation to a Th17 response is still not understood, it is evident that the bacteria can modulate host response by engaging with TLR4.

Modulation of immune response by changing LOS/LPS structure has also been observed in other species. The oral pathogen *Porphyromonas gingivalis* expresses diverse forms of LPS which have been observed to activate both TLR2 and TLR4 in mice (193). *P. aeruginosa* has been found to change between penta-acylated and hexa-acylated versions of LPS in cystic fibrosis and bronchiectasis respectively. This difference in LPS structure was detected by human TLR4 (194). Commensal bacteria, which also express LOS, are continually sampled by the immune cells in the lymphoid tissue but, paradoxically, fail to elicit a sustained local inflammatory response (195). Jarvis *et al* showed that neisserial pathogens induce significantly higher levels of TNFα from human monocytic cells in comparison to commensal species (35). The differences in the physical structure of LOS between commensals and pathogens could be one of the reasons for the difference in their inflammatory potential. Expression of LOS with different physical structure, combined with TLR4

polymorphism, can also explain the wide variations in clinical presentation of gonorrhea.

The biosynthetic pathway of LPS has been well characterized, and the enzymes involved have been targets for developing novel antibiotics. In *Neisseriaceae,* LOS has been observed to undergo high frequency variation in structure due to phase variation of LOS biosynthetic genes and the relaxed specificity of some of the enzymes involved in LOS biosynthesis (24, 27). This variation has been implicated in disease pathogenesis as well as immune evasion. While LOS biosynthesis on neisserial pathogens has been well studied, not much is known about LOS structure in the commensals. Limited studies on commensal LOS suggest that LOS variability is higher in commensals than among the pathogenic strains. Our lab had earlier showed that one of the commensal strains was able to express an LPS, unlike any of the other neisserial species. The O-repeats were identified as disaccharide repeats of rhamnose and GlcNAc (196). While *N. sicca* is a commensal species, this particular strain was isolated from a fatal case of endocarditis. It is possible that production of an O-repeat could have increased the pathogenicity of this strain. More importantly, this highlights the heterogeneity of LOS structure in neisserial commensals.

I took a bioinformatic approach to identify the LOS biosynthetic genes in neisserial commensals. In this study, I used bioinformatic tools to identify genes involved in LOS biosynthesis in various *Neisseriaceae* to predict the diversity of LOS structure among the different species. I also identified patterns of gene distribution that correlated with empirical data about the corresponding bacteria-host interaction, which led me to identify commensal-restricted LOS biosynthetic genes. I

hypothesized that those commensal-restricted genes could be responsible for attenuating TLR4-mediated immune response. In order to test this hypothesis, I knocked out one of the commensal-restricted genes and one of the pathogen-restricted genes and used those mutants to compare TLR4 response before and after gene knockouts.

This study is important for understanding LOS biosynthesis among commensal species in *Neisseriaceae*. In addition to demonstrating the diversity of LOS structure among the various species I also attempted to define the structure-function relationship of neisserial LOS using the LOS inner core as proof-of-concept. Identifying structure-function relationship of LOS will help us design better vaccines. This work was published in PloS One in 2011 in a paper titled "Sequence –based predictions of lipooligosaccharide diversity in *Neisseriaceae* and their implication in pathogenicity". While the sequencing, assembly of sequencing reads and identification of ORFs was performed by Clint Miller and Daniel Sommer, I generated all the figures shown in this chapter.

2.2 Materials and Methods

2.2.1 Commensal *Neisseria* sequencing

Chromosomal DNA for *N. sicca* 4320, *N. sicca* DS1, *N. polysaccharea* 342, *N. sicca* NS19, and *N. meningitidis* 44 was isolated using the method described in Maniatis et al. The DNA sequence of each of these samples was determined by the Genomics Resource Center at the University of Maryland School of Medicine, Baltimore, MD. The sequence reads were assembled using Newbler to generate a set

of contigs for each commensal (197). The sequence reads were assembled by Daniel Sommer and curated for submission tin NCBI by me. The genomic sequences were submitted to NCBI: *N. sicca* 4320, GenomeProject ID #60861; *N. sicca* DS1, GenomeProject ID #60863; *N. polysaccharea*e 342, GenomeProject ID #60865; *N. lactamica* NS19, GenomeProject ID #60867; and *N. meningitidis* 44, GenomeProject ID #60869.

2.2.2 Mummer alignments

Unique regions of the contig sequences of *N. sicca* 4320 were determined by alignment with the contig sequences of the other identified commensals using Nucmer, a component of the Mummer software package (198). The alignments were filtered to remove repeat alignments leaving a 1-to-1 alignment between *N. sicca* 4320 and the other sequenced commensal. Regions not aligning were identified as unique to *N. sicca* 4320. This work was performed by Clint Miller and Daniel Sommer.

2.2.3 Geneious

The contig sequences of the commensals were imported into the program Geneious (Geneious Inc, New Zealand). Custom BLAST databases of each sequenced commensal were compiled to use for further analysis. Geneious was used to manage all sequences and to construct the sequence figures found in this manuscript.

2.2.4 ORF annotation

The contig sequences were analyzed by the program Glimmer3 and putative

ORFs predicted. The amino acid sequences of the predicted ORFs were extracted and used as queries in BLAST searches of the various NCBI and custom databases. The ORFs were annotated by Clint Miller and me.

2.2.5 Bioinformatic BLAST screen of *Neisserial* sequence

The custom commensal BLAST databases were searched to identify sequences with similarity to glycosyltransferases. ORFs showing similarity to the sequences were used as a query in a BLAST search of the nr database to confirm similarity to putative O-repeat biosynthesis genes.

2.2.6 THP1Xblue Assay

THP1Xblue cells were obtained from Invivogen and grown in appropriate selection media to maintain the transfection. The cells have alkaline phosphatase gene under NFκB-promoter. The cells were seeded at 10e5 cells per well in 96 well plate and treated with bacteria at an MOI of 10 for 6 hours. The supernatant was collected and assayed using the Quantiblue (Invivogen) substrate, which turns blue if alkaline phosphatase is expressed as a result of NF-κB activation (199). The results are measure using a spectrophotometer and relative levels compared.

2.3 Results

2.3.1 Analysis of KDO transferases in *Neisseriaceae*

Lipid A is connected to the hydrophilic portion of LOS by KDO, which is added by KDO transferase while the lipid IVA moiety is still attached to the inner leaflet and facing the cytoplasm of the bacteria. Lipid IVA refers to the lipid A

structure with four fatty acid tails instead of the usual six. The other two tails are added after the two KDO groups are added to lipid IVA, resulting in what is referred to as KDO_2-Lipid A. The KDO transferase in *E. coli* has been shown by Raetz *et al* to be bifunctional, meaning it can add both the KDOs onto lipid IVA before the 'late' acyltransferases begin acting (200). The 'late' acyltransferases add the final two fatty acid tail to KDO_2-Lipid IVA. However, bacteria such as *H. influenzae* have a KDO transferase that is monofunctional and *C. trachomatis* possesses a trifunctional KDO transferase (183).

Since KDO transferase's functionality cannot be determined by sequence analysis, I used sequence of *kdkA* gene, which encodes the second KDO transferase in *Haemophilus* spp. to search for the second KDO transferase among all the available neisserial genomes. I did not identify any sequence with homology to *kdkA*, which led us to conclude that neisserial KDO transferase or WaaA protein is a bifunctional enzyme. The data in figure 2.2, shows that all neisserial species have a highly conserved *waaA* gene as seen by the consensus logo for neisserial pathogens, commensals *N. cinerea* and *N. lactamica*, and the other neisserial commensals. Consensus logos are a graphical representation of sequence alignment where the relative size of each letter (representing an amino acid) corresponds to the frequency of the amino acid among all the sequences used to generate the logo. The pathogens all have uniformly large sized letters representing their amino acids implying very little variability between the two species and the many strains used for sequence alignment. The commensals appear to have conserved in most parts of the protein, except the C-terminal where there is significant variability as seen by the variable size

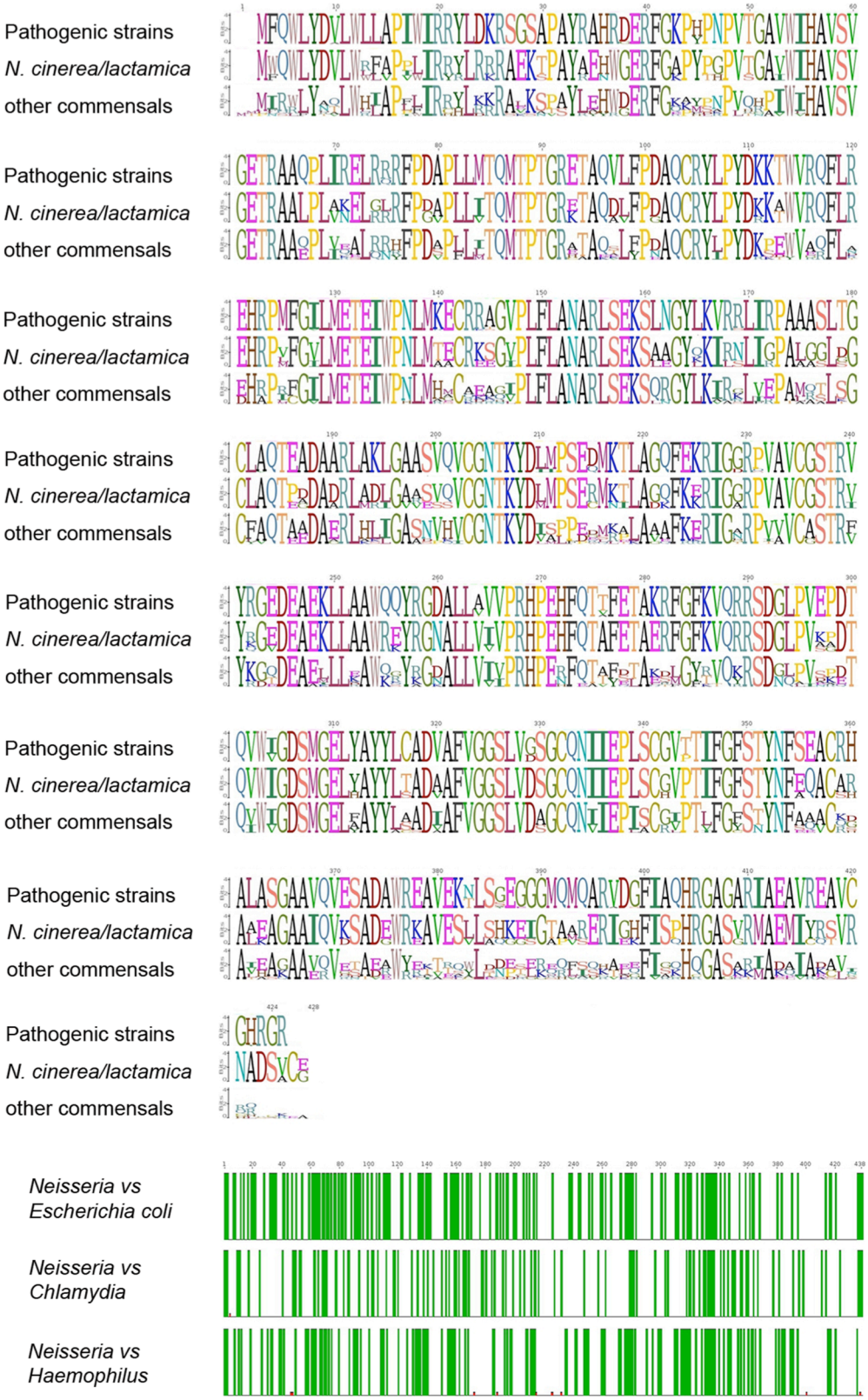
Pathogenic strains
N. cinerea/lactamica
other commensals
Pathogenic strains
N. cinerea/lactamica
other commensals
Pathogenic strains
N. cinerea/lactamica
other commensals
Pathogenic strains
N. cinerea/lactamica
other commensals
Pathogenic strains
N. cinerea/lactamica
other commensals
Pathogenic strains
N. cinerea/lactamica
other commensals
Pathogenic strains
N. cinerea/lactamica
other commensals
Neisseria vs Escherichia coli
Neisseria vs Chlamydia
Neisseria vs Haemophilus

Figure 2.2 WaaA is highly conserved within *Neisseriaceae*. (A) Consensus logo created using the translated sequence of *waaA* from various neisserial strains. (B) Neisserial *rfaC* sequence compared against the *E. coli waaA, C. trachomatis waaA* and *H. influenzae waaA*. Neisserial *waaA* had highest similarity with the bifunctional *waaA* sequence from *E. coli*. The DNA sequences used for this analysis were *N. gonorrhoeae* strains; 1291, 35/02, DGI18, DGI2, F62, FA19, FA6140, MS11, PID1, PID18, PID24-1, PID332, SK-92-679 and SK-93-1035 [sequences from the Broad Institute], FA1090 (accession no. AE004969); and NCCP11945 (accession no. CP001050). DNA sequences for *N. meningitidis* were NS44 (DCS, unpublished sequence), 053442 (accession no. CP000381), FAM18 (accession no. AM421808), 22491 (accession no. AL157959-1), MC58 (accession no. AE002098.2), alpha 14 (accession no. AM889136.1). Commensal Neisserial strain DNA sequences were: *Neisseria cinerea* ATCC 14685 (accession no. ACDY00000000); *N. elongata* subsp. glycolytica ATCC 29315 (accession no. ADBF00000000); *N. flavescens* strain SK114 (accession no. ACQV00000000) and strain NRL30031/H210 (accession no. ACEN00000000); *N. lactamica* ATCC 23970 (accession no. ACEQ00000000), Y92-1009 (Project ID: 50739), ST-640 (Project ID: 13472), and strain NS19 (DCS, unpublished sequence); *N. mucosa* ATCC 25996 (accession no. ACDX00000000) and C102 (Project ID: 38747); *N. polysaccharea* ATCC 43768 (accession no. ADBE00000000) and NS342 (DCS, unpublished sequence); *N. sicca* ATCC 29256 (accession no. ACKO00000000), 4320 (DCS, unpublished sequence) and DS1 (DCS, unpublished sequence); *Neisseria sp.* oral taxon 014 str. F0314 (Project ID: 49701); and *N. subflava* NJ9703 (accession no. ACEO00000000). The DNA sequences for *Haemophilus influenzae* were: Rd KW20 (accession no. L42023), 86-028NP (accession no. CP000057), PittEE (accession no. CP000671) and PittGG (accession no. CP000672. The DNA sequences for *Chlamydia trachomatis* were: strain 434/Bu (accession no. B0B9V8) and strain A/HAR-13 (accession no. Q3KMF4).

of each amino acid. Comparison of neisserial *waaA* sequence against *waaA* sequence from *E. coli, C. trachomatis* and *H. influenzae* (panel B, figure 2.2) shows that neisserial *waaA* has highest similarity with the monofunctional *waaA* from *E. coli.* This suggests that all neisserial species should express LOS with two KDO molecules connecting the inner core to lipid A, which is supported by previous analysis of neisserial LOS using mass spectrometry.

2.3.2 Distribution of heptosyl transferases among Neisseriaceae

Structural analysis of gonococcal and meningococcal LOS shows that they have two heptose molecules in the inner core, which have the α- and β-chain linked to each one of them. The first heptose is added by RfaC and the second heptose is added by RfaF. In order to study the distribution of *rfaC* gene among neisserial commensals, I identified putative *rfaC* from all the neisserial genomes by BLAST and aligned them. As shown in panel A of figure 2.3, the synteny was identical in all the neisserial genomes. While there was virtually no variation among gonococcal strains in the RfaC protein sequence as seen in the consensus logo representation of gonococcal RfaC, meningococcal strains differed significantly within only 8 strains (panel B of figure 2.3). The sequences in *N. lactamica* and other commensals exhibited even more variability. In addition, mutations in the stop codon in commensals resulted in the readthrough adding between 4 and 16 amino acids to the protein. The reason for the high degree of conservation in gonococcus unlike the other neisserial species is not very clear.

I looked at distribution of *rfaF* gene among the neisserial species by BLAST analysis of the genomes using *rfaF* sequence of GC as the input sequence. All strains

A


pncA
rfaC
etfB

B

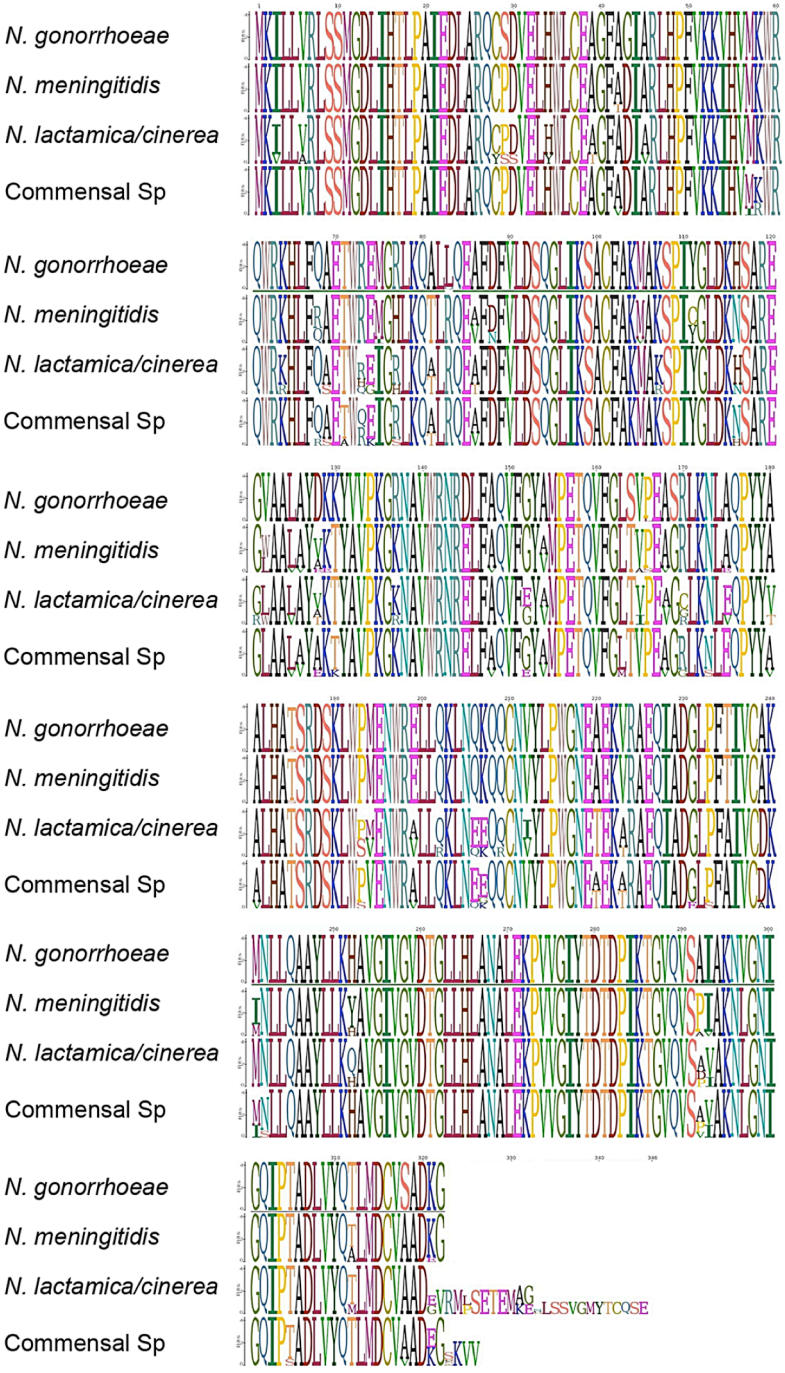
N. gonorrhoeae
N. meningitidis
N. lactamica/cinerea
Commensal Sp
N. gonorrhoeae
N. meningitidis
N. lactamica/cinerea
Commensal Sp
N. gonorrhoeae
N. meningitidis
N. lactamica/cinerea
Commensal Sp
N. gonorrhoeae
N. meningitidis
N. lactamica/cinerea
Commensal Sp
N. gonorrhoeae
N. meningitidis
N. lactamica/cinerea
Commensal Sp
N. gonorrhoeae
N. meningitidis
N. lactamica/cinerea
Commensal Sp

Figure 2.3 Analysis of the *rfaC* region in the *Neisseriaceae*. (A) Genomic organization of the *rfaC* region. The gene *pncA* encodes pyrazinamidase/nicotinamidase, and the gene etfB encodes an electron transfer protein. (B) Sequence logo alignment of the *rfaC* gene from the various sequenced neisserial strains. Same strains as listed the previous figure were used for the analysis.

possessed an ORF with greater than 88% similarity across the entire sequence, suggesting that all neisserial species possess at least two heptose residues in their LOS as well as the selective pressure acting on maintenance of *rfaF* gene. The selective pressure is further highlighted when I looked at the synteny which was highly different between the pathogens and commensals and even within the various commensal strains, as represented in figure 2.4.

2.3.3 Elongation of alpha chain with lgtF in *Neisseriaceae*

The alpha chain begins by adding the first sugar on to the first heptose in the core region. This sugar, glucose, is added by LgtF. The genomic organization of *lgtF* was analyzed among all the neisserial species. In pathogenic species, *lgtF* and *rfaK*, which encodes a N-acetylglucosamine transferase, form a two gene cluster. Experimentally it has been demonstrated that LgtF can add a glucose to the first heptose only after RfaK has finished transferring a GlcNAc to the second heptose. This could explain the reason the two genes are clustered together in the genome.

Pathogenic species and the closely related commensals, *N. lactamica* and *N. cinerea,* possessed both *lgtF* and *rfaK*. Figure 2.5 shows that the synteny was also conserved among the multiple strains in the four species. However, *N. elongata* has an ORF encoding N-acetyl galactosamine transferase instead of *rfaK.* The other commensal neisserial species encoded a fusion protein with the N-terminal half being similar to LgtF and the C-terminal half being similar to the GalNAc transferase seen in *N. elongata.* Since the function of the novel two-domain glycosyltransferase has not yet been determined, it needs to be verified if the commensal neisserial species

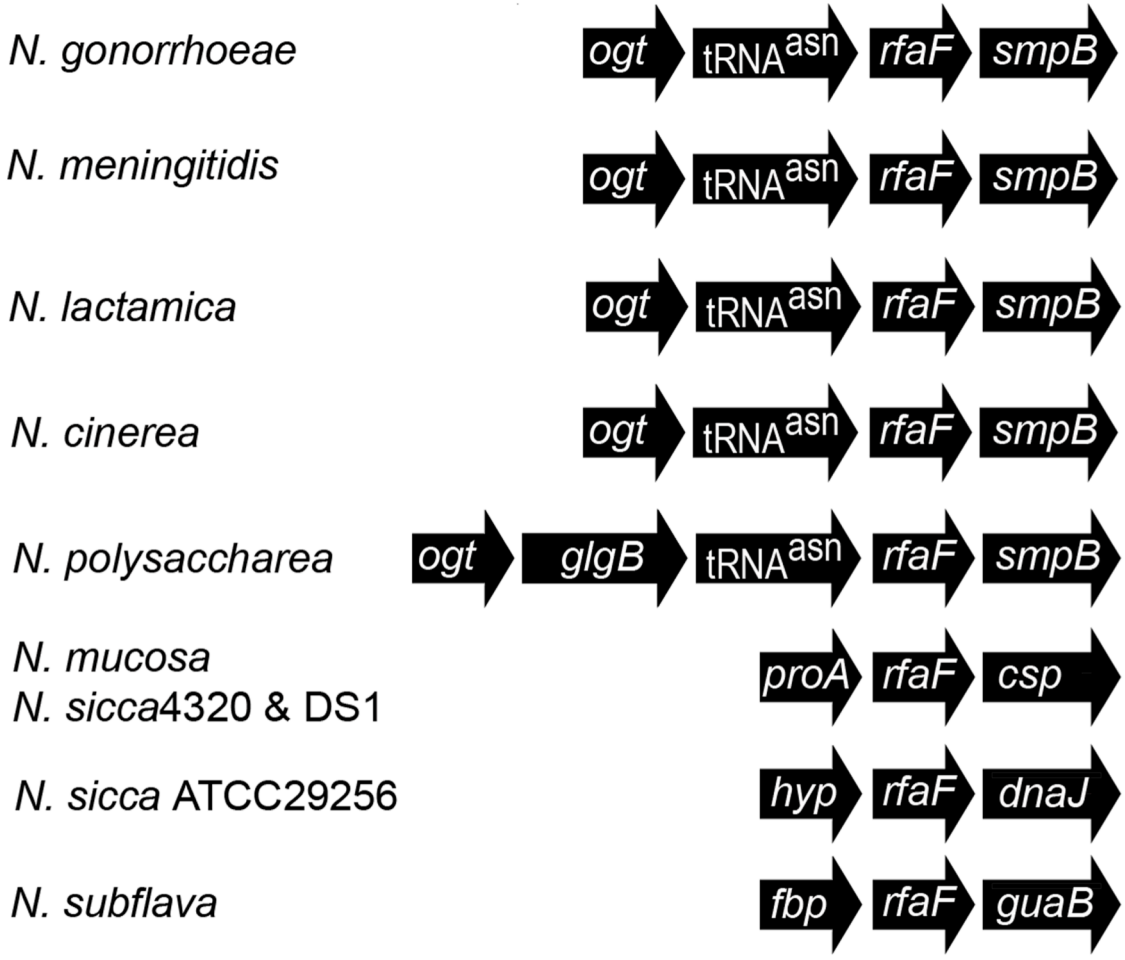

Figure 2.4 Analysis of the *rfaF* region in the *Neisseriaceae*. The flanking genes encode: *ogt*, O-methylguanine-DNA methyltransferase; *smpB*, *RNA-binding protein*, *proA*, gamma-glutamylphosphate reductase, *csp*, cold shock protein; *hyp*, hypothetical protein; *dnaJ*, Heat shock protein; *flp*, recombinase; and *guaB*, inosine 5′-monophosphate dehydrogenase.

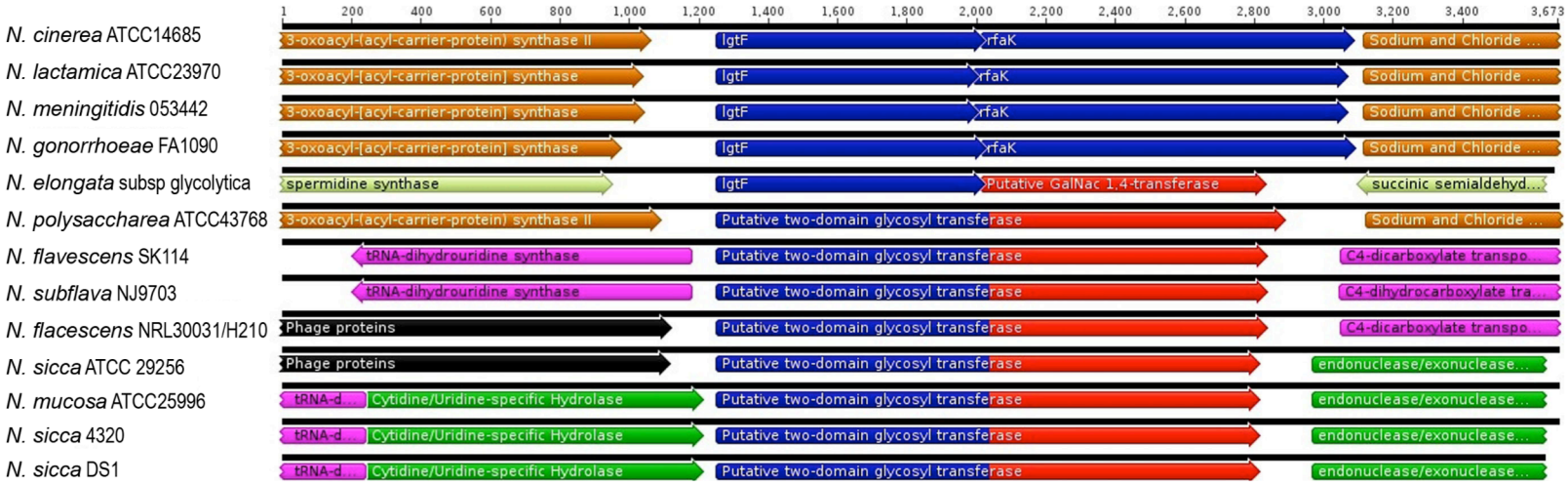

Figure 2.5 Genomic organization of genes involved in initiating alpha-chain synthesis. While all neisserial pathogens and the closely related commensals, *N. lactamica* and *N. cinerea*, have *lgtF*, *N. elongata* has an *lgtF* and a putative GalNAc transferase instead of *rfaK* (a GlcNAc transferase). The rest of the commensals have an ORF representing an LgtF-GalNAc transferase fusion protein.

elongate their LOS via a sugar other than glucose. We also observed that the synteny was variable within the rest of neisserial commensals.

2.3.4 Distribution of gamma chain N-acetylglucosamine transferase

I examined the distribution of *rfaK* gene among the different strains by performing BLAST analysis using *N. gonorrhoeae* FA1090 *rfaK* as template. I discovered that all the pathogenic species possessed an ORF with 100% identity to our template. The closely related commensals *N. lactamica, N. cinerea,* and one strain of *N. polysaccharea* possessed an ORF with 95% identity. All strains that possessed both *lgtF* and *rfaK* had the same genomic organization, with the same flanking genes. None of the other commensals had any sequence with similarity to RfaK. Searching the commensals using sequence information of the flanking genes showed that the two genes were not linked. This suggests that the ability to extend a gamma chain by adding GlcNAc was limited to pathogens and closely-related commensal *Neisseriaceae.*

Previous experimental work in our lab has shown that LOS from *N. sicca* 4320 had three heptose residues instead of two. This suggested that commensals could possibly extend their gamma chain by adding a third heptose instead of GlcNAc. I used the *E. coli* DH1 heptosyl transferase (WaaQ) sequence to search the neisserial genomes, since *E. coli* has been shown to have three heptoses in the inner core region of its LPS. All the neisserial strains that lacked RfaK, possessed a gene that encoded a putative protein with >50 % identity to *E. coli* WaaQ. Table 2.1 shows the distribution of *rfaK* and *waaQ* among all the neisserial genomes, since they both

Strain	*rfaK*	*rfaC*	*waaQ*
Gonococci	+	+	−
Meningococci	+	+	−
N. cinerea ATCC 14685	+	+	−
N. lactamica ATCC 23970	+	+	−
N. lactamica NS19	+	+	−
N. lactamica (Sanger)	+	+	−
N. polysaccharea	+	+	−
N. polysaccharea NS342	−	+	+
N. mucosa ATCC 25996	−	+	+
N. flavescens NRL30031/H210	−	+	+
N. sicca 4320	−	+	+
N. sicca DS1	−	+	+
N. sicca ATCC 29256	−	+	+
N. subflava NJ9703	−	+	+

[+]Presence of gene sequence.
[−]Absence of gene sequence.

Table 2.1 Distribution of genes involved in LOS core synthesis. Distribution of genes involved in addition of LOS core heptose (*rfaC*) and extension of gamma chain (*rfaK* and *waaQ*) is shown above. Members of *Neisseriaceae* appear to have two different ways to extend their gamma chain; addition of GlcNAc or a third heptose.

target the same acceptor molecule. The mutually exclusive distribution of these two genes suggests that neisserial strains possess two basic core structures; one with a gamma GlcNAc and one with a third heptose in place of GlcNAc.

2.3.5 Modification of LOS with sialic acid

As described earlier, sialylation of LOS protects the bacteria from complement-mediated killing and leads to increased virulence. However, GC does not possess the synthetic machinery to make sialic acid and instead scavenges CMP-NANA from the host, and uses a sialyltransferase (Lst). I screened all the neisserial genomes using Lst sequence from FA 1090. All the pathogenic strains possessed Lst with more than 97% identity to FA 1090 Lst. Among the commensals, except *N. lactamica* and *N. polysaccharea*, there was no hit matching Lst sequence. Figure 2.6 shows the genomic organization of *lst.* For strains that did not contain Lst, the genomes were screened with flanking ORFs from *N. gonorrhoeae* FA 1090 (both cytochrome c and isocitrate dehydrogenase). It appears that *N. cinerea* had deleted *lst* gene since it has the same flanking sequence as *N. lactamica* and *N. polysaccharea.* None of the other commensals possessed *lst* gene as shown, possibly due to the lack of selective pressure for sialylation in the absence of a strong immune response against the commensals.

2.3.6 Distribution of other putative LOS biosynthetic gene

The alpha chain is elongated by a series of enzymes that are clustered together. These enzymes LgtA, LgtB, LgtC, LgtD and LgtE have been well characterized and their functions identified as shown in figure 3. All of these five genes are present in

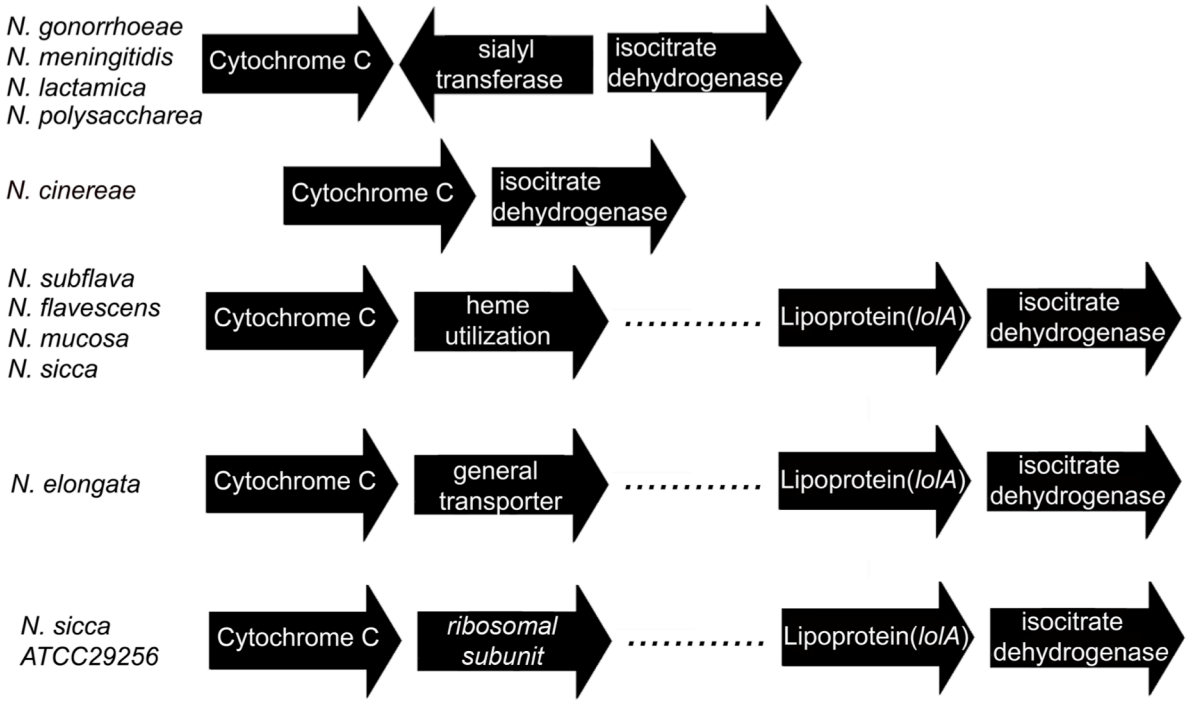

Figure 2.6 Genomic organization of regions that might contain *lst*. The presence of the *lst* gene in the genomes of the various neisserial strains was determined by search the genomes with the gonococcal Lst protein sequence (Accession number AAC44539). In strains that did not contain the Lst protein, the genomes were search with the flanking ORFs (cytochrome c or isocitrate dehygrogenase). These genes were identified in the genomes, and the flanking gene to these identified by performing a Blast search of the adjacent ORF

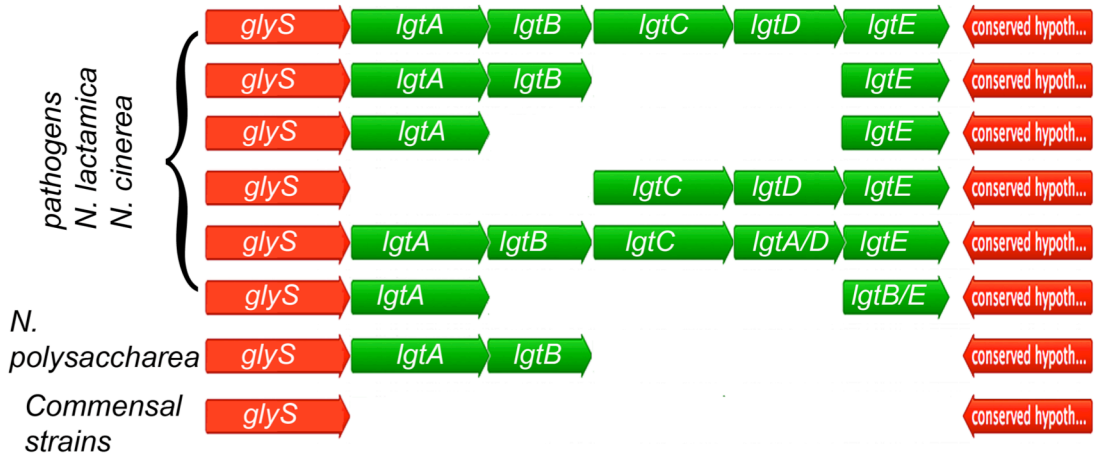

Figure 2.7 Genomic organization of the *lgt* gene cluster. The *lgt* gene cluster in *N. gonorrhoeae* strain F62 is flanked by two ORFs, glycyl tRNA synthase and a conserved hypothetical protein. Various organizational structures of the *lgt* genes were found in the pathogens, *N. lactamica*, *N. cinerea*, and *N. polysaccharea*, as indicated. All other commensal organisms tested had the glycyl tRNA synthase and a conserved hypothetical protein adjacent to each other**.**

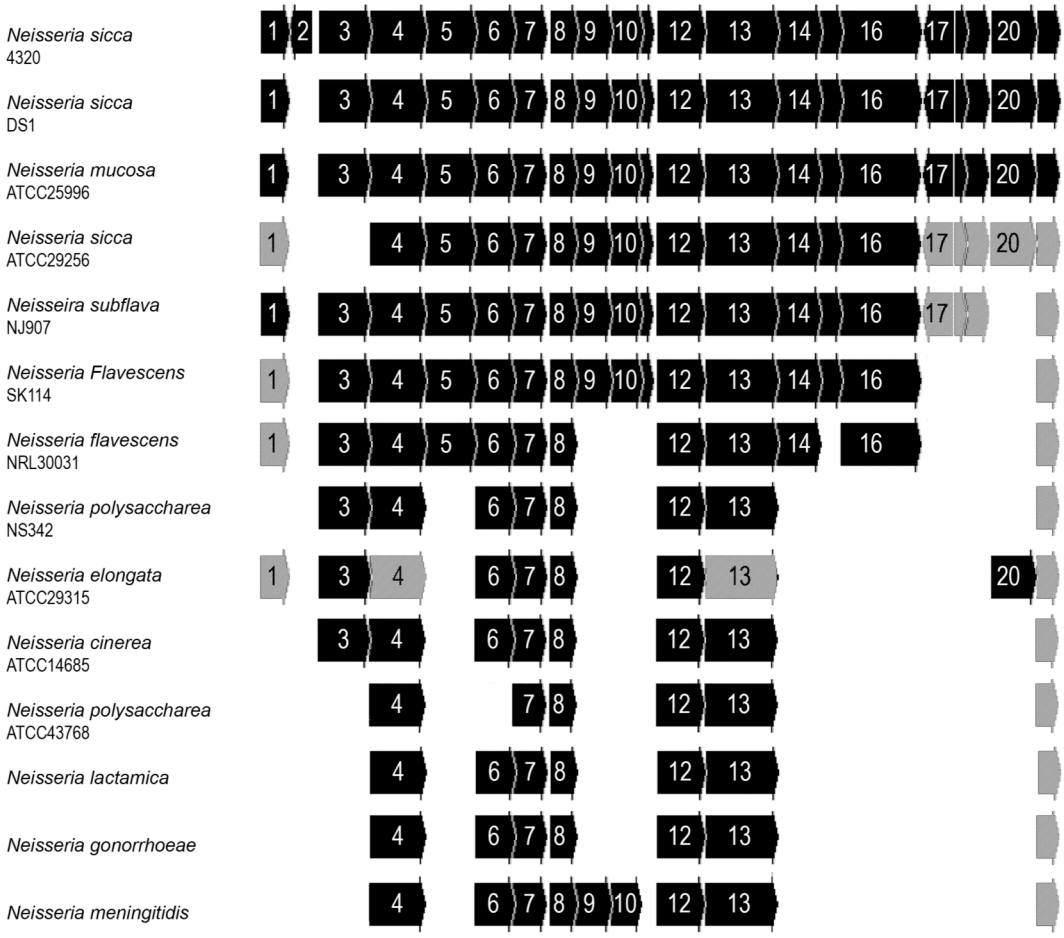
Neisseria sicca
4320
Neisseria sicca
DS1
Neisseria mucosa
ATCC25996
Neisseria sicca
ATCC29256
Neisseira subflava
NJ907
Neisseria Flavescens
SK114
Neisseria flavescens
NRL30031
Neisseria polysaccharea
NS342
Neisseria elongata
ATCC29315
Neisseria cinerea
ATCC14685
Neisseria polysaccharea
ATCC43768
Neisseria lactamica
Neisseria gonorrhoeae
Neisseria meningitidis

Figure 2.8 Genomic organization of the putative LPS biosynthetic gene cluster. The numbers contained within the arrows refer to open reading frames found on Contig 56 that contained genes with homology to LPS biosynthetic genes. There were 21 ORFs. ORFs 11, 15, 18, 19 and 21 are not numbered as they were too small to fit in the figure. All ORFs were compared to the organization as seen in strain *N. sicca* 4320. A solid black ORF indicates that the gene was found in the designated location. A stippled ORF indicates that the gene is present in a location elsewhere on the chromosome. Missing ORFs were not found in the test organism. All strains are as designated. If no species identification is present, it indicates that all strains from that species had the same genomic organization. The numbers located in the ORFs refer to the following proteins, which had the highest degree of similarity to proteins found in the NCBI database: 1) Rrf2-linked NADH-flavin reductase; 2) Unknown protein; 3) UDP-glucose dehydrogenase; 4) Lipopolysaccharide biosynthesis translocase; 5) Oligosaccharide repeat unit polymerase Wzy; 6) Capsular polysaccharide biosynthesis glycosyl transferase capM; 7) Glycosyl transferase); 8) UDP N-acetyl galactosaminyl transferase; 9) Putative carbamoyl phosphate synthase large subunit, short form; 10) 2-Haloalkanoic acid dehalogenase; 11) Methionyl tRNA formyl transferase; 12) Pleiotropic regulatory protein; 13) Nucleoside-diphosphate sugar epimerase; 14) Polysaccharide export protein (Wza); 15) Low-molecular weight protein-tyrosine phosphatase (Wzb); 16) Tyrosine-protein kinase (Wzc); 17) CidA-associated membrane protein CidB; 18) Holin-like protein CidA; 19) LysR family regulatory protein CidR); 20) Oxidoreductase; 21) Oxygen-insensitive NAD(P)H nitroreductase/Dihydropteridine reductase.

both gonococci and meningococci, whereas various combinations of these five genes have been observed in *N. lactamica, N. cinerea* and *N. polysaccharea.* None of the other commensals had any of the genes in the *lgt* cluster, although the flanking genes were present (figure 2.7). This suggests that the commensal LOS has a different structure compared to pathogens, consistent with published data. Of recent interest in our lab has been *N. sicca* 4320 since I showed that it expressed a lipopolysaccharide unlike other members of *Neisseriaceae* (196). The LPS bands show up as a ladder pattern in tris-tricine gel because of variations in O-antigen length. Comparison of *N. sicca* 4320 genomic DNA with meningococcal and gonococcal genomes resulted in identification of ORFs unique to *N. sicca* 4320, as shown in figure 2.8. The putative LPS biosynthetic genes also showed significant identity to those seen in *E. coli.* Figure 2.8 also lists the name of each of those ORFs while table 2.2 shows the genomic similarity of those LPS biosynthetic genes from *N. sicca* 4320 compared with other neisserial species. I observed that a majority of those genes are missing in the pathogens while still being present in other commensals. The identification of these LPS biosynthetic genes provides the theoretical basis to explain the LPS O-antigen observed in *N. sicca* 4320.

2.3.7 Structure function relationship of neisserial LOS

Comparative bioinformatic analysis of commensal and pathogenic neisserial genomes showed us the distribution of various genes and their correlation with bacteria-host interaction. I determined that the lack of O-antigen biosynthetic genes correlates with the greater immunostimulatory behavior since none of the pathogens possessed those genes. Also, the inner core structure between neisserial commensals

and pathogens differed significantly. Table 2.3 shows the distribution of various genes involved in biosynthesis of LOS core region. While all pathogens and closely related commensals *N. lactamica, N. polysaccharea* and *N. cinerea* possessed RfaK protein, the other commensals had WaaQ protein, suggesting that inner core in commensals has a third heptose instead of a GlcNAc attached to the second heptose. I also observed that genes for Lpt3 and Lpt6, which add a PEA moiety to the second heptose in the core region, are absent in a majority of the commensals but present in all the pathogenic strains. Inner core substitution of GlcNAc with heptose, and lack of inner core decoration with PEA strongly correlated with commensal behavior, as did expression of O-antigen instead of a truncated alpha chain.

Commensal LOS is less immunoreactive in comparison to LOS from neisserial pathogens (35). Figure 2.9 (Panel A) shows that the pathogenic bacteria *N. gonorrhoeae* was able to induce more TNF- α than the commensals *N. lactamica* and *N. subflava.* PEA decoration of lipid A was observed to be responsible for the difference in immunoreactivity between pathogens and commensals. I hypothesized that differences in overall charge in inner core can also affect LOS immunoreactive potential by modulating LOS extraction the bacterial outer membrane. Computer simulation models of LPS and experimental data show the role of divalent cations in increasing LPS stability (figure 2.10). Divalent cations have been observed to form adducts that act as glue to bind neighboring LOS molecules, stabilizing the outer membrane. I hypothesized that an increase in overall negative charge of LOS core regions would mean a stronger cation-mediated cross-linking, making it harder for host LPS-binding proteins (LBPs) to extract LOS from outer membrane. Since

Organism	O-antigen polymerase/ligase	Chain length determinant protein	Putative Wza(Complex polysaccharide trasnport)	LPS biosynthesis translocase	LPS biosynthesis oxidoreductase	4-a-glucano transferase	1,4-a-glucan-branching enzyme
N. sicca DS1	96.3%[a]	97.6%	93.8%	97.4%	94.4%	93.9%	95.6%
N. sicca ATCC 29256	76.9%	85.0%	96.6%	70.8%	94.4%	94.0%	99.0%
N. mucosa ATCC 25996	96.6%	97.4%	93.8%	96.9%	94.1%	93.8%	96.2%
N. subflava NJ9703	70.1%	76.4%	78.9%	72.0%	NP[b]	76.0%	86.0%
N. flavescens NRL30031/ H210	71.6%	76.8%	47.0%	76.8%	NP	85.6%	85.6%
N. polysaccharea NS342	NP	NP	NP	NP	NP	81.7%	88.3%
N. lactamica NS19	NP	NP	NP	NP	NP	NP	NP
N. cinerea ATCC 14685	NP	NP	NP	NP	NP	NP	NP
N. meningitidis	NP	NP	NP	NP	NP	NP	NP
N. gonorrhoeae	NP	NP	NP	NP	NP	NP	NP

[a]Percentage listed is the degree of similarity between the tested gene and the homolog found in *N. sicca* 4320.
[b]NP indicates that no homolog was present.

Table 2.2 Genomic similarities of putative LPS biosynthetic genes.

	rfaC	*rfaF*	*rfaK*	*waaQ*	*lpt3*	*lpt6*	*lgtG*	*lgtF*	*lgtE*	*lgtA*	*lgtB*	*lgtD*
N.meningtidis	■	■	■		■	■	■	■	■	■	■	■
N.gonorrhoeae	■	■	■		■	■	■	■	■	■	■	■
N.lactamica	■	■	■		1/5	1/5		■	■	■	■	■
N.polysaccharea	■	■	■							■	■	
N.cinerea	■	■	■							■	■	
Other commensals	■	■		■	3/10							

Table 2.3 LOS biosynthetic gene distributions among Neisseria species. Filled boxes correspond to a gene that is present in all the completed genomes available for that species. Numbers, for example 1/5, mean 1 out of the 5 available genomes of that species possess the gene.

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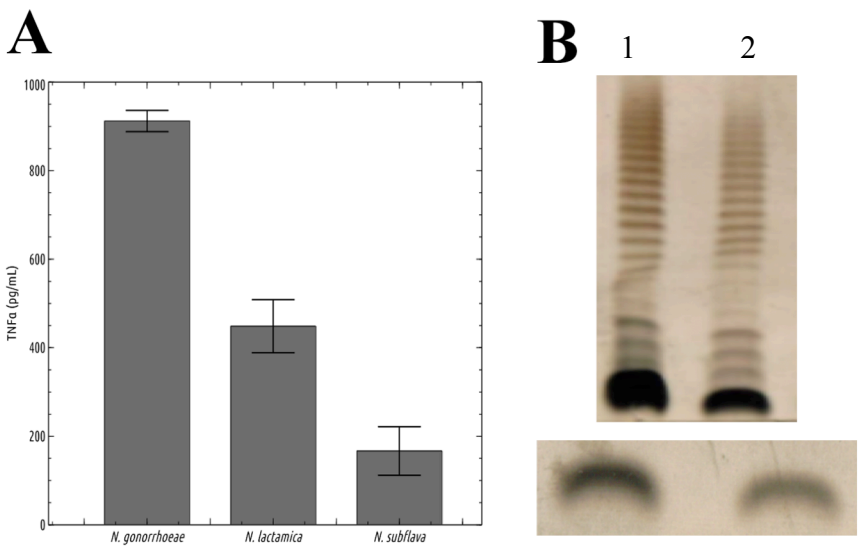
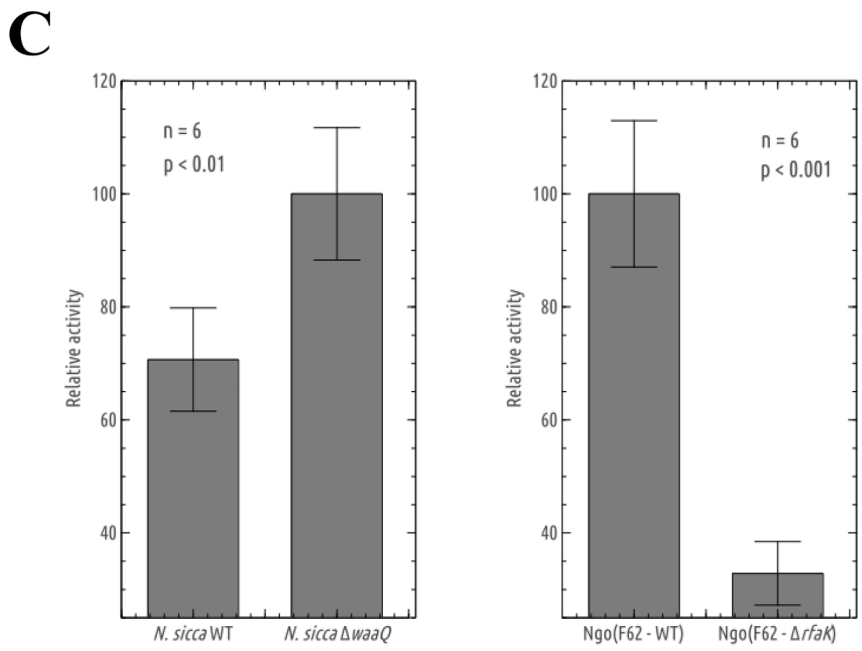
commensal LOS core region has three heptose molecules as opposed to two in neisserial pathogens, the commensal LOS core region has a relatively more negative charge with respect to neisserial pathogens. I knocked out *waaQ* gene in *N. sicca* 4320 and *rfaK* gene in *N. gonorrhoeae* F62. *N. sicca* 4320 Δ*waaQ* resulted in an LOS that appeared to be shifted slightly down compared to wildtype, suggesting loss of a heptose (panel B figure 2.9). Unlike neisserial pathogens where loss of *rfaK* results in loss of alpha chain elongation as well, there was no such effect on the O-antigen elongation in *N. sicca* 4320 on deleting *waaQ* (panel B figure 2.9).

The immunostimulatory potential was compared by incubating wildtype bacteria and the corresponding isogenic mutant with THP-1Xblue cells, which express an NF-κB/AP-1-inducible reporter system to monitor TLR-induced NF-κB/AP-1 activation. Figure 2.9 shows the relative levels of activation by each strain. I observed that there was a statistically significant increase in immunostimulation by *N. sicca* 4320 Δ*waaQ* compared to the wildtype strain, and knocking out *rfaK* gene reduced NF-κB/AP-1 activation in our human monocytic cell line in comparison to *N. gonorrhoeae* F62 wildtype strain. One possible explanation for the evolutionary selection for an LOS that is more immunostimulatory in pathogens is that having a less negative core region makes it harder for cationic antimicrobial peptides to interlace themselves between the LOS molecules, making pathogenic strains less sensitive to CAMP-mediated killing compared to commensal strains. Not having a highly immunostimulatory LOS also allows the commensal strains to escape host immune response, which they are not well equipped to survive. I tested all four strains for their sensitivity to polymyxin. Polymyxin acts similar to CAMPs by intercalating





Polymyxin MIC	0.4	0.8	>50	12.5	(μg mL^{-1})

Figure 2.9 Differences in LOS correlate with TLR4 activation. (A) TNF- α produced by THP-1 cells after incubation with *N. gonorrhoeae, N. lactamica*, and *N. subflava* for 6 hours were measured by ELISA. (B) 16.% Tris-tricine gel of LOS preparation from *N. sicca* wildtype (lane 1) and *waaQ* deletion strain (lane 2). Inset image corresponds to the lower most band on LOS gel. This inset makes no sense as it does not look like the top panel. Ge trid of it. (C) Relative levels of NF-κB activation measured using THP-1Xblue cell line, which secretes alkaline phosphatase (SEAP) on NF- κB activation. THP1-Xblue cells were treated with *N. sicca* 4320 wildtype, *N. sicca* 4320 Δ*waaQ*, *N. gonorrhoeae* F62 wildtype or *N. gonorrhoeae* F62 Δ*rfaK*. The supernatant were collected and SEAP activity estimated by using a substrate that turns blue on cleavage by SEAP. Shown below the graphs is corresponding polymyxin MICs for each strain.

itself between neighboring LPS molecules in a stoichiometric fashion. This allowed me to indirectly measure the overall charge of the LOS core and lipid A region in each strain, since strains with a lower polymyxin MIC would mean a more negatively charged core region which makes it easier for polymyxin to intercalate. Figure 2.9 (Panel C) shows that while knocking out *waaQ* only slightly increased the MIC in commensals, *N. gonorrhoeae* F62 Δ*rfaK* had a fourfold decrease in MIC compared to wildtype strain.

2.4 Discussion

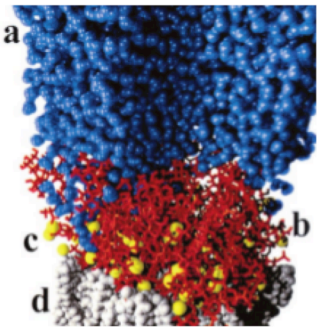
This study was undertaken to determine the genetic potential of the *Neisseriaceae* with respect to LPS/LOS expression making use of advances in genomic sequencing. The data indicate that neisserial LOS possesses two basic core structures; one that possesses a gamma chain N-acetylglucosamine and one that contains a third heptose. The presence of the third heptose was found in strains that rarely caused disease, while the presence of the gamma chain N-acetylglucosamine was associated with pathogens. However, two commensal strains expressed a gamma chain N-acetylglucosamine, *N. lactamica* and *N. cinerea*. The inability of *N. lactamica* (most closely related to *N. meningitidis*) to cause disease relates to its inability to express a capsule. Colonization of nasopharyngeal mucosa by *N. lactamica* has been observed to correlate with protection against development of meningococcal meningitis, suggesting development of cross-reactive protective immunity. With regard to *N. cinerea,* the inability of *N. cinerea* to cause disease could be related to its inability to sialylate its LOS, however other factors may also be involved and or responsible for the avirulence of this strain. This is the first study to

look at diversity of LOS profile in *Neisseriaceae* using a bioinformatics approach.

The importance of LOS in neisserial pathogenesis is illustrated by the high degree of sequence conservation in the genes needed for LOS biosynthesis in the pathogens. It is further substantiated by the presence of homopolymeric runs of guanines in key genes involved in LOS biosynthesis, which leads to phase variable expression of LOS. I was unable to identify any homopolymeric runs, or other repeated sequences in commensal LOS biosynthetic genes, suggesting that the expression of LOS in commensal strains is invariant. The alteration in LOS structure seen in the commensal organisms suggests that the third heptose modification may play an important role in modulation LOS/LPS mediated toxicity. In order to test this hypothesis, I created a mutant strain of *N. sicca* 4320 unable to add a third heptose to its LOS inner core. Similarly, a gonococcal strain lacking the ability to add a GlcNAc in its LOS core region was created as well. Using these strains, I show that changes in LOS inner core can alter TLR4 stimulation in host cells. It is possible the difference in TLR4 stimulation is at the level of LOS extraction from bacterial outer membrane by LPS-binding proteins, rather than at the level of LOS-TLR4 binding since it is the lipid A portion that interacts with TLR4 rather than inner core portion of LOS.

LOS molecules on the surface of outer membrane form a tight network of sugar molecules that use divalent cations as adducts to stabilize the outer membrane (201).

N. gonorrhoeae elicits a Th17 response in the mouse model of infection. Th17 response is characterized by neutrophilia and secretion of cationic antimicrobial peptides (CAMP). A highly negative LOS inner core would make *N. gonorrhoeae* highly susceptible to CAMP-mediated lysis, which the bacteria bypass by adding a



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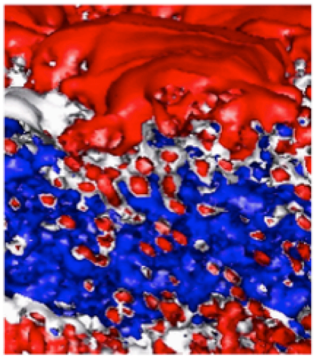

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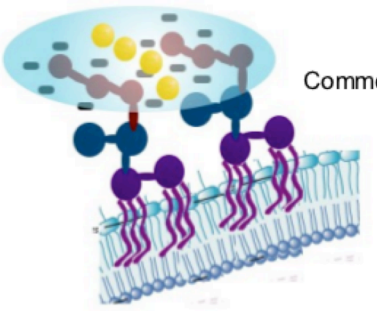
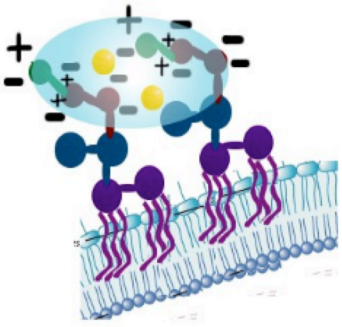


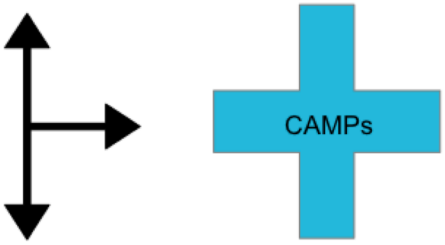
Commensal

Pathogen



The three heptoses in the inner core are heavily negatively-charged; divalent cations form an adduct between neighboring LOS molecules and cross-link them.

The inner core has two heptoses, a N-acetyl glucosamine & one to three PEA group(s), resulting in reduction of overall negative charge, requiring fewer divalent cations & increasing resistance against CAMPs.



Increased susceptibility to CAMPs possibly due to the higher inner core negative charge but commensals do not appear to elicit any Th17 response *in vivo*.

Inner core has less affinity for CAMP binding which increases chances of survival against a Th17 response. Gonococci has been shown to elicit a Th17 response.

The stronger inner core cross-linking,would mean reduced extractability of LOS by LBP, which in turn reduces the LOS bioactivity.

The weaker inner core cross-Linking by cations, results in Increased extractability by LBP, increasing the LOS bioactivity

C

Figure 2.10 Role of LOS inner core charge and LOS extractability. (A and B) Computer simulations of the LOS layer in *P. aeruginosa* which show how divalent cations play a crucial role in improving stability by acting as adducts between neighboring LOS molecules on the surface (From (201)). (C) Neisserial pathogens appear to have a less negative LOS core region compared to LOS core of neisserial commensals.

Charge neutral GlcNAc instead of a negatively-charged third heptose in the LOS core region. My experiments with polymyxin MIC suggest that the loss of GlcNAc makes the bacteria more susceptible to polymyxin-mediated killing. In the commensal strain, the loss of the third heptose only slightly increased the polymyxin MIC. Expressing the RfaK protein in the *N. sicca* 4320 *waaQ* mutant strain would further help us understand the relationship between inner core charge distribution and TLR4 stimulation. While this is a preliminary result, further experiments are needed to test if the extractability of LOS has changed. This can be tested by using BODIPY-tagged LOS molecules and purified human LPS-binding protein (LBP) or soluble CD14 (sCD14). Since LOS tends to form micelles in water, the BODIPY-fluorescence is quenched. In the presence of LBP or sCD14, LOS is extracted from the micelles which makes the BODIPY tags to fluoresce. Measuring the fluorescence would help us understand how inner core charge distribution can affect extractability. These experiments are the first step towards establishing a structure-function relationship for the neisserial LOS inner core. Understanding the role of inner core structure in LOS-TLR4 interaction is important for developing vaccines that can use LOS as adjuvant as well as an antigenic component.

Chapter 3: Role of gonococcal surface glycosylation in bacteria-bacteria interaction

3.1 Introduction

Gonorrhea, caused by the exclusive human pathogen *N. gonorrhoeae,* is a major public health problem. This is highlighted by the recent rise in the incidence of antibiotic-resistant strains prompting CDC to dub this bacterium an "immediate public health threat that requires urgent and aggressive action". While laboratory studies on gonococci have contributed to a significant understanding of the mechanistic underpinnings of gonococcal interactions with the host, during natural infection the organism probably exists on mucosal surfaces as a biofilm (87). This leads to questions about the potential differences between planktonic bacteria versus sessile bacteria during their interaction with host cells. Studies of biofilm formation are also important in the context of emergence of cefixime and ceftriaxone-resistant strains (5).

Biofilm formation is important for gonorrhea pathogenesis, persistence in the female reproductive tract, and development of drug resistance. Biofilm formation begins with bacterial attachment to each other and to the surface. The bacteria then form a microcolony, continue to replicate and establish a mature biofilm. GC has been shown to form a biofilm on glass, primary and transformed human cervical epithelial cells (88, 118). However, little is known about how gonococci form biofilm, the signals that regulate biofilm production or the mechanism by which

gonococci disperse from the biofilm. DNA plays a critical role in organizing neisserial biofilm matrix with secretion of single-stranded DNA playing an important role in the initiation of gonococcal biofilms (80). The presence of DNase can impair biofilm formation and promote bacterial dispersal (118). Bacterial surface properties such as their hydrophobicity have also been observed to affect biofilm formation, as observed in *N. meningitidis* (114). Increased surface hydrophobicity allows the bacteria to aggregate and form microcolonies which seed biofilm formation. Similarly, the presence of polyamines such as spermine, which are present in genital secretions and coat the mucosal surface, also impair biofilm formation (202). These could also aid in bacterial escape from a biofilm, thereby promoting transmission as well as aiding in ascending and invasive infection. Studies on biofilms produced by other organisms have suggested several mechanisms of dispersal, including erosion (continuous release of single cells or small clusters of cells) and sloughing (rapid detachment of large portions of the biofilm) (110, 111). Erosion and sloughing can result from bacterial metabolism-associated processes, such as secretion of glycosidases and nucleases.

Gonococci in biofilms appear to be intimately associated with each other through unknown mechanisms. Previous work with other biofilm forming bacteria such as *P. aeruginosa*, *E. coli*, and *S. aureus* has established the role of surface proteins in influencing biofilm formation. Our lab had previously established the role of neisserial opacity-associated proteins (Opa) in causing bacterial aggregation by functioning as a bacterial adhesin (39). Our lab demonstrated that the lack of Opa proteins on the bacterial surface allowed GC to invade and transmigrate human

epithelial cell monolayer faster than bacteria expressing Opa proteins on their surface. This shows that bacterial escape from microcolonies could promote invasive infection.

Opa proteins can act as lectins and bind to LOS from neighboring cells (38). This bacteria-bacteria interaction promotes initial microcolony formation. Using confocal microscopy and scanning electron microscopy I have shown the importance of Opa in gonococcal aggregation and biofilm formation. In order to determine the effect of LOS truncations on biofilm formation, I compared biofilm formation between GC FA1090 WT strain and GC FA1090 lacking *rfaK* gene (which expresses a truncated LOS molecule lacking the terminal lactoneotetraose and GlcNAc in core region). Biofilm formation was markedly reduced in the presence of a truncated LOS molecule (see Appendix). This suggests that gonococcal biofilm formation is dependent on surface glycoconjugates as well as surface lectins such as Opa.

While phase variation of Opa and LOS can modulate biofilm formation *in vivo*, the modification of glycoconjugates on the surface by glycosidases resulting in modulation of biofilm formation has not been clearly understood. Modification of surface glycoconjugates by glycosidases would also be a faster way to alter glycosylation than phase variation of glycosyltransferases. I hypothesized that modification of bacterial surface glycosylation could affect bacterial escape from biofilms. To see if this can happen *in vivo* I used bioinformatics to identify possible glycosylhydrolases where I identified a glucosaminidase that I characterized as NagZ. I demonstrated its role in biofilm dispersal.

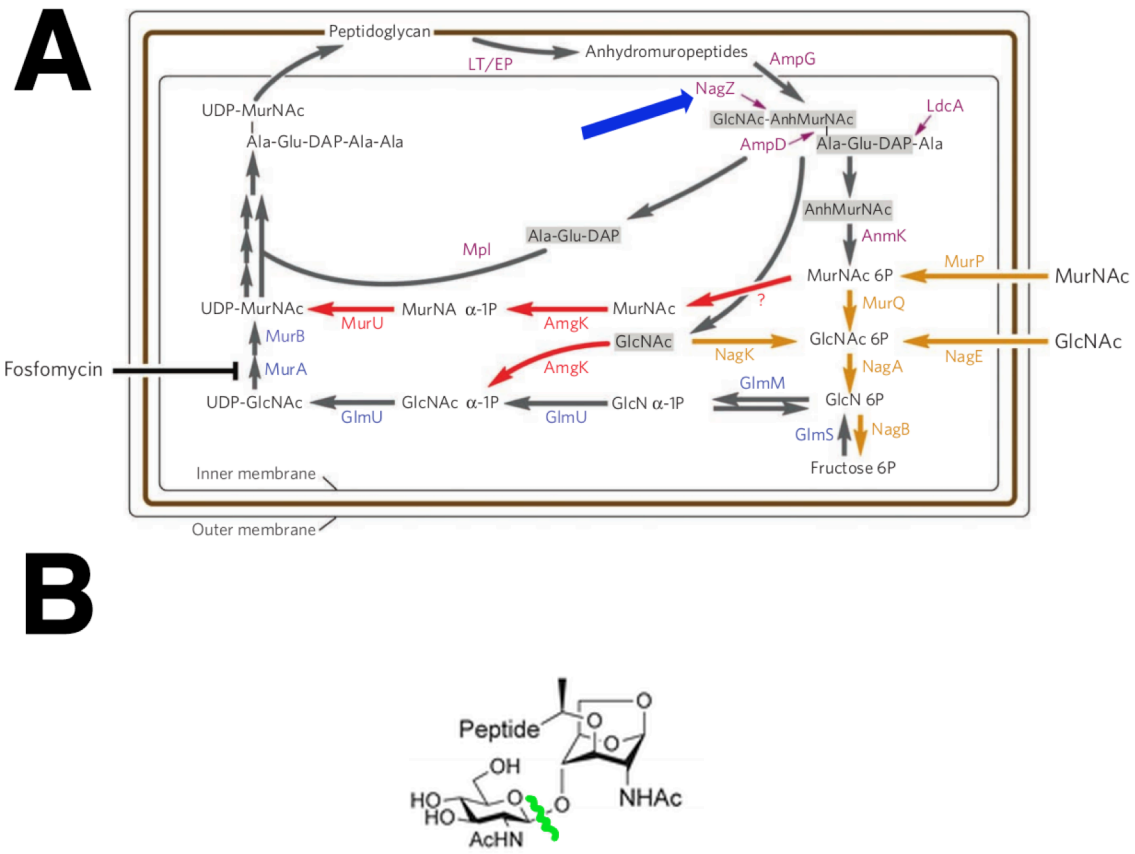


Figure 3.1 NagZ involved in peptidoglycan recovery in *Pseudomonas spp.* and *E. coli*.

(A) Schematic of peptidoglycan recovery pathway from (243). NagZ is indicated by the blue arrow. (B) Substrate for NagZ as characterized in *P. aeruginosa* and *E. coli*. Site of cleavage by NagZ is indicated by wavy green line. Modified from (244)

NagZ has been established to be involved in peptidoglycan recovery in *E. coli* and *P. aeruginosa* (Figure 3.1). NagZ has been characterized as a exoglycosidase that removes the non-reducing GlcNAc residue to liberate 1,6-anhydroMurNAc peptides from peptidoglycan monomers (203). This is the first study to characterize the biochemical function of NagZ in gonococci and to identify a role for NagZ in biofilm modulation.

3.2 Materials and Methods

3.2.1 Bacterial strains and plasmids

N. gonorrhoeae (FA1090, MS11, and F62) were grown in standard gonococcal medium (Difco, MI), designated GCP if used as phosphate-buffered broth, and designated GCK if used with agar, supplemented with 1% Kellogg's supplement [48], at 37º C and 5% CO_2. *E. coli* BL21(DE3) and *E. coli* DH5αMCR (Life Technologies, MD) were grown in L broth (LB) at 37° C or 30° C [49]. Antibiotics included in media were used at the following final concentrations (μg ml^{-1}): ampicillin 100, kanamycin 50. Plasmid pUC19 (New England Biolabs) and pET28a(+) (Novagen) were used to make deletions in various *N. gonorrhoeae* strains and to express NagZ protein, respectively. *S. aureus* SH1000 strain was obtained from Dr. Jeffery Kaplan, George Washington University.

3.2.2 Chemicals and Reagents

All reagents were purchased from Sigma-Aldrich, unless stated otherwise. Polymerase chain reactions were performed using Pfu polymerase (Thermo Scientific Molecular Biology, Pittsburgh, PA) or GoTaq polymerase (Promega, Madison, WI),

and carried out according to the manufacturer's instructions. All restriction enzymes were sourced from New England Biolabs (Beverly, MA). A sample of Dispersin B was obtained from Dr. Jeffery Kaplan, George Washington University.

3.2.3 Construction of mutants

A list of all primers used is provided in Table 3.1. A DNA fragment carrying the *nagZ* gene of *N. gonorrhoeae* FA1090 along with ~850 bp of right and left flanking DNA sequences was amplified using primers hexoF and hexoR. The resulting amplicon (~2700 bp) was cloned into the pUC19 EcoRI and HindIII sites and transformed into *E. coli* DH5αMCR, resulting in the formation of pUC19::nagZ. One of the isolated clones was used for further experiments. To disrupt *nagZ* in the gonococcus, a Kanamycin (Kan) resistance gene was excised from pK18 with SmaI, the DNA fragment purified from agarose gel and inserted into the EcoRV site of pUC19::nagZ. A plasmid from a presumptive *E. coli* clone carrying pUC19::nagZ::Kan was isolated and the construct was verified by DNA sequencing (Macrogen, USA). This plasmid DNA was cleaved with EcoRV and ligated with double-strand oligo DNA prepared from DUSF and DUSR, which contain two EcoRV sites and *N. gonorrhoeae* DNA uptake sequence, after cleaving with EcoRV. Kanamycin resistant transformants were selected, the plasmid DNA isolated and checked for the presence of the uptake sequence. Plasmid DNA from such clones was used for transformation of *N. gonorrhoeae* strains by liquid transformation method and selected on GCK plates containing kanamycin (50 μg/ml). *nagZ* was deleted by a spot transformation technique in *N. gonorrhoeae* FA1090, *N. gonorrhoeae* F62, and *N. gonorrhoeae* MS11Δopa.

Primer Name	Sequence
HexoF	GCGAATTCACAGCCGCATCTCGATAC
HexoR	GCAAGCTTTTCCGCCATGATCTACAC
DUSF	ATGCGATATCGCCGTCTGAAGAATTCGATATCATCGAT
DUSR	ATCGATGATATCGAATTCTTCAGACGGCGATATCGCAT
Ngo0135F	CTAGCTAGCATGACCGTCCCCCATATTCC
Ngo0135R	CCGCTCGAGTTAAAAGGCCTCTCCGACTTTTA

Table 3.1 List of primers used in this study.

3.2.4 Expression and purification of NagZ protein

A DNA fragment encoding *nagZ* isolated from *N. gonorrhoeae* FA1090 was amplified using primers Ngo0135F and Ngo0135R and the resulting amplicon (1300 bp) was cloned into pET28a(+) using NheI and XhoI, resulting in the formation of plasmids pET28a(+)::nagZ. To purify NagZ, a single colony generated by transformation of *E. coli* BL21(DE3) with pET28a(+)::nagZ was used to inoculate 100 ml of LB broth with kanamycin. Cultures were incubated at 37 ^{o}C, and when the OD_{600} of the culture reached 0.6, IPTG was added to a final concentration of 1 mM. Incubation was continued at 30 ^{o}C for an additional 12 hr. Cultures were centrifuged and bacterial pellets were resuspended in 10 ml buffer A containing 50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 20 mM imidazole, 10 mM -mercaptoethanol, 0.1% (v/v) Tween 20, 100 μM PMSF. After sonication, the cellular debris was removed by centrifugation at 40 000 *g* for 1 hr and then the supernatant applied to a 3 ml Ni-NTA agarose column previously equilibrated with 100 ml of above buffer. The column was washed with 100 ml of buffer B containing 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole and 10% (v/v) glycerol and the proteins eluted with a 6 ml of buffer B containing increased concentration of imidazole (20 mM, 50 mM, 100 mM, 150 mM, 200 mM and 250 mM). N-acetyl-β-D-hexaminidase eluted at 150 mM -200 mM. The fractions containing the purified proteins were pooled and dialyzed against buffer B and reapplied to a Ni-NTA agarose column. The purity of the enzyme was determined by running an aliquot on a 15% SDS-PAGE. PageRuler^{TM} prestained protein ladder (170, 130, 100, 70, 55, 40, 35, 25, 15 and 10 kDa) (Fermentas) was used as protein molecular weight marker.

3.2.5 LOS purification and analysis

LOS was purified from broth-grown cells by hot phenol-water method (204). LOS was then concentrated by lyophilization and extraction with hot phenol-water continued until the preparation obtained minimal absorbance at 200nm. LOS was analyzed on a 16.5% Tris-Tricine gel at a fixed current of 0.03 mA in an ice-cooled chamber, and visualized by silver staining (196).

3.2.6 Hexosaminidase assay

Enzyme activity was assayed using various p-nitrophenol tagged saccharides as substrate. The standard reaction mixture consisted of 20 µl of substrate solution at a final concentration of 1.2 mM, 20 µl of 1 M KPO_4 buffer pH 8.0 (final concentration of 200 mM), 50 µl of water and 2 µg of enzyme (final concentration of 380 nM). After 30 min incubation at 37^{o} C, the reaction was stopped by addition of 50 µl of 200 mM glycine NaOH buffer pH 10. Water (860 µl) was then added and the release of p-nitrophenol from the substrate was monitored at 405 nm. One unit (U) of enzyme was defined as the amount of enzyme that released 1 µmol of p-nitrophenol from pNP-GlcNAc per min at 37^{o} C.

3.2.7 Biofilm formation

To prepare biofilms**,** bacteria from overnight cultures grown on GCK agar plates were suspended in GCP with sodium bicarbonate and Kellogg's supplement to a final concentration of 10^8 bacteria per mL. Dynamic biofilms were grown in new heat-sterilized glass tubes by adding 1 mL of the bacterial suspension per tube, and incubating in a rotary shaker for 48 hr. Static biofilms were obtained by making a

suspension of bacteria to a Klett of 100 in GCP and adding an aliquot to culture plates using published methods (114). The biofilms were then quantitated by staining with 1% w/v crystal violet. The crystal violet stained biofilm was eluted out by dissolving in 30% acetic acid and the absorbance read at 590 nm in a spectrophotometer.

3.2.8 Confocal Microscopy analysis

Static biofilms were grown in 35 mm glass bottom microwell dish with No. 15 coverglass (MatTek, MA) for 48 hr (114). Biofilms were washed with PBS three times and stained with Hoechst stain or propium iodide as appropriate for 15 min fixed with 4% PFA for 20 min visualized with Leica TCS SP5 X confocal microscope (Leica Microsystems, IL, USA).

3.2.9 Scanning electron microscopy

Static biofilms were grown for 36 hr over a glass coverslip placed inside a 24-well cell culture plate. Briefly, the coverslips were gently washed three times with PBS, and fixed with 2% glutaraldehyde for 60 min at RT and then overnight at 4^{o} C. The coverslips were fixed the next day using 1% osmium tetroxide, dehydrated by a series of washes with increasing concentrations of ethanol, dried by critical point drying method, and finally coated with gold-palladium alloy (205). Samples were visualized with Amray 1820D microscope (20 kV) and/or Hitachi S4700 microscope (5 kV).

3.2.10 Statistical analysis:

Experiments were repeated as three independent trials and data analysed using Graphpad Prism 6 (GraphPad Software, La Jolla California USA). p-values were

determined by Student's t-test unless specified (Graphpad Prism 6). Error bars shown are standard errors of mean, unless specified otherwise.

3.3 Results

3.3.1 Identification of putative glycosylhydrolases in the neisserial genome

I performed a bioinformatic screen on *N. gonorrhoeae* genomic DNA sequences using the glycosylhydrolase library in Carbohydrate-Active enZYmes database (CAZY) and created a list of putative glycosidases, listed in Table 3.2, which also shows the accession number for the corresponding protein in NCBI and the glycosylhydrolase family number. The majority of them were annotated in NCBI as enzymes involved in the peptidoglycan recovery pathway. I focused on ORF0135 because it encoded a putative hexosaminidase. Since the terminal sugar in LOS is a hexosamine, I hypothesized that ORF0135 could be able to act upon LOS and truncate the outer core. In addition, Apicella *et al* showed the presence of hexosamine in neisserial biofilm using lectin-binding studies, which suggested that hexosamines play a critical role in bacterial aggregation (88). ORF0135 was also present in all the neisserial genomes available on NCBI, including commensal species which suggests an essential role for the enzyme.

3.3.2 ORF0135 encodes a cytoplasmic hexosaminidase

Using the CAZY database, I determined that ORF0135 in *N. gonorrhoeae* FA1090 encoded a putative beta-hexosaminidase (EC 3.2.1.52) belonging to glycosyl hydrolase (GH3) superfamily. GH3 family of glycosylhydrolases hydrolyze terminal

non-reducing N-acetyl-D-hexosamine residues using retaining hydrolysis mechanism. To identify the protein localization I analyzed the protein sequence using the web-based protein analytical software MESSA (MEta Server for Sequence Analysis) (206). MESSA is an integrated tool that uses multiple other tools such as PSIPRED (for secondary structure prediction), DISEMBL (for predicting coils and loops), and TOPRED (for transmembrane helix identification) to determine the protein structure, location and function for a given protein sequence. I predicted that ORF0135 is a cytoplasmic protein with hexosamindase activity. Figure 3.2 shows a sequence logo representation of the active site for GH3 family of hexosaminidases.

The consensus pattern is [LIVM](2)-[KR]-x-[EQKRD]-x(4)-G-[LIVMFTC]-[LIVT]-[LIVMF]-[ST]-D-x(2)-[SGADNIT], where D is the active site residue. The top panel in figure 3.3 shows the synteny of *orf0135* in *N. gonorrhoeae* FA 1090. The synteny was identical in all the gonococcal genomes available, but varied in commensal neisserial species. Comparison of ORF0135 protein from all other neisserial species showed that the protein is present in all neisserial species and highly conserved as shown in the panel A of figure 3.3, where height of the green bars corresponds to degree of identity at the nucleotide level among all sequenced gonococcal genomes. I propose renaming *orf0135* as *nagZ* because of the high sequence similarity with *nagZ* gene in other gram-negative bacteria. This is shown in panel B of figure 3.3, comparing the NagZ protein sequence in *N. gonorrhoeae, E. coli,* and *P. aeruginosa.* The purple arrow in the C-terminal half of the protein shows the location of GH3 consensus sequence in all three proteins.

ORF/Gene	Accession Number	Glycosylhydrolase Family
Sialyltransferase	AAW89748.1	GT52
Lytic transglycosylase	AAW90656.1	GH102
NGO0086	AAW88847.1	GT4
NGO0087	AAW88848.1	GT4
NGO0135	AAW88893.1	GH3
NGO0207	AAW88960.1	GT2
NGO0418 (fragment)	AAW89161.1	GT4
NGO0419 (fragment)	AAW89162.1	GT4
NGO0532	AAW89270.1	CEnc
NGO0600 (possible fragment)	AAW89329.1	CEnc
NGO0608	AAW89336.1	GH23
NGO0626	AAW89354.1	GH103
NGO0987	AAW89672.1	GT9
NGO1033	AAW89709.1	GH23
NGO1056	AAW89727.1	CBM50
NGO1136 (fragment)	AAW89803.1	GT2
NGO1353	AAW90002.1	GT2
NGO1354	AAW90003.1	GT4
NGO1533	AAW90169.1	GT28
NGO1603	AAW90231.1	GT51
NGO1782	AAW90401.1	GT19
NGO1915	AAW90528.1	GT30
NGO1934	AAW90547.1	GT9
NGO2065	AAW90668.1	CE11
NGO2072	AAW90673.1	GT4
NGO2135	AAW90732.1	GH23
NGO2156	AAW90751.1	GT25
NGO2158	AAW90752.1	GT2
NGO2159	AAW90753.1	GT25
Pilin α-galactosyltransferase	AAM15778.1	GT4

Table 3.2 List of putative glycosylhydrolases in *N. gonorrhoeae* FA 1090 The accession number for the corresponding protein, from NCBI and the glycosylhydrolase /glycosyltransferase family number, derived from CAZY database, are also shown.

3.3.3 Biochemical characterization of NagZ

Using PCR amplification, a fragment of DNA encoding *nagZ* was cloned into the expression vector pET28a, transformed into *E. coli* BL21(DE3), and the expression of *nagZ* was induced with IPTG. After purification on a nickel column, an aliquot of the protein was analyzed by SDS-PAGE. The molecular mass of NagZ as determined by SDS-PAGE (47 kDa) was consistent with the predicted mass for this protein (Figure 3.4 Panel A). I determined the optimal enzymatic conditions for NagZ using *p*-Nitrophenyl N-acetyl-β-D-glucosaminide as substrate. The purified enzyme showed no dependence on divalent ions. The enzyme showed highest activity at 200 to 400 mM of potassium phosphate buffer. The optimal conditions were determined to be 37ºC, in potassium phosphate buffer (200 to 400 mM) at a pH of 8.0 (Panels B and C, Figure 3.4). The enzyme retained activity even after 48 hours incubation at room temperature. The kinetic parameters *K*m and Vmax against pNP-GlcNAc were determined as 3.2 mM and 64 μmol min^{-1} mg^{-1} respectively. These values are in the same order of magnitude as those for several other hexosaminidases. I further screened for activity of this enzyme using pNP conjugated to a variety of other sugar moieties as listed in figure 3.3, panel D. Of the substrates that produced detectable cleavage products, NagZ had maximal activity on *p*-Nitrophenyl N-acetyl-β-D-glucosaminide. I also observed some activity against β-D-N',N-bisacetylchitobiose. There was negligible activity against α- and β-forms of both glucopyranoside and galactopyranoside, as well as against acetylated forms of galactosaminide. This suggests that NagZ is predominantly an exoglycosidase but also has some endoglycosidase activity as seen by its activity on β-D-N',N-bisacetylchitobiose.

Figure 3.2 Sequence logo of the consensus sequence type 3 glycosylhydrolases. The active site sequence was extracted from all the known glycosylhydrolase (GH3) type 3 proteins in the CAZy database and aligned. The most abundant amino acid residue(s) at each position are shown. The height of the amino acid at each position corresponds to their relative frequency among the sequences compared. The D at 15th position is the active residue. The consensus pattern corresponds to [LIVM](2)-[KR]-x-[EQKRD]-x(4)-G-[LIVMFTC]-[LIVT]-[LIVMF]-[ST]-D-x(2)-[SGADNIT] for GH3 proteins.

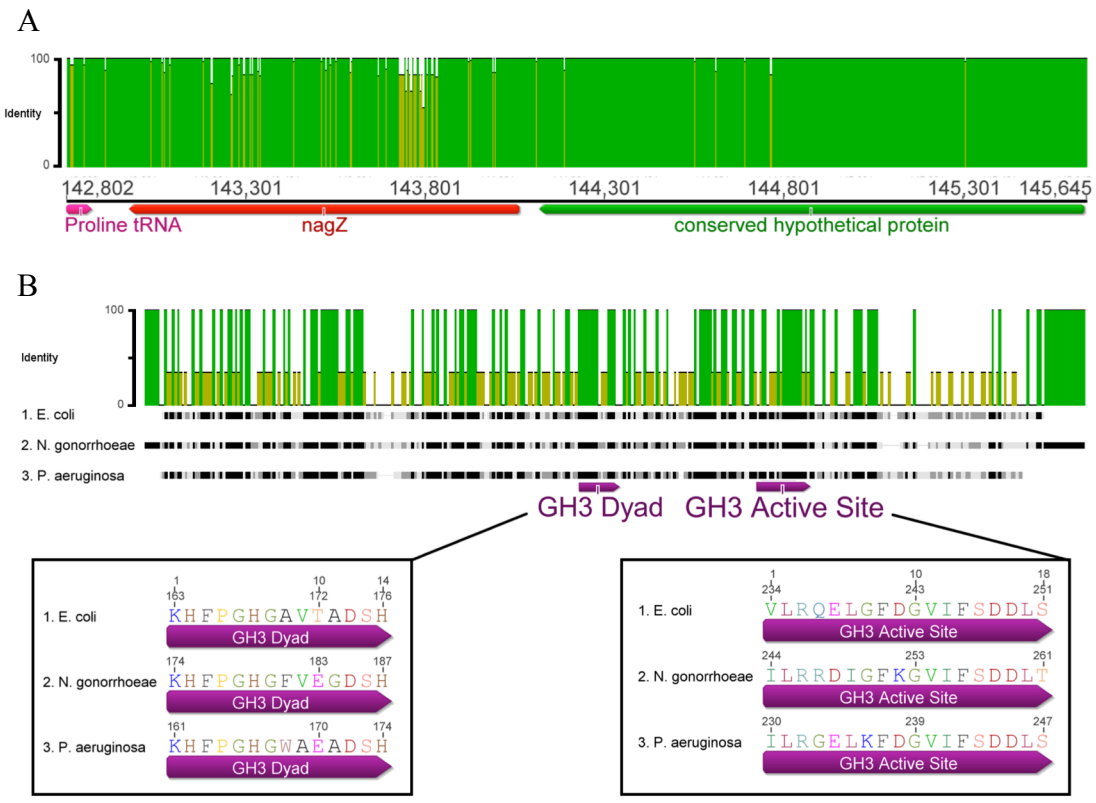


Figure 3.3 Bioinformatic analysis of *nagZ* gene. (A) Nucleotide alignment and genomic organization of *nagZ* in gonococcal genomes (NC_002946.2, NC_011035.1, ABZF01000028.1, ABZG01000030.1, ABZH01000027.1, ABZI01000026.1, ABZL01000026.1, ABZM01000035.1, ABZN01000023.1, ABZO01000044.1, ABZP01000042.1, ABZQ01000043.1, ACIG01000102.1, ADAA01000005.1, NC_017511.1, AKCG01000001.1, AKCH01000001.1, ABZJ02000001.1, and NC_022240.1). The height of green bars correspond to degree of identity among the different strains. (B) Amino acid alignment of NagZ protein sequence from *N. gonorrhoeae*, *E. coli* and *P. aeruginosa.* The position of GH3 family active site and dyad site are highlighted. Gonococcal NagZ is 39% identical and 55% similar to *E. coli* NagZ, and 41% identical and 56% similar to *P. aeruginosa* NagZ. The tall green bars represent identical amino acids, and smaller yellow bars represent similar amino acids. All analyses were performed using Geneious software.

To determine if NagZ can act as a β-1,6-hexosaminidase, I used *Staphylococcus aureus* strain that produces an extracellular polymeric substance (EPS) predominantly composed of poly β-1,6-*N*-acetyl-glucosamine (PNAG). I used dispersin B, which is a β-1,6-hexosaminidase as a positive control. The biofilm was stained after washing with crystal violet and quantified as described in the methods section. Figure 3.4 (Panel E) shows the comparison of biofilm without and with enzymatic treatment. NagZ treatment had a very slight reduction on *S. aureus* biofilm, whereas dispersin B dramatically reduced the biomass of the bioiflm. Dispersin B treatment of gonococcal biofilm also had a negligible impact on its biofilm, showing that gonococcal biofilm is not composed of significant amounts of PNAG. I conclude, from these data, that NagZ lacked endo-β-1,6-hexosaminidase activity. Since even prolonged treatment with NagZ had no impact on the *S. aureus* biofilm, I conclude that NagZ lacks exo-β-1,6-hexosaminidase activity.

3.3.4 NagZ is not secreted and it is not essential

Because NagZ is needed for recovery of monosaccharides from the PG monomers released during the cytosolic steps of the muropeptide-recycling pathway in *E. coli*, I made a NagZ knockout to see if NagZ is essential for survival to see if the accumulating PG fragments affect gonococcal viability. I constructed a NagZ deletion strain by transforming pili-positive wild type strain of *N. gonorrhoeae* with a DNA fragment constructed to contain a kanamycin cassette instead of *nagZ* gene, and selected for kanamycin-resistance transformants. The deletion was verified by DNA sequence analysis after PCR amplification of the region containing the deletion. I also measured the growth rate of the mutant strain, and found that the rate is identical to

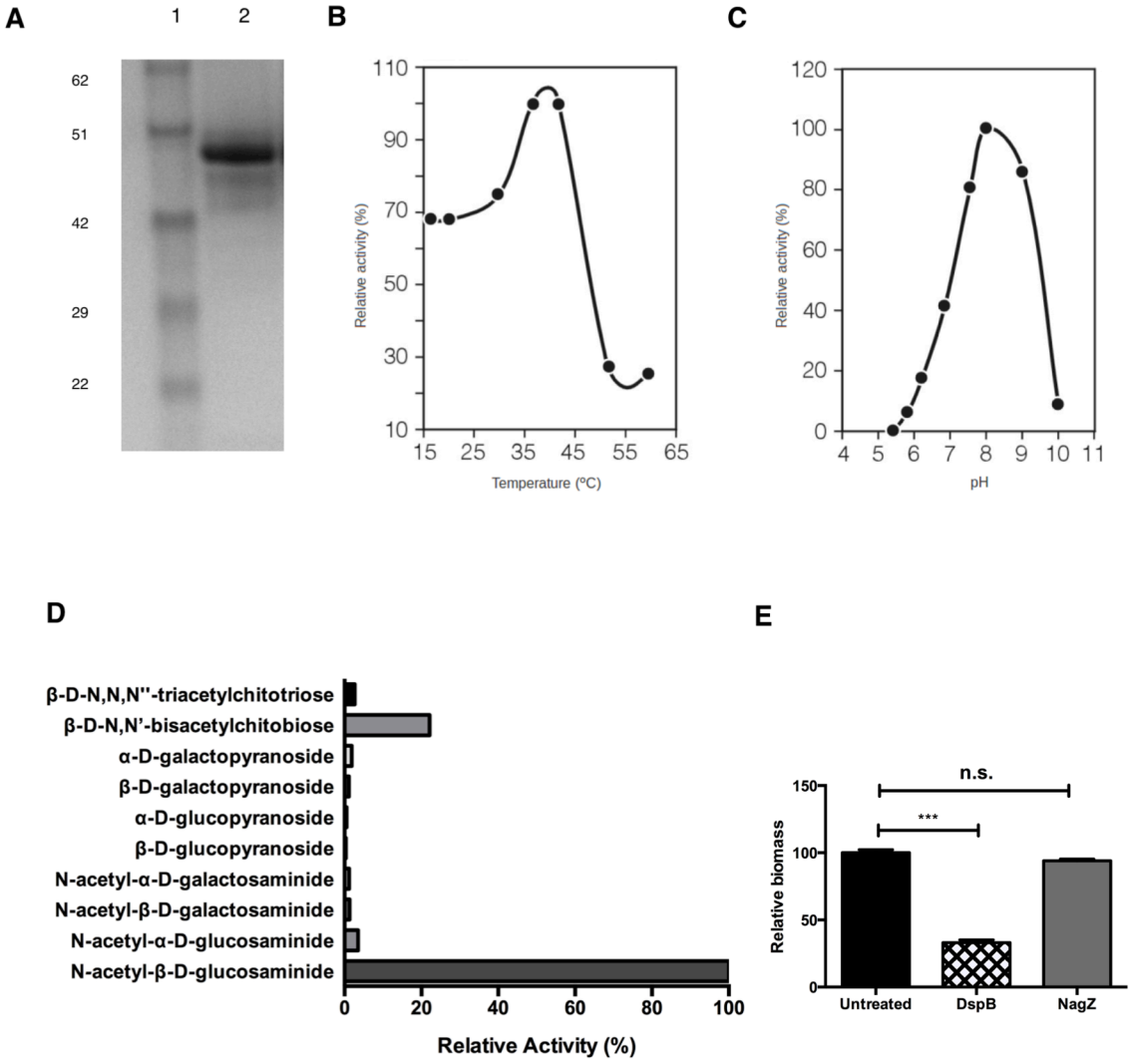

A
1
2
62
51
42
29
22
B
Relative activity (%)
Temperature (ºC)
C
Relative activity (%)
pH
D
β-D-N,N,N''-triacetylchitotriose
β-D-N,N'-bisacetylchitobiose
α-D-galactopyranoside
β-D-galactopyranoside
α-D-glucopyranoside
β-D-glucopyranoside
N-acetyl-α-D-galactosaminide
N-acetyl-β-D-galactosaminide
N-acetyl-α-D-glucosaminide
N-acetyl-β-D-glucosaminide
Relative Activity (%)
E
n.s.
***
Relative biomass
Untreated
DspB
NagZ

Figure 3.4 Biochemical analysis of NagZ. (A) SDS-PAGE gel of purified NagZ. Lane 1 is the molecular weight standard (mass of each band is indicated on the left of the gel, in kDa) and lane 2 is purified NagZ. (B) The activity profile for NagZ as assayed over a variety of temperatures. (C) The activity profile for NagZ as assayed over a variety of pHs. For panels B and C, the activity of NagZ used p-Nitrophenyl N-acetyl-β(1-4)-D-glucosaminide as a substrate. (D) The relative activity of NagZ for various substrates measured at 37ºC at a pH 8.0. (E) Biofilm of *S. aureus* SH1000 was incubated with Dispersin B (10 μg) or NagZ (20 μg), and the amount of biofilm remaining in the well determined by measuring the biomass. Data represents mean values (± SE) of three independent experiments performed in triplicate. Statistical significance was determined using two-tailed t-test assuming unequal variance. (n = 3, ***$p < 0.001$, n.s. stands for not significant and error bars are SEM)

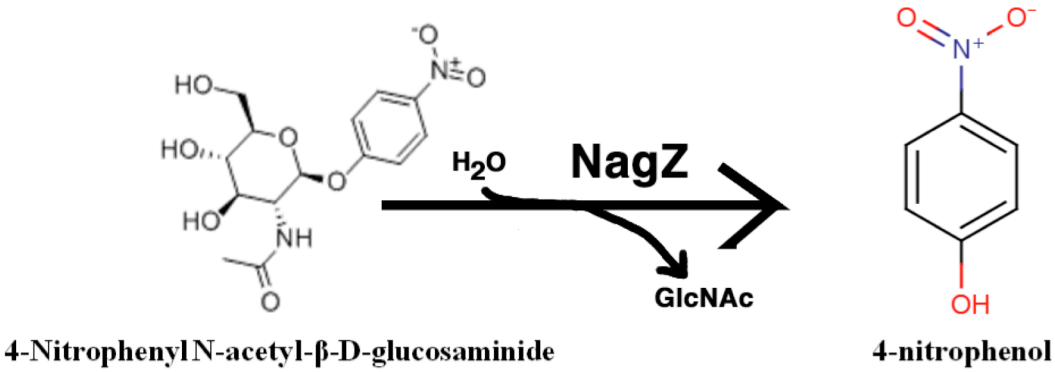


Figure 3.5 Cleavage of pNP-GlcNAc by NagZ. A cartoon representation of cleavage of 4-nitrophenyl N-acetyl-B-D-glucosaminide by NagZ releasing free N-acetyl glucosamine and 4-nitrophenol whose absorbance can be measured at 405 nm.

the growth rate of the wild type strain as shown in panel A of figure 3.6. NagZ is not essential for GC survival as I was able to make the mutant.

To see if NagZ is secreted, GC were grown to mid-log phase and bacterial growth media was collected to separate supernatant and bacterial pellet. After lysing the bacterial pellet, I tested for NagZ activity in both the bacterial lysate as well as the supernatant. I did not detect NagZ activity in the supernatant, unlike bacterial pellet as shown in panel B of figure 3.6. This suggests that NagZ is not secreted by the bacteria, as predicted by MESSA analysis. NagZ protein is not a secreted protein in other bacterial as well (207).

3.3.5 NagZ is present extracellularly

Gonococci express an autolysin *atlA,* which encodes a peptidoglycan hydrolase. Dillard and Seifert have shown that AtlA is responsible for bacterial lysis seen during stationary phase (208). I hypothesized that even though NagZ is predicted to be a cytoplasmic enzyme, I should be able detect the presence of NagZ activity in the supernatant after GC start lysing during the stationary phase. Bacteria were grown in broth, and aliquots of the culture supernatant tested for NagZ activity at various time points. Panel A in figure 3.6 shows the relative levels of NagZ activity at various time points in the supernatant and bacterial lysate, in comparison with the growth curve. I also tested the *nagZ* deletion strain for NagZ activity. There was no detectable activity in the deletion strain, confirming that the gene has been knocked out and that there are no other hexosaminidases present in *N.* gonorrhoeae FA 1090 (Panel B Figure 3.6). Figure 3.6 shows that NagZ activity begins to increase as the culture enters stationary phase. After overnight growth, the increase in detectable NagZ

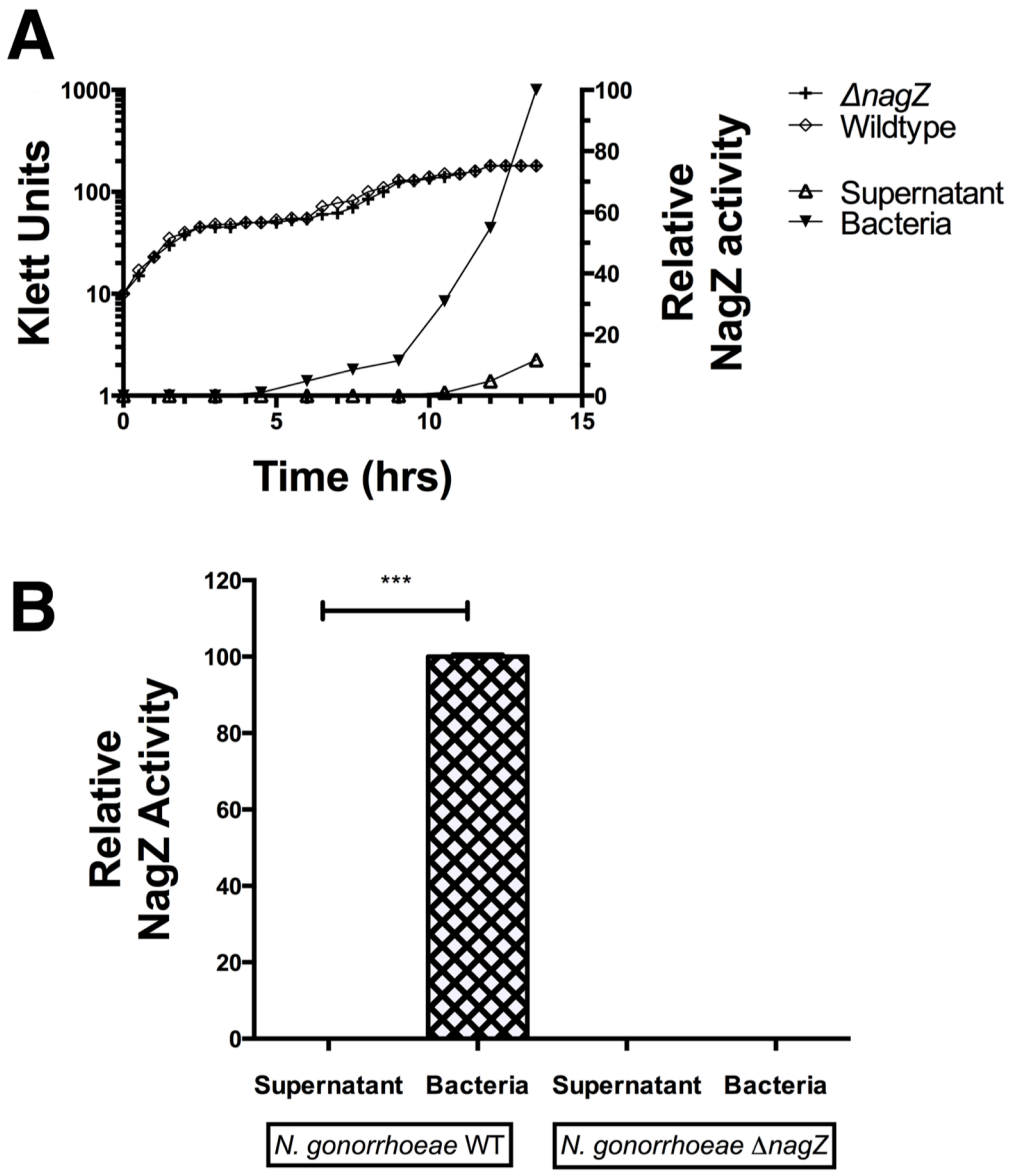


Figure 3.6 NagZ is found in culture supernatants of stationary phase grown gonococci. (A) Growth of FA1090 and FA1090 Δ*nagZ* was monitored hourly by measuring the turbidity using a Klett-Summerson colorimeter. Liquid broth (1 ml) was collected every 2 hours and assayed for presence of NagZ, using the methods described in the materials and methods. (B) Broth from a 6 hour culture of FA1090 and FA1090 Δ*nagZ* was spun down and NagZ activity was tested as described in methods section by running the reaction for 1 hour in both supernatant as well as bacterial lysate.

activity in the supernatant corresponded to the reduction in bacterial viability.

3.3.6. *nagZ* knockout strain forms thicker biofilm

Gonococcal strains growing in biofilms are under significant metabolic stress. Since I have shown in the previous experiments that NagZ can be detected in the extracellular milieu after stationary phase, I further hypothesized that the extracellular NagZ could play a role in modulating interaction between individual bacteria and hence affect biofilm formation. To test this hypothesize, I compared the wild type and *nagZ* knockout strains of *N. gonorrhoeae* (FA 1090, MS11, and F62) for biofilm formation. Since presence of pili increases biofilm formation by promoting bacterial aggregation I used non-piliated bacteria for all my experiments in order to eliminate the effect of pili-mediated aggregation.

The biofilms were formed under both static and dynamic conditions. Static biofilms were formed by incubating bacteria in GCP with Kellogg's supplement in 24-well tissue culture plates in an incubator, and dynamic biofilms were developed at the liquid-air interface on the inner surface of culture tubes placed in a rotary shaker at 37º C. After 48 hours, the supernatant media along with planktonic bacteria was aspirated and the biofilm washed three times and allowed to dry. Biofilm formation was measured by staining with crystal violet, removing the biomass from the glass surface by washing with acetic acid, and measuring the absorbance at 590 nm. Figure 3.7 shows an example of dynamic biofilm formed by the two different strains after 48 hours (Panel A). The biofilms were quantitated and shown in panel B figure 3.7. I observed that there was a significant increase in the biofilm formation by the *nagZ* deletion strain in comparison to the wild type, and this difference was seen across the

three strains of GC used in this experiment.

Because the greatest difference was seen in *N. gonorrhoeae* FA 1090 strain compared to other gonococcal strains, I used FA 1090 for most of the experiments on biofilm formation. *N. gonorrhoeae* FA1090 also served as a better model to study the role of surface sugar in biofilm formation since it does not contain Type IV secretion system encoded in a 57-kb region labeled gonococcal genomic island (GGI) (75). This precludes confounding by secreted DNA affecting biofilm formation. I also complemented the ΔnagZ strain by reintroducing *nagZ* into the mutant. Biofilm made by the complemented strain was compared with both wildtype and the mutant strain as shown in figure 3.7 panel B. It can be seen that complementation restores the original wildtype phenotype in the mutant and forms less biofilm.

3.3.7 Biofilm evolution over time

In order to understand how the two biofilms evolve over time the biofilm levels were measured at various time points. The biofilm formed by wildtype and *nagZ* mutant strains were compared at each time point as shown in figure 3.8. The corresponding static biofilms were also imaged using SEM (Figure 3.9). I found that while the biofilm formation between the two strains was not significantly different at 6 hrs after incubation, the mutant strain started forming a thicker biofilm after the 12 hr time point. The biofilm formed by the mutant strain kept increasing in quantity until 72 hrs whereas the wildtype biofilm increased until 24 hrs, plateaued at 48 hrs, and even slightly degraded at 72 hrs. This could be possible because bacterial lysis-mediated release of NagZ is preventing further increase in biofilm formation and even

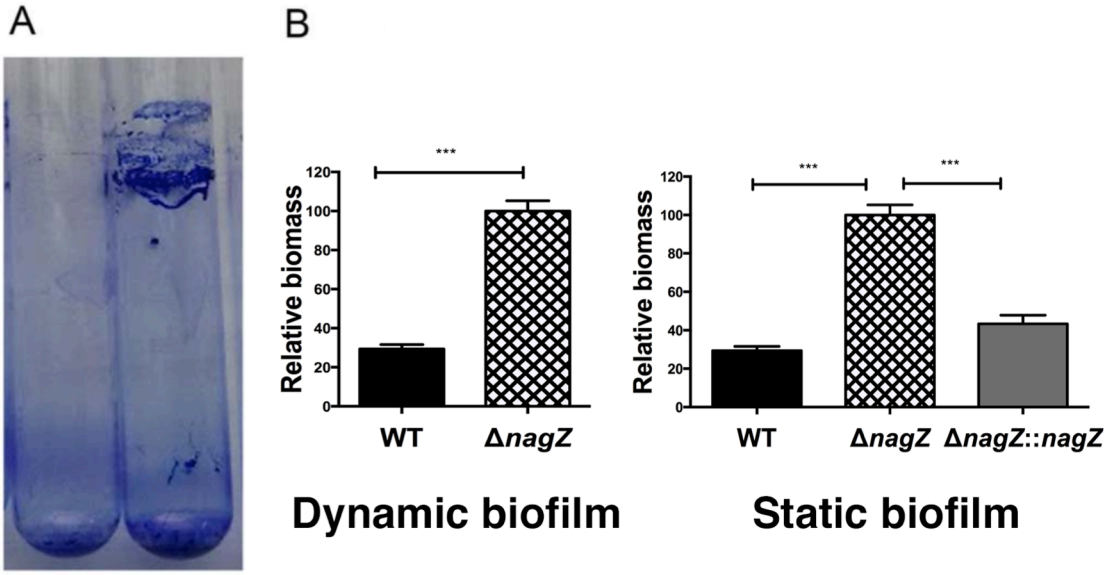


Figure 3.7 Biofilm formation by gonococcal strains. (A) Photograph of dynamic biofilm formed by wildtype bacteria (left) and the Δ*nagZ* mutant strain (right) after 24 hrs. (B) Quantification of dynamic biofilm formation by FA 1090 wildtype and FA 1090 Δ*nagZ* on the left (n = 6) and static biofilm formed by the same strains on the right, using crystal violet staining and measuring absorbance to estimate relative biomass as described in the methods. Complementation of *nag*Z in the knock out strain reduced the ability of the mutant to form a thicker biofilm in a static biofilm (n = 5, ** $p<0.01$ and *** $p<0.001$)

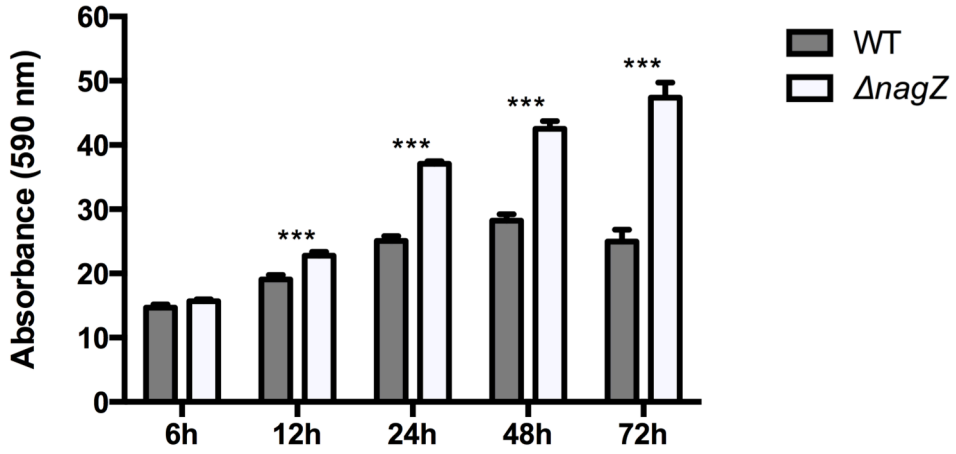


Figure 3.8 Biofilm evolution over time by wildtype and Δ*nagZ* strains. (A) Dynamic biofilms were prepared, and the biomass of biofilm determined at various time points by crystal violet staining method as described in the methods section. Statistics were analyzed by two-tailed t-test assuming unequal variance. Data shown is mean(+/- SEM) of 3 independent experiments (*** $p<0.001$).

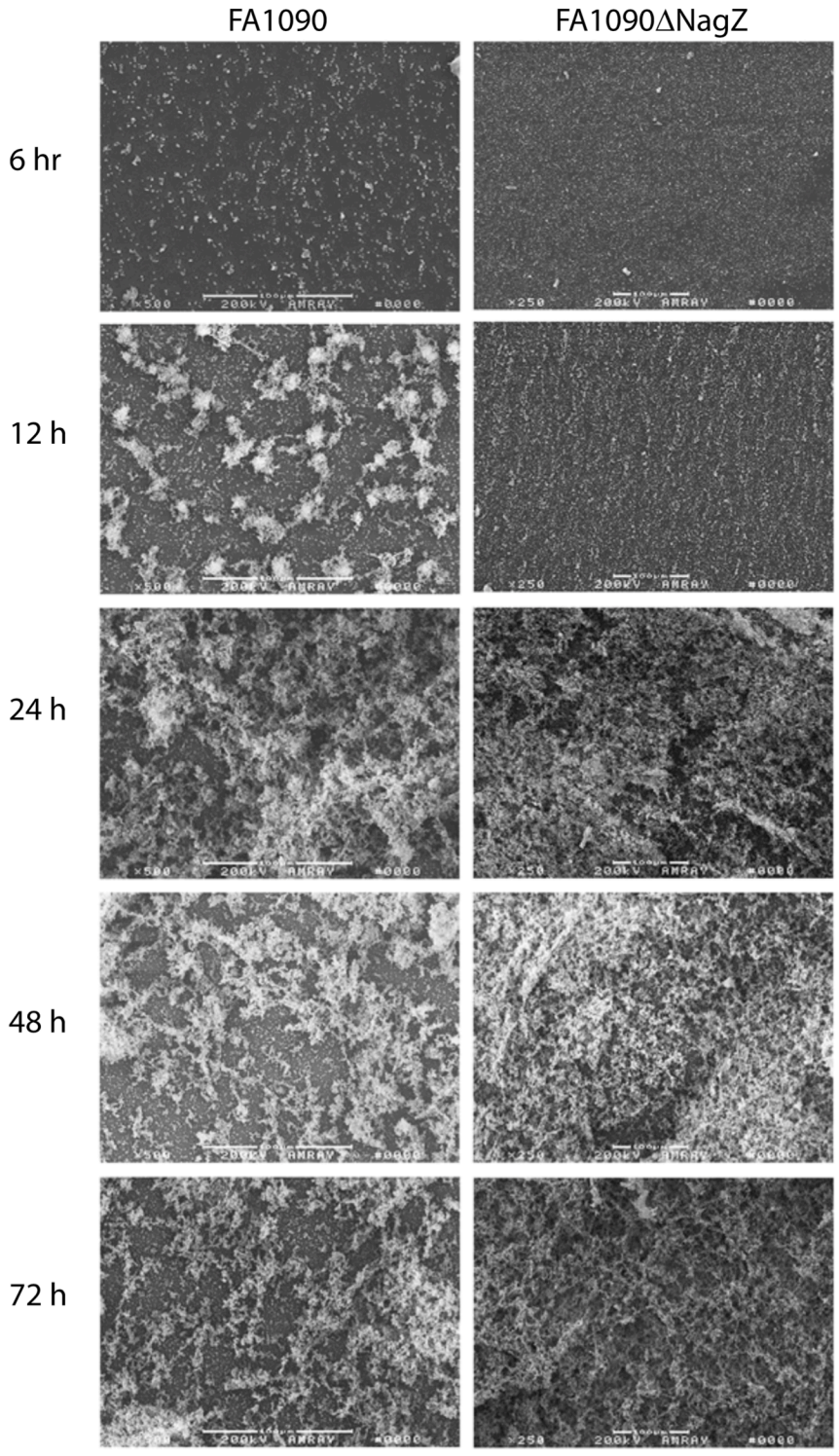


Figure 3.9 SEM of gonococcal biofilm. Static biofilm by *N. gonorrhoeae* FA 1090 WT and *N. gonorrhoeae* FA 1090 Δ*nagZ* visualized by SEM at various time points (250x magnification).

promoting biofilm degradation, possibly also in association with release of other intracellular biofilm degrading agents such as nuclease. The absence of NagZ could explain the continuously increasing biofilm being formed the deletion strain. Since the growth curve of the two strains looks identical it is unlikely that a defect in bacterial survival could be the cause for increased biofilm formation.

3.3.8 Visualization of the biofilm

In order to understand the biofilm architecture, I used microscopic techniques to visualize the biofilm formed by the two strains. I used confocal microscopy on the static biofilms grown for 36 hours, using Hoechst stain to visualize the biofilm. The biofilms were grown in MaTeK glass bottom dishes. The mutant strain formed a denser and taller biofilm (~4 fold increase), consistent with qualification of biomass using crystal violet method, as shown in figure 3.10. In addition I also observed that the wildtype biofilm has bacteria escaping the biofilm in small clusters while this is seldom seen in the mutant biofilm. This suggests a defect in bacterial dispersal from biofilm caused by *nagZ* deletion.

In order to see if there was an alteration in bacterial survival in the biofilm, I stained the biofilm with Hoechst and propidium iodide (PI) to analyze the proportion of live and dead bacteria in the biofilm. Hoechst stains both dead and live bacteria whereas PI only permeates the membranes of dead bacteria. The data in figure 3.11. shows the double-stained biofilms visualized by confocal microscopy. Panel B shows quantification by comparing the mean fluorescence intensity ratio (FIR) of Hoechst staining to PI staining. I observed no difference in proportion of live to dead bacteria

in both kinds of biofilm, although the amount of dead bacteria increased over time as seen by an increase of fluorescence intensity of PI.

I visualized the surface of the biofilm using a SEM. Figure 3.9 shows the biofilm formation over a glass slide visualized at various time points. The SEM image shows a very similar-looking biofilm at 6 hour in both strains whereas the wildtype strain appears to form sites of enucleation at 12 hours in contrast to the mutant which is spread out and begins forming a diffuse biofilm. The *nagZ* knockout strains forms a denser biofilm and continues to increase in density until 48 hours and shows a slight degradation whereas the wildtype biofilm begins to start degrading after 48 hours. Analysis of older biofilms suggested that FA 1090 was lysing, releasing intracellular contents such as DNase. This was evident by reduced amount of DNA visible in the older FA 1090 biofilms as compared to FA 1090 Δ*nagZ* biofilms.

Since all of the previous experiments were carried out on abiotic surfaces, I tried to understand biofilm formation on biotic surface using both human cell lines and human cervical tissue explants. Cervical tissues were obtained from consenting women undergoing uterine surgeries and sourced by NDRI. The tissue was cut into small pieces of endocervical tissue and ectocervical tissue, and grown in CMRL media in 24-well plates. Each piece of tissue was approximately 3 millimeter by 3 millimeter. The tissue was infected with approximately 10^9 bacteria per tissue piece, with both wildtype and mutant strain for various times. Map tiling function was used to take images of small sections of the tissue as a Z-stack, and all the images were

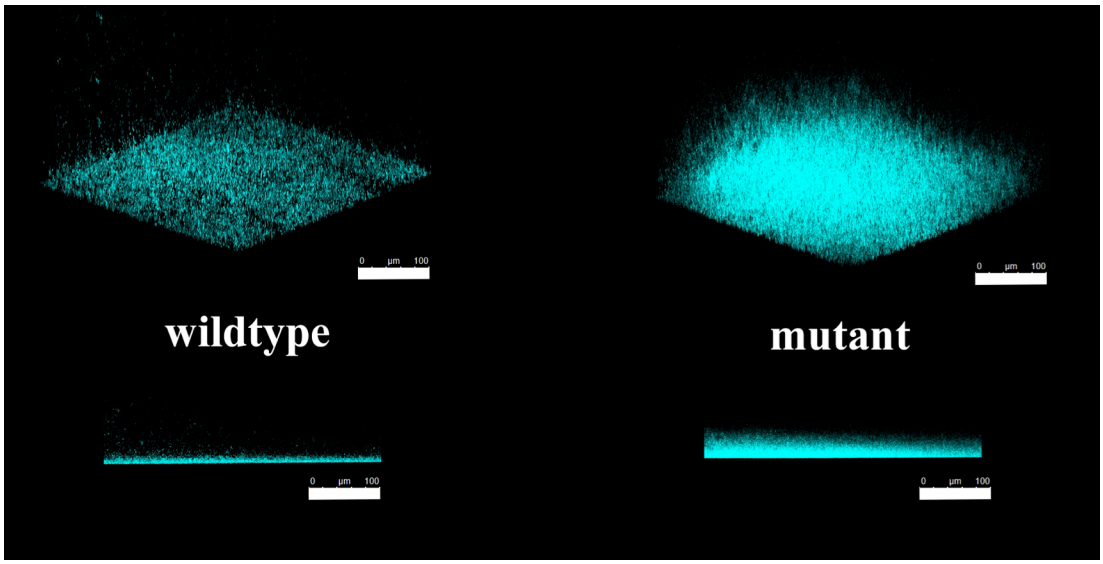


Figure 3.10 Confocal imagine of biofilm formation by gonococcal strains. Static biofilm formed by *N. gonorrhoeae* FA 1090 wildtype (left) and *N. gonorrhoeae* FA 1090 Δ*nagZ* (right) visualized by confocal microscopy, after staining with Hoescht. The view on top shows a tilted x-y projection and view on bottom is x-z representation of the same biofilm field. Scale bar represents 100 microns.

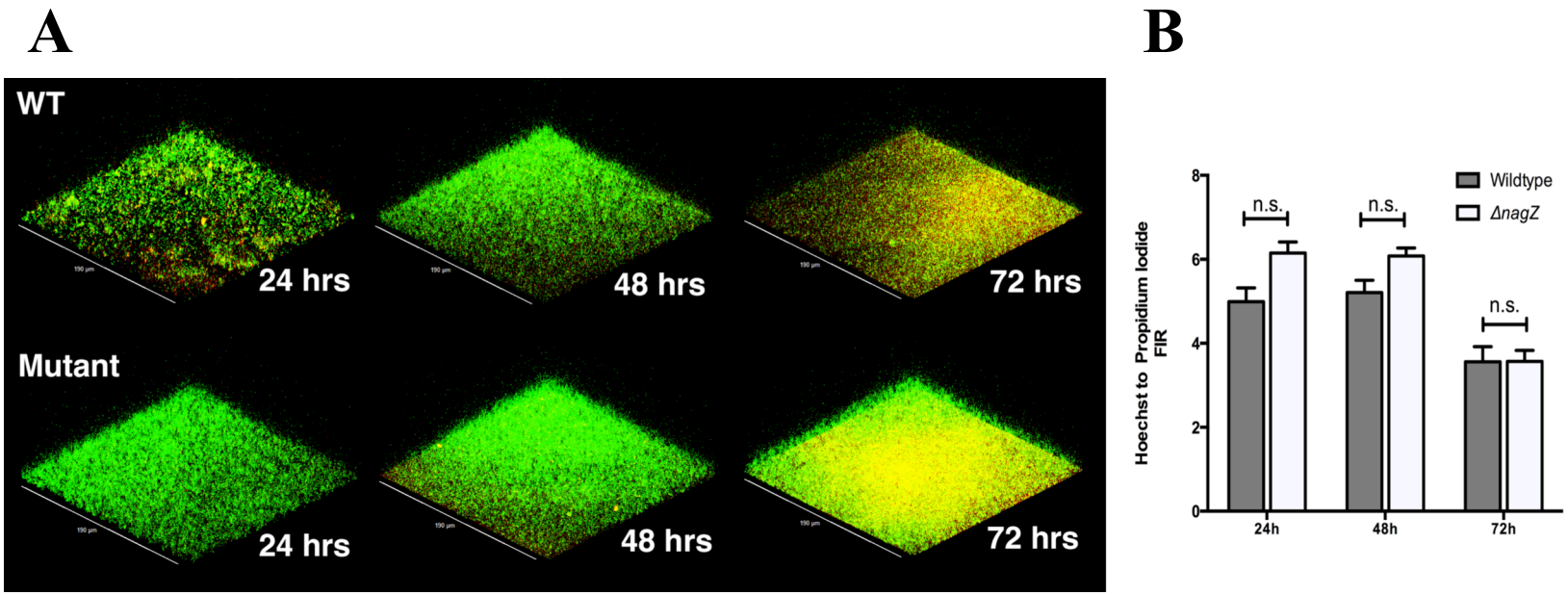


Figure 3.11 Live – dead analysis of gonococcal biofilm. (A) Static biofilms were formed with FA 1090 wildtype and FA 1090 Δ*nagZ* strains for different time points, and visualized by confocal microscope after staining with propidium iodide (red) and Hoechst (green). (B) The fluorescence intensity ratio (FIR)between Hoechst and PI was measured for both strains at the three time points. Shown in panel A are representative images and average FIR (with SEM) from three independent experiments. (n.s. not significant)

tiled using automated software to create a Z-stack of the entire cervix specimen. The stacks were Z-projected as shown in figure 3.12. Confocal imaging showed that the mutant strains were able to form a better biofilm on the surface of cervical tissue than the wildtype strain (Figure 3.12). I used ImageJ to quantify the fluorescence intensity in proportion to the area of tissue as well as the ratio of biofilm fluorescence intensity to fluorescence intensity of Hoescht staining since it should be representation of the total number of cells in the tissue slice. I observed that the proportions in both measures were higher in the *nagZ* experiment compared to wildtype strain, which is in agreement with experiments performed abiotic surface (Figure 3.10). Biofilms formed over cervical tissue explant were also visualized by SEM. The data in figure 3.14 shows biofilms formed by both strains over cervical explants. Similar to previous experiments on glass surface, I observed that the mutant strain forms a denser biofilm over cervical explants as evident by the increase in bacterial density as well as cervical epithelial cell damage. The experiments were performed by forming biofilm for 48 hours on the surface of cervical tissue explants. Figure 3.15 also shows biofilm formation by the two strains on the apical surface of polarized T84 cells, which is a colonic epithelial cell line. The biofilm formation above the apical surface was marked out using ImageJ (the gaps within biofilm were excluded) and the proportion of biofilm area to tissue area was calculated using 2500x images taken from random fields. Figure 3.16 shows the comparison of the quantification of biofilms formed by wildtype and ΔnagZ strains. This data is consistent with our previous findings that *nagZ* knockout strain forms a thicker biofilm in comparison to wildtype strain.

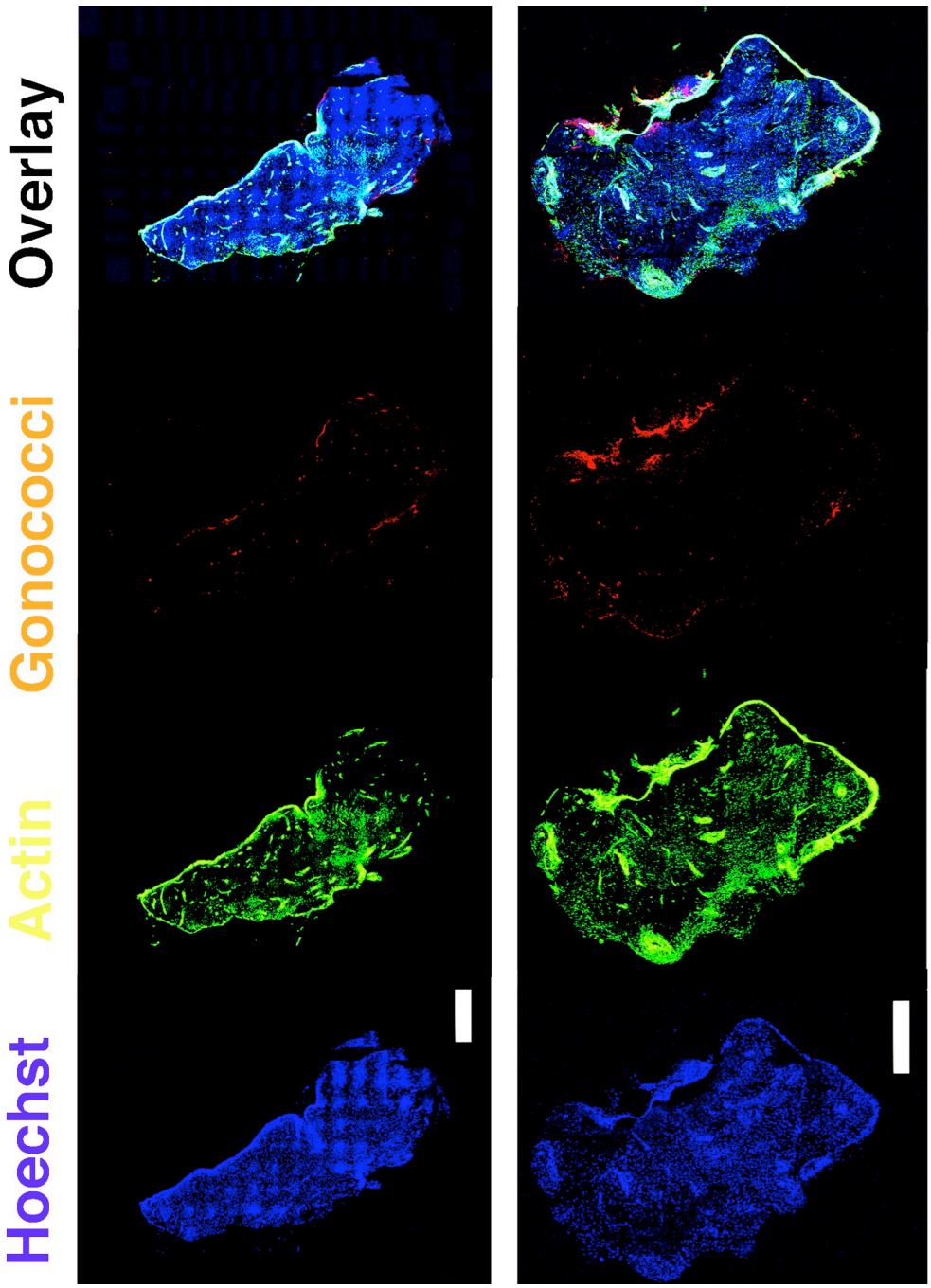

Hoechst
Actin
Gonococci
Overlay

Figure 3.12 Gonococcal biofilm formation on cervical explant tissue. Biofilm formed on the surface of human cervix tissue explant by *N. gonorrhoeae* FA 1090 WT (on top row) and *N. gonorrhoeae* FA 1090 Δ*nagZ* (on bottom row) was fixed after 48 hours and visualized by confocal microscopy. The tissue was stained for nucleus (Hoechst - blue), actin (phalloidin - green), and gonococci (anti-GC antibody - red). The right most panel shows an overlay of all channels. Scale bar corresponds to 500 μm.

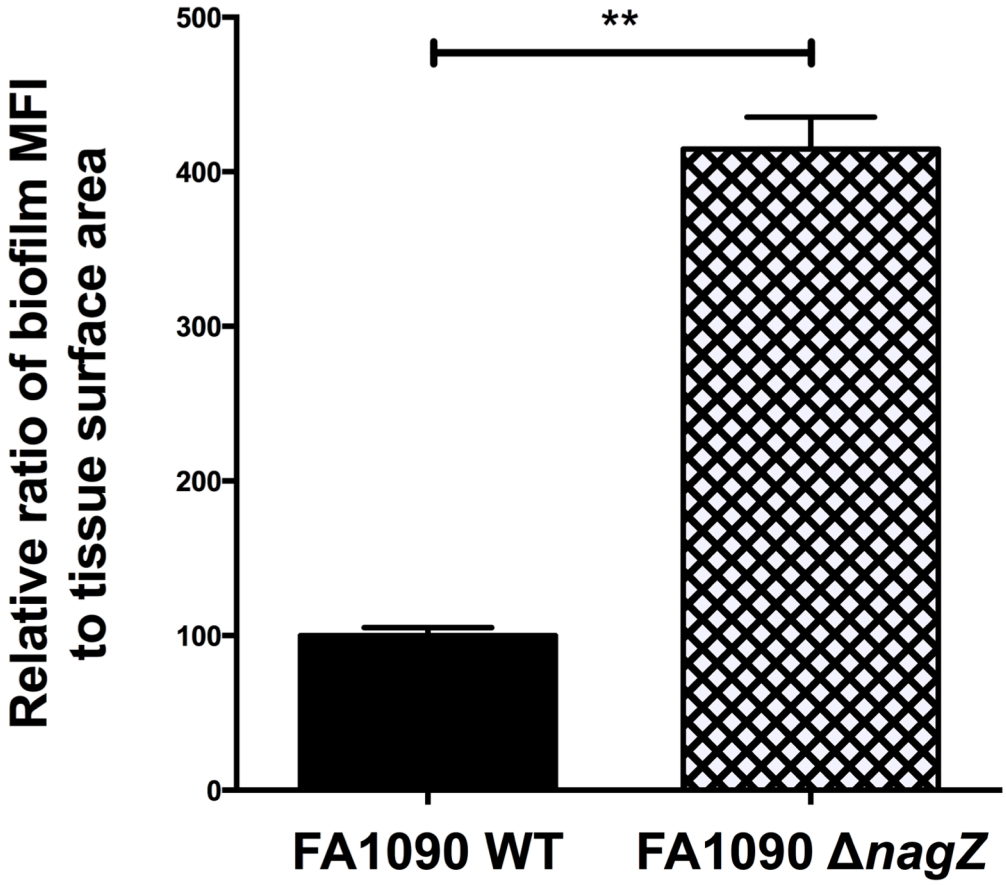


Figure 3.13 Quantification of gonococcal biofilm formation on cervical explant tissue. Normalized ratio of mean fluorescence intensity (MFI) of gonococcal biofilm to tissue surface area determined by ImageJ for FA 1090 wildtype and FA 1090 ΔnagZ strain. Results are average (with SEM) of two independent experiments.

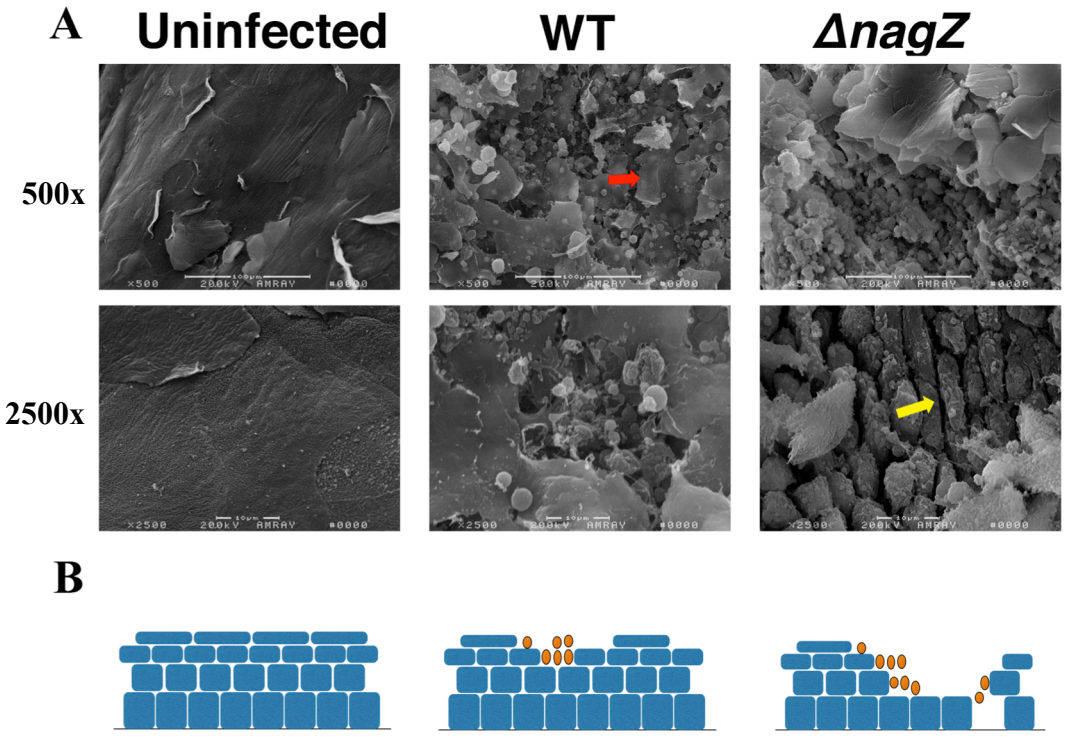


Figure 3.14 SEM of gonococcal biofilm on cervical explant tissue. (A) SEM images showing biofilm formation (after 96 hours) by wildtype and ΔnagZ strains of *N. gonorrhoeae* FA1090 on the surface of human cervix tissue along with uninfected cervix sample on the left side, at 250x and 2500x. Red arrow shows squamous epithelial cells that are being sloughed off and yellow arrow points at cuboidal cells in bottom layer that are now exposed. (B) Cartoon representation of stratified squamous epithelial layer in uninfected sample and epithelial shedding seen in infected samples.

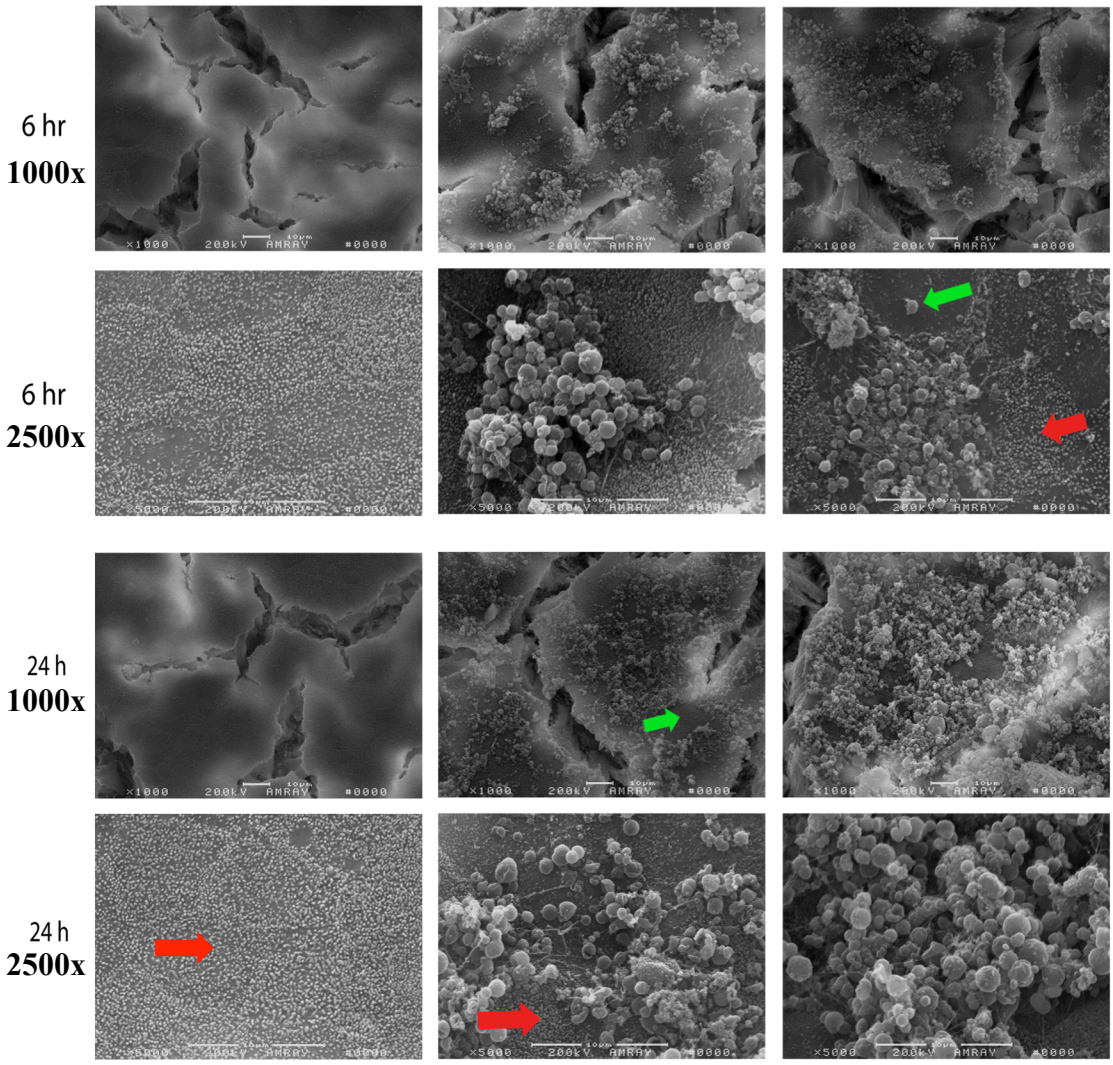


Figure 3.15 SEM of gonococcal biofilm on polarized T84 cells. Gonococcal biofilm was formed on the apical surface of polarized T84 epithelial cells by *N. gonorrhoeae* FA 1090 WT and Δ*nagZ* strains at 6 hours and 24 hours. Images taken at two different magnification (1000x and 2500x) are shown for each biofilm. The left most images are taken from uninfected T84 epithelial cells. Green arrows point to apical surface of epithelial cells and red arrows point at microvilli on the apical surface.

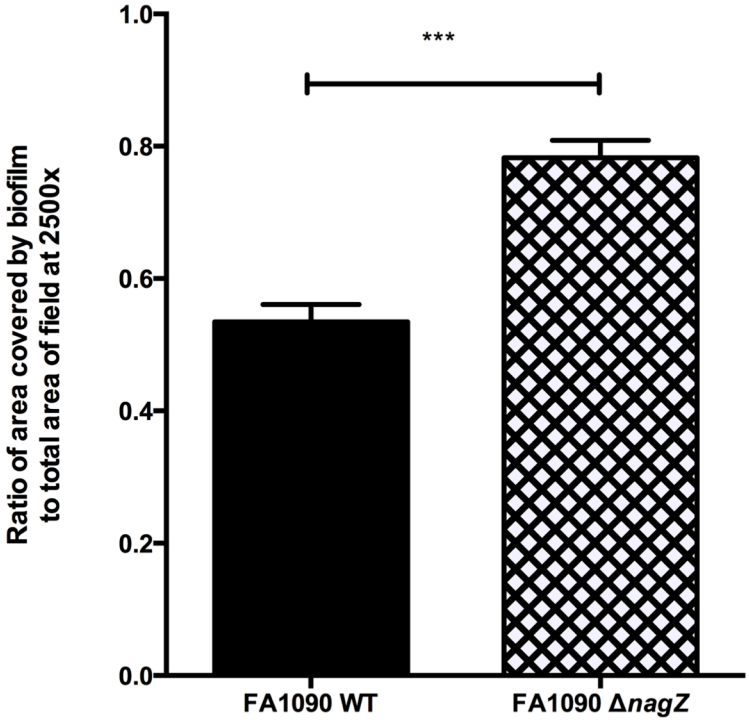


Figure 3.16 Quantification of gonococcal biofilm on epithelial cells. SEM images were taken at random fields at 2500x magnification and the area of epithelial cell surface covered by biofilm measured manually using ImageJ. The ratio of area covered by biofilm to the total area of the image was calculated for both wildtype and ΔnagZ strains. Two-tailed Student's t-test was used to determine statistical significance. (*** $p < 0.001$)

3.3.9 *nagZ* deletion does not alter blebbing

N. gonorrhoeae generates outer membrane vesicles. Alterations in vesicle formation has been observed in bacteria with defects in peptidoglycan metabolism, membrane protein synthesis and the σ^E envelope stress response (209). Since NagZ is involved in peptidoglycan recycling, it is possible that NagZ could affect biofilm formation indirectly by increasing the release of outer membrane vesicles (210). To determine if deletion of *nagZ* resulted in a change in the ultrastructure of bacteria in biofilm, high resolution scanning electron microscopy (SEM) was used to visualize the biofilm architecture and the ultrastructure of bacteria within the biofilm. SEM can also be used to identify any defects in EPS matrix components which could explain the difference in biofilm formation between the two strains. Biofilms were grown as described earlier, under static conditions and samples fixed for SEM microscopy. Figure 3.17 shows membrane blebbing on the surface of individual bacteria in both wild type and knockout strains at all time points.

I measured the distribution of membrane blebs using ImageJ. Images of random fields were taken at 20000x magnification and blebs were identified by defining their size as less than 250 nm (210). Figure 3.18 shows that there was no difference in bleb distribution between the two strains, which rules out membrane blebbing defect as cause for difference in biofilm formation. SEM images also show what appears to be DNA entangling the bacterial aggregates in a random fashion. The source of DNA is possible bacterial lysis since the strain I used (FA 1090) does not secrete DNA, unlike MS11 or other strains possessing the GGI. The DNA strands were disappeared after treatment with DNase but were unaffected by proteinase K treatment of biofilm

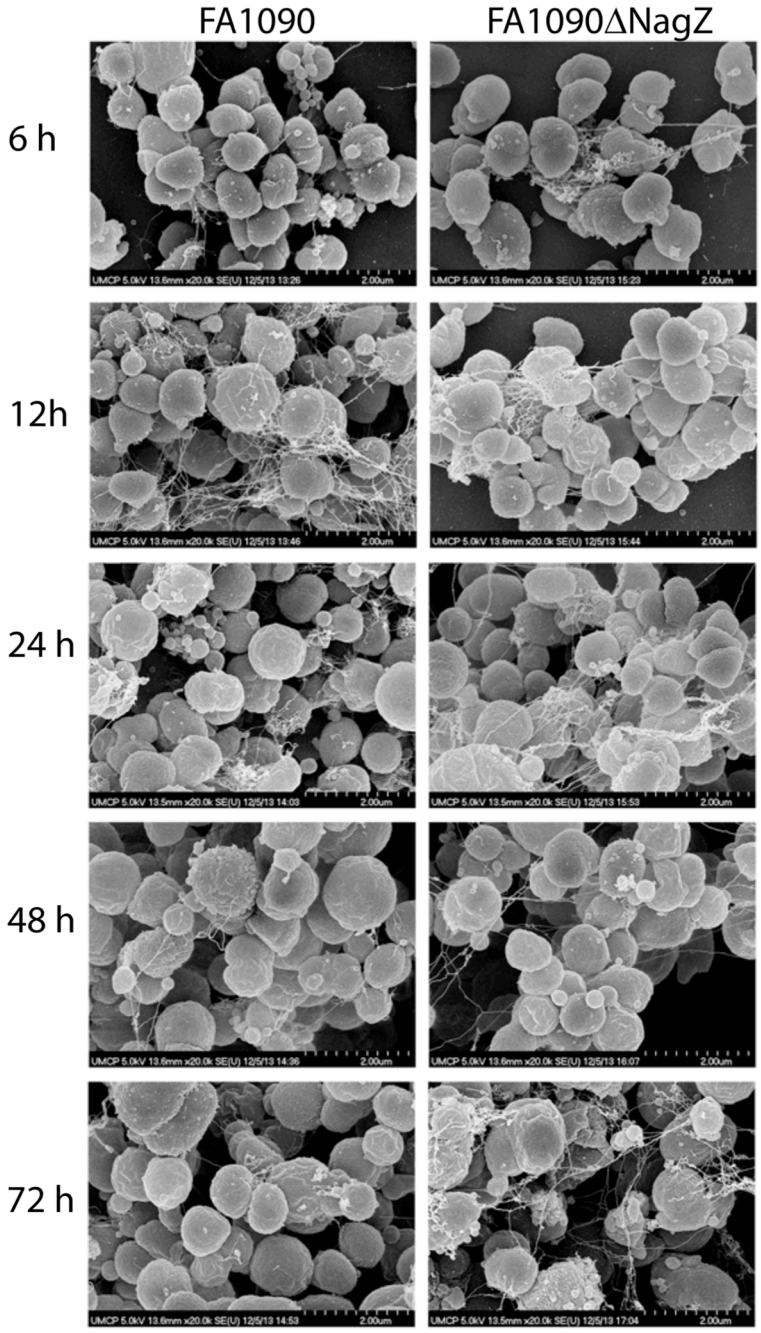


Figure 3.17 High resolution SEM of gonococcal biofilm. High magnification image of gonococcal clusters found within the biofilm at different time points in wildtype and ΔnagZ strains (20,000x). These images are representative of bacteria when they are contained within a cluster.

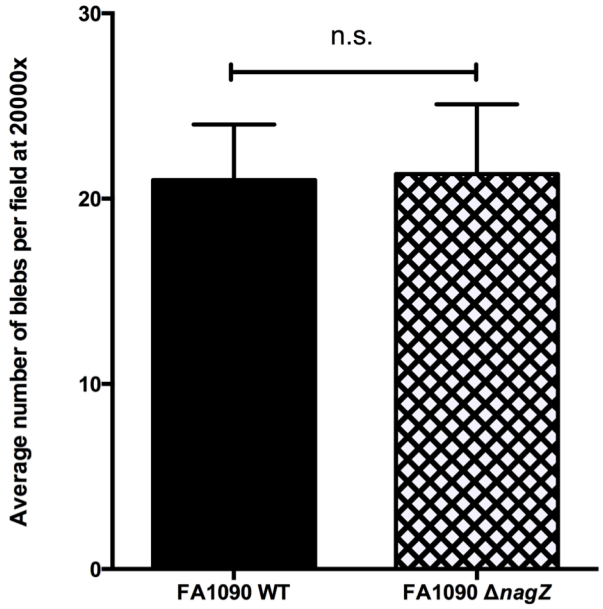


Figure 3.18 Quantification of blebbing. Average number of OMV blebs per field in biofilm formed by wildtype and ΔnagZ strain. Multiple SEM images of 48 hour biofilm were taken at 20000x magnification of wildtype and ΔnagZ strains. Membrane blebs were defined as anything less than 250nm and counted using ImageJ software. (n.s. – not significant)

suggesting they are composed of nucleic acid. The presence of DNA, in spite of the absence of a known secretion system, shows that intracellular contents are released following bacterial death and that bacterial lysis serves a vital purposes in biofilm formation. I did not observe any differences in extracellular DNA (eDNA) or membrane blebbing in either strain, showing that the differences in biofilm formation are not due to any alteration in vesiculation or eDNA availability.

3.3.10 DNA contributes to structural integrity of both WT and mutant biofilm

Zweig M *et al* have shown that secreted single-stranded DNA (ssDNA) plays a role in the initial phases of biofilm formation, but it is not incorporated in mature biofilms in *N. gonorrhoeae* MS11 (80). To understand the role of eDNA in biofilm stability in *N. gonorrhoeae* FA1090, which releases eDNA predominantly by lysis, I measured the amount of biofilm formed in the presence of DNase at varying time points by both wildtype and mutant strains. The biofilms were stained and measured the same way as described before. Figure 3.19 shows that both wildtype and mutant strains appear to be equally affected by presence of DNase with regard to biofilm formation. Further, there was no difference in effect of DNase co-incubation between biofilms formed at 24 hours, 48 hours, and 72 hours, suggesting that eDNA in GC FA1090 biofilm plays a structural role both in early biofilm and mature biofilm. This experiment highlights the vital role played by eDNA in GC biofilm.

3.3.11 Biofilm dispersal by adding exogenous NagZ

In order to test my hypothesis that NagZ acts extracellularly by promoting

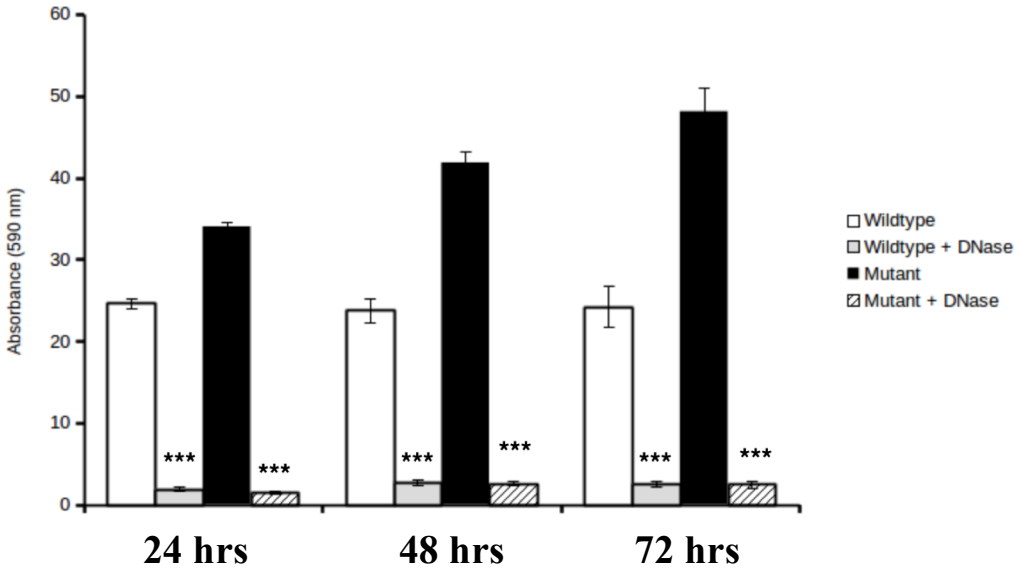


Figure 3.19 Role of DNA in gonococcal biofilm. Absorbance of biofilm formed by wildtype and ΔnagZ strains, with and without DNase incubation, measured at 590 nm after staining with crystal violet as described in methods. Data represents mean values (± SEM) of three independent experiments performed in triplicate. Statistics were analyzed by two tailed Student's t-test (*** $p < 0.001$).

biofilm dispersal, I added purified NagZ on biofilm formed by gonococci. Static biofilms were formed and treated with NagZ protein for 3 hrs at 37ºC. Biofilms were either quantified by crystal violet method or visualized by SEM, after treatment.

The data in Figure 3.20 shows that NagZ treatment reduces the thickness and density of biofilm formed by the mutant, making its appearance more reminiscent of the biofilm formed by wildtype strain. NagZ-treated biofilms still show presence of intact strands of genetic material which disappeared on treating with DNase. Although NagZ is not a secreted protein, it can have a direct effect on biofilm formation through extracellular release following autolysis, along with other intracellular contents that can potentially modulate biofilm formation such as the Nuc thermonuclease. Our experiments showing NagZ activity in the supernatant (stationary phase) of broth culture but not in mid-log phase supernatant support this idea.

3.3.12 NagZ does not act upon LOS

Previous work from our lab and other labs has shown the importance of LOS in bacteria-bacteria interaction. Deletion of *rfaK* in *N. gonorrhoeae* FA 1090, which results in loss of lactoneotetraose as well as the GlcNAc in the LOS core region, results in significantly impaired biofilm formation. Since it appears that exogenously added NagZ can impair biofilm formation, I wanted to investigate if NagZ can act upon LOS. In addition, NagZ was able to cleave a variety of substrates containing N-acetylglucosamine, and neisserial LOS contains N-acetylglucosamine in a variety of conformations. LOS was purified from *N. gonorrhoeae* F62 using hot phenol-water method, digested with NagZ and its mobility in a Tris-tricine gel compared with that

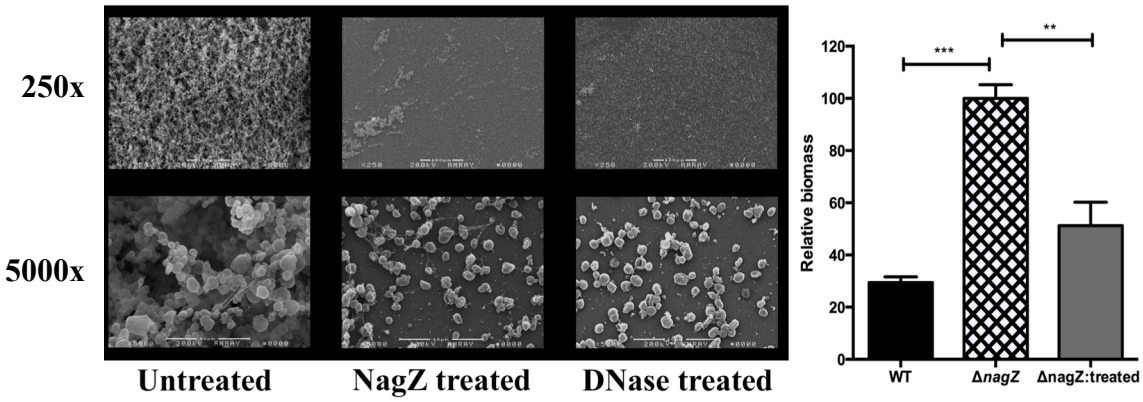


Figure 3.20 Biofilm destruction by exogenously added NagZ. (A) SEM showing differences between untreated ΔnagZ biofilm (first column), ΔnagZ biofilm treated with NagZ (second column), and ΔnagZ biofilm treated with DNAse (third column) at 250x and 5000x magnifications. The DNA strands disappear on treatment with DNAse in the third column, while NagZ almost completely destroys biofilm. (B) Quantification of biofilm formed by untreated mutant strain, and mutant strain biofilm treated with purified NagZ, using crystal violet method. There is a statistically significant reduction in biofilm on treatment with NagZ (**$p < 0.01$). Two-tailed t-test assuming unequal variance was employed to determine statistical significance.

of undigested LOS. Panel A in figure 3.21 shows the 16.5% Tris-tricine gel, silver-stained, where lane 1 and 2 are control lanes of *N. gonorrhoeae* F62 Δ8-1 and *N. gonorrhoeae* F62 wildtype strain respectively. The structures corresponding to each band in the gel are marked as A, B or C and are represented in the bottom panel. Lane 3 shows LOS from *N. gonorrhoeae* F62 Δ*nagZ* which does not show any difference in comparison to lane 3 and 4. Lane 4 is the same LOS as in lane 3 but treated with purified NagZ *in vitro.* Since lanes 2, 3 and 4 are virtually identical, I concluded that NagZ does not act upon LOS even though there are terminal and internal GlcNAc molecules in various forms. This showed that NagZ lacks β-1-3 exoglycosidase activity and α-1-2 endoglycosidase activity.

3.3.13 NagZ acts on peptidoglycan

NagZ in other organisms such as *E. coli* and *P. aeruginosa* function in peptidoglycan recovery by cleaving GlcNAc from PG monomers. NagZ was tested for its ability to digest peptidoglycan fragments in the extracellular milieu. As gonococci grow they recycle most of the peptidoglycan fragments removed from the sacculus, but they release a significant portion of these fragments into the medium. Our collaborators at University of Wisconsin, Jonathan Lenz and Joe Dillard carried out the following experiment. *N.gonorrhoeae* was pulse-labeled with [6-^{3}H]glucosamine to label the N-acetyl glucosamine and N-acetylmuramic acid in peptidoglycan in the cell wall. After a 2.5 hr chase period, the supernatant was collected and the ability of purified NagZ to digest peptidoglycan fragments in the supernatant was examined. The addition of boiled NagZ had no apparent effect on the peptidoglycan fragments in the supernatant, and the profile of peptidoglycan

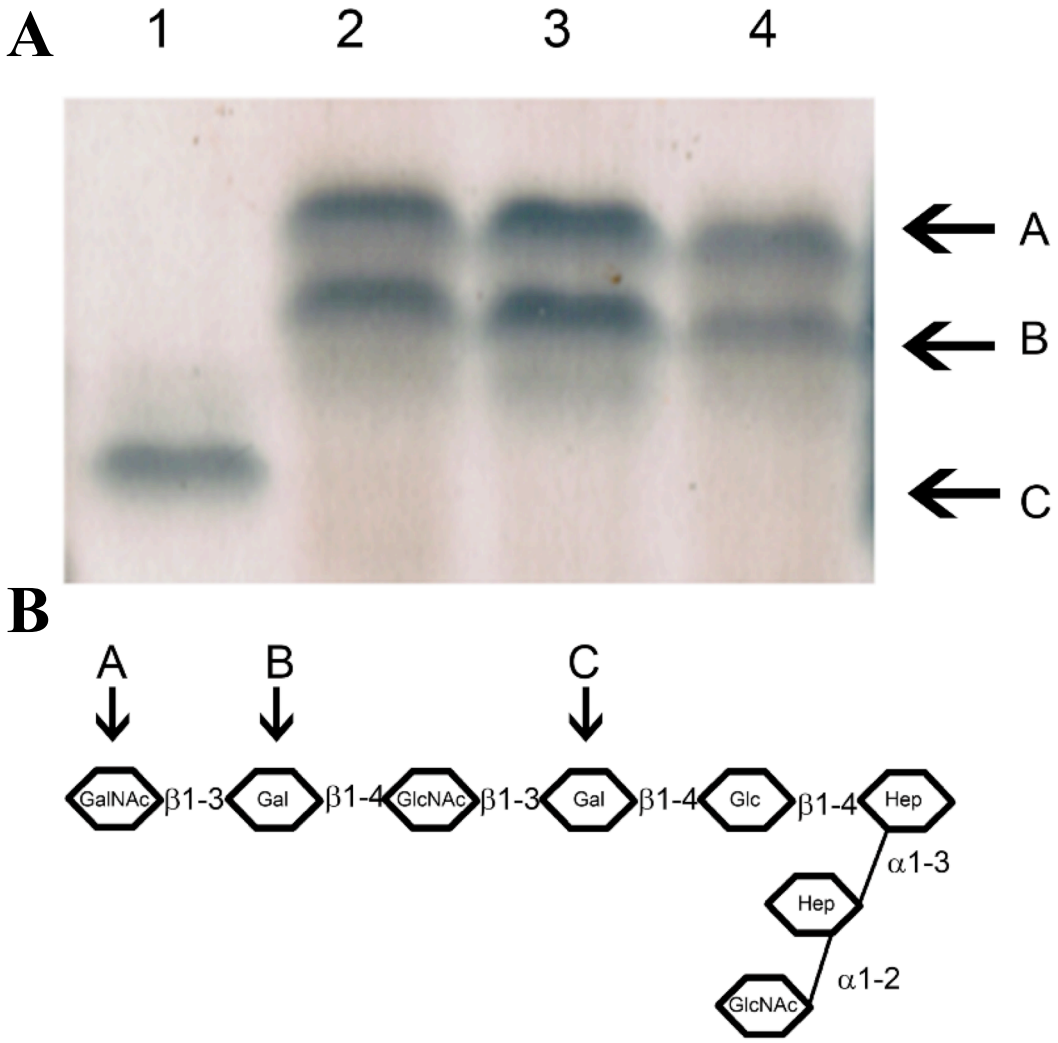


Figure 3.21 NagZ does not act upon gonococcal LOS. (A) SDS-PAGE gel of LOS isolated from: 1) *N. gonorrhoeae* F62 Δ8-1; 2) *N. gonorrhoeae* F62; 3) *N. gonorrhoeae* F62Δ*nagZ*; and 4) *N. gonorrhoeae* F62Δ*nagZ* treated with NagZ. (B) Graphical representation of the oligosaccharide portion of LOS isolated from F62. Letters in the LOS schematic correspond to the LOS bands seen in the SDS-PAGE gel.

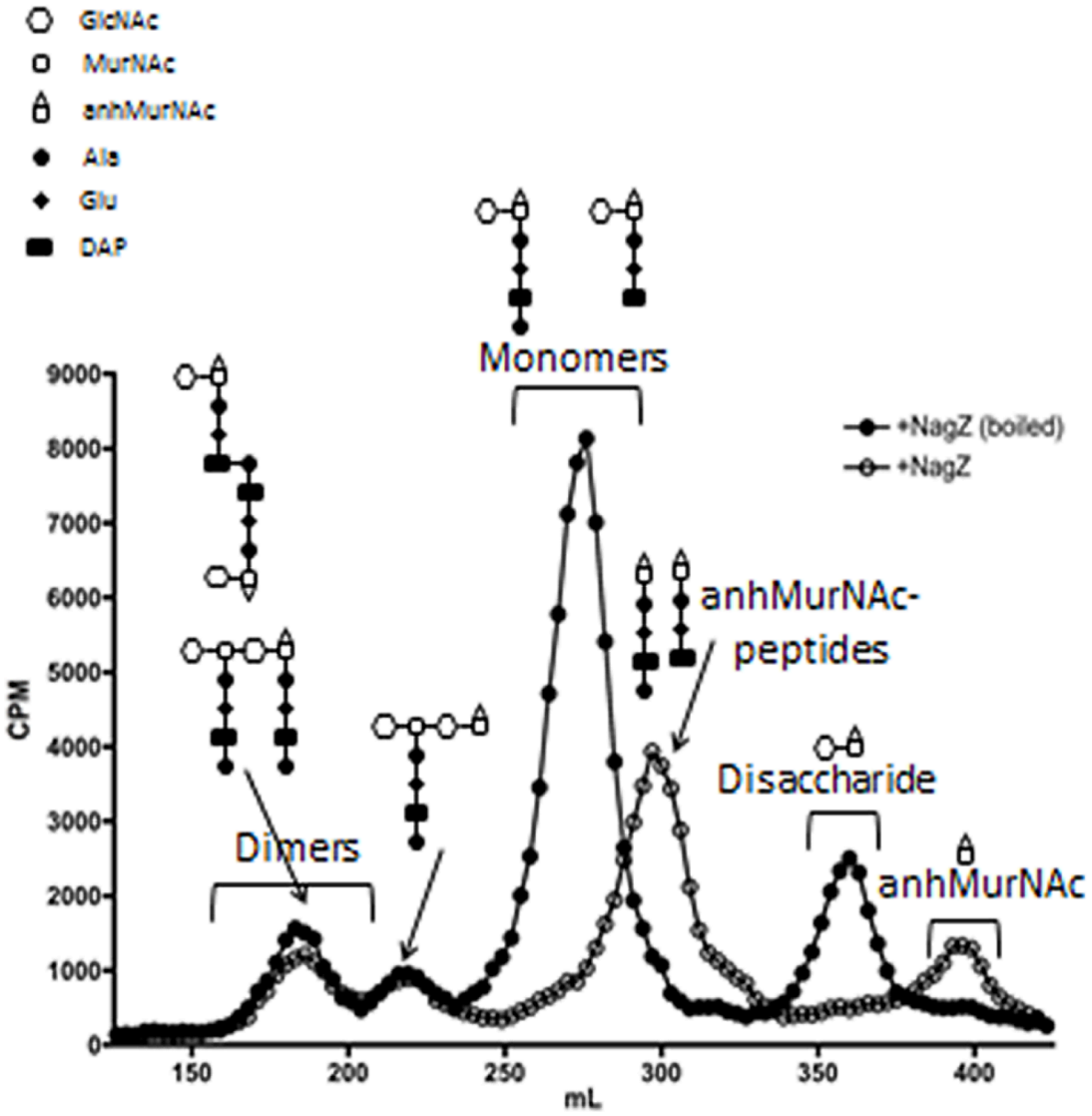


Figure 3.22 NagZ acts on peptidoglycan monomers. Gonococci were metabolically labeled with [6-^{3}H]glucosamine. After a chase period, supernatants were collected and treated with purified NagZ. Radiolabeled fragments were separated by size exclusion chromatography and detected by liquid scintillation counting. Gonococcal peptidoglycan contains PG dimers, monomers, free disaccharide, and anhydro-*N*-acetylmuramic acid. GlcNAc, *N*-acetylglucosamine; MurNAc, *N*-acetylmuramic acid; anhMurNAc, anhydro-*N*-acetylmuramic acid.

fragments showed the presence of dimers, monomers, and free disaccharide as has been observed in multiple studies of peptidoglycan fragment release from gonococci (Figure 3.22). Addition of active NagZ changed the profile of peptidoglycan fragments. The peptidoglycan monomer peak shifted to later fractions (smaller size) from the sizing columns, consistent with the removal of N-acetyl glucosamine from the peptidoglycan monomers. Similarly, the free disaccharide peak was eliminated and a peak appeared for free 1,6-anhydro-N-acetylmuramic acid. No significant changes were seen to the peaks for peptidoglycan dimers and tetrasaccharide-peptide, suggesting that these molecules are not substrates of NagZ. These data are consistent with the known activity NagZ in other bacteria, acting to remove N-acetylglucosamine from peptidoglycan monomers and free disaccharide. In addition, adding NagZ to live bacteria had no effect on their viability unlike lysozyme which has endoglucosaminidase activity. This further confirmed that NagZ lacked endoglycosidase activity.

3.3.14 NagZ-mediated biofilm degradation is affected by surface glycosylation

Goodwin *et al* in a recent paper demonstrated how *E. coli* sabotages production of O-linked-GlcNAc modified proteins *in vivo* (207). NagZ was identified to be the molecular saboteur responsible for cleaving O-linked GlcNAc from proteins. This further supports the idea that NagZ could alter protein glycosylation on the surface of GC, and hence alter biofilm formation. In order to test this idea, I constructed a *pglC* deletion in *N. gonorrhoeae* FA1090 and FA1090Δ*nagZ*. Vik *et al* demonstrated the

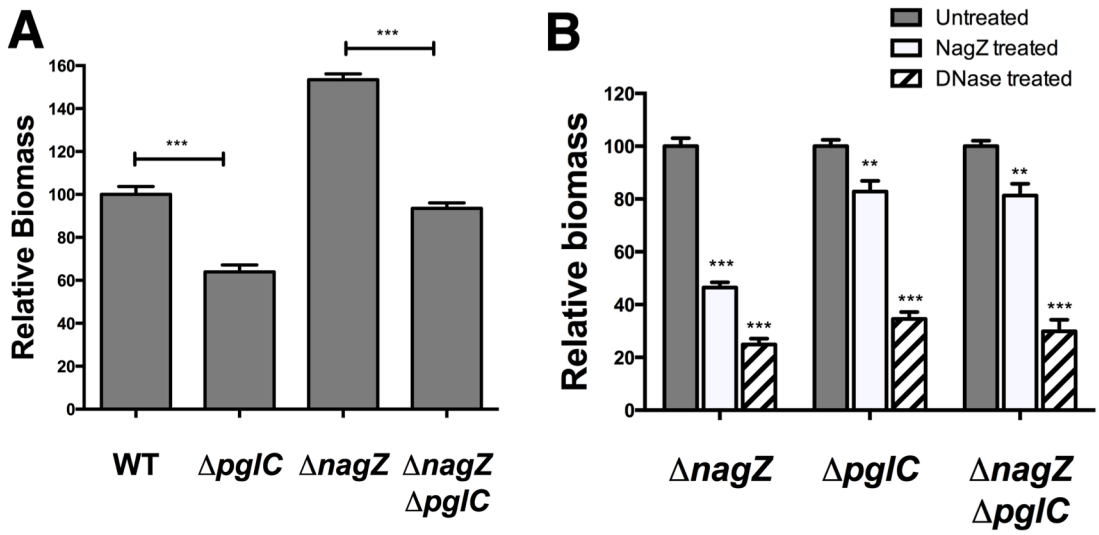


Figure 3.23 Role of PglC-dependent glycosylation in gonococcal biofilm formation. (A) Relative biomass of static biofilm formed by FA1090 wildtype, Δ*pglC, ΔnagZ,* and ΔnagZΔ*pglC* double mutant strain. (B) Relative biomass of static biofilm formed by Δ*pglC, ΔnagZ,* and ΔnagZΔ*pglC* double mutant strain before and after treatment with purified NagZ and DNase. Data represents mean values (± SE) of three independent experiments performed in triplicate. Statistics were analyzed by two tailed Student's t-test (** $p < 0.01$, *** $p < 0.001$).

promiscuity of the pilin glycosylation system showing that more than 15 proteins were glycosylated in a PglC-dependent manner. I compared the biofilm formation between FA1090 and FA1090Δ*pglC*. Figure 3.23 (Panel A) shows that loss of *pglC* resulted in a significant reduction of biofilm formation of *N. gonorrhoeae* FA 1090. The same effect was observed on Δ*nagZ* background. Loss of *pglC* resulted in abrogation of the increased in biofilm formation caused by deletion of *nagZ.* Figure 3.23 (Panel B) shows the effect of NagZ and DNase treatment on *N. gonorrhoeae* FA 1090 Δ*nagZ*, Δ*pglC* and Δ*pglC nagZ* strains. While DNase has a very potent effect on all three strains in degrading biofilm, NagZ has maximal effect only on Δ*nagZ* strain. While NagZ treatment causes statistically significant reduction in the other two strains, the reduction is minimal suggesting that NagZ requires the bacterial surface to be glycosylated to have its full effect.

3.4 Discussion

N. gonorrhoeae and *N. meningitidis* can form biofilms *in vivo* and *in vitro*, however the mechanism by which they are formed, and the processes that mediate their dispersal remain unclear. Zweig and coworkers demonstrated that secreted single-stranded DNA is involved but not required in the initial phase of biofilm formation and is not retained in the biofilm as it matures (80). Furthermore, they showed that the organizational role of ssDNA is taken over by other components in the later stages of biofilm formation. Steichen and coworkers showed that gonococcal biofilms contain DNA and that Nuc nuclease appears to play a crucial role in biofilm formation and remodeling (118). Consistent with these observations, I observed that

exogenously added DNase significantly impacts gonococcal biofilm. My studies also indicate that exogenously added NagZ results in destruction of preformed biofilms, and in its absence, the biofilms continue to accumulate over time. Since NagZ is cytosolic, and only seen in culture supernatants of stationary phase gonococci, this further supports the conclusion that older gonococcal biofilms underdo significant decay due to autolysis. While NagZ has been shown to play a role in peptidoglycan (PG) turnover in other organisms, this report is the first to demonstrate its ability to degrade a biofilm. Since strain FA1090 lacks the type IV secretion system common to most gonococcal strains, this raises the question as to the source of extracellular DNA in young biofilms (74). The data in figure 3.9 shows a striking difference between early biofilms produced by FA1090 and FA1090ΔNagZ. In *N. meningitidis*, early DNA release from a small fraction of cells was mediated by cell wall-modifying enzymes and was crucial for initial biofilm formation (76). In FA1090ΔNagZ, biofilm organization does not differ from the wildtype until some of the bacteria in the culture until 24 to 48 hours suggesting that the increase in biofilm formation depends on DNA released by autolysis. The presence of DNA in FA1090ΔNagZ biofilms was readily visualized in the biofilm by SEM.

My SEM data corresponds to a relatively young biofilm (36 hrs), made by strain FA1090. This biofilm appears similar to those made by *N. gonorrhoeae* FA19 but significantly different from biofilms formed by strain 1291 after 48 hrs (87, 118, 202). In this older 1291 biofilm, gonococci appear to have a significant degree of membrane blebbing and cellular debris, more in line with our 72 hr biofilms (66). Since these older biofilms are under significant metabolic stress, it suggests that these

images are reflective of a dying biofilm, with the observed debris due to cellular lysis (66). Greiner *et al.* indicated that when gonococci form biofilms in the absence of CMP-Neu5ac, the terminal sugar of the biofilm matrix contains either a β-linked N-acetylglucosamine or galactose (88). Because NagZ was unable to cleave neisserial LOS, the glycosylated moiety of the matrix cannot be LOS. Vik *et al*. have shown that *N. gonorrhoeae* possesses a wide repertoire of glycosylated proteins aided in part by a set of promiscuous glycosyltransferases (121). In subsequent work they expanded this to a total of 19 known glycoproteins (211). Faridmoayer *et al.* demonstrated that the meningococcal protein PglL, the protein responsible for oligosaccharyl transfer of sugars to pilin is a promiscuous enzyme able to mediate the transfer of a variety of carbohydrates, including peptidoglycan fragments, to pili (212). Since *pglL* is an ortholog of the gonococcal gene *pglO*, this would suggest that peptidoglycan fragments and other carbohydrates could be added to a variety of surface expressed proteins, which could serve as the source of β-linked N-acetylglucosamine. Further, allelic variations in *pglH*, which encodes a protein involved in pilin glycosylation downstream of pglC, was observed to result in addition of N-acetylglucosamine, which is the primary substrate for NagZ (122).

Since many of these proteins are surface expressed, they could promote bacteria-bacteria interactions. Glycosylation of surface proteins has been shown to promote bacterial aggregation in other species as well (123). I hypothesize that NagZ could be acting on some of these proteins, functionally acting as a biofilm dispersing agent. NagZ has been shown to be able to act upon glycoproteins (207). In my working model, I propose that decoration of bacterial surface proteins with glycoside

moieties by various promiscuous glycosyltransferases can promote bacteria-bacteria interaction and this interaction is interrupted by NagZ that destroys peptidoglycan monomers (Figure 3.22). The glycan diversity observed on gonococcal surface is further compounded by the observation that NagZ exhibits endoglycosidase activity in addition to its exoglycosidase activity (Figure 3.1). Previously another hexosaminidase (dispersin B), belonging to GH 20 family, has been shown to cause dispersal of *Actinobacillus actinomycetemcomitans* biofilm (213). Dispersin B was found to cleave a polymer of N-acetylglucosamine with β-(1,6) linkage, present in the extracellular polysaccharide (EPS) matrix of *A. actinomycetemcomitans*. However, Dispersin B had no effect on gonococcal biofilms, indicating that the GlcNAc on gonococcal surface was not β-(1,6) linked.

I also created an *ex vivo* model to study biofilm formation using human cervix tissue obtained from women undergoing hysterectomy. Using SEM this is the first study showing biofilm formation on human cervix tissue as well as polarized epithelial cells. The biofilm formation on abiotic surfaces as well as biotic surface correlated in this study suggesting that interaction with epithelial cells did not modify biofilm formation significantly. However, quantifying biofilm formation on cervix tissue is technically challenging because of mucus secretion and sloughing of epithelial cells. Using SEM on cervix explants, I observed significant epithelial sloughing which suggests that gonococci seldom form biofilm *in vivo* without causing significant damage to epithelial cells. It is possible that damage to epithelial cells is limited by biofilm dispersal mediated by enzymes such as nuclease and NagZ. One of the critical questions that remains unanswered in the neisserial field is how gonococci

are able to produce asymptomatic infections in women, especially since it appears to be growing as a biofilm on cervical specimens. While the gonococcus possesses all of the requisite surface molecules needed to induce an innate immune response, they fail to induce a strong proinflammatory cytokine response in infected women (214). Studies of cytokine induction by the gonococcus show that in fallopian tube tissue, there is an increase in IL-1β, IL-8, and tumor necrosis factor (TNF)-α (215). TNF-α was also found to correlate with tissue damage that occurs during infection (176, 216).

Since N-acetylglucosamine suppresses neutrophil functions and proinflammatory cytokine induction, and NagZ has been shown to be capable of removing N-acetylglucosamine from glycosylated proteins, this would suggest that liberation of N-acetylglucosamine from host and bacterial proteins by NagZ can serve to suppress the immunological signaling when the gonococcus colonizes cervical mucosa (217, 218). This was evident when the wildtype strain appeared to cause more sloughing of epithelial cell than *nagZ* mutant strain. In the gonococcus, I propose a model for biofilm formation that is regulated by interplay of biofilm promoting factors such as glycosyltransferases, Type IV secretion system (which releases eDNA), pilin, Opa and anti-biofilm factors such as NagZ and Nuc DNase. Bacterial dispersal from biofilm is not only important for disease transmission but is necessary for causing invasive and/or ascending infection, because released bacteria are now free to cross the epithelial monolayer in female endocervix.

Chapter 4: *N. gonorrhoeae* interaction with epithelial cells induces activation of inflammasome and inflammasome-dependent cytokine production

4.1 Introduction

Epithelial cells also express pattern-recognition receptors such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs) (128). TLRs are evolutionarily conserved transmembrane proteins that recognize either exogenous PAMPs or endogenous danger-associated molecular patterns (DAMPs). Activation of TLRs by extracellular or membrane-bound PAMPs results in expression of NF-κB- and AP-1-mediated cytokine production. Inflammasome is a multiprotein complex that is formed in response to cytosolic danger signals and induces maturation and release of inflammatory cytokines such as IL-18 and IL-1β. Inflammasome activation requires two signals. An initial priming signal, mediated by TLRs, induces expression of NLRs, pro-IL-1β and pro-IL-18. The second signal, generated by an unidentified mechanism, triggers the assembly of NLRs, ASCs and pro-caspase 1. This assembly activates caspase 1 that cleaves pro-IL-1β and pro-IL-18 into their active forms. Inflammasome activation results in a highly inflammatory response, characterized by neutrophil infiltration.

Symptomatic gonorrhea is an inflammatory disease characterized by neutrophil infiltration and purulent discharge. However, numerous patients exhibit no symptoms (177). While there is a strong inflammatory response at the mucosal level,

immunosuppressive signaling pathways are activated in B and T lymphocytes resulting in lack of protective immunity (219). Studies on human subjects are limited due to ethical and financial concerns. Limited studies on male subjects have shown detectable levels of IL-1β in urine, suggesting the involvement of inflammasome activation in GC-induced inflammation. Similar observations were made in urethral cell lines and immortalized human epithelial cells from the FRT. The paradoxical observation between a locally inflammatory response and a lack of protective immunity post GC infection are not yet understood.

This study aims to understand the role of inflammasome activation in epithelial cell-mediated cytokine response against gonococci. Duncan *et al* have shown that *N. gonorrhoeae* can activate NLRP3 inflammasome in monocytes by causing activation of the cysteine protease cathepsin B by an unknown mechanism (174). LOS was implicated as an activator of NLRP3 inflammasome. While human studies have showed that IL-1β levels in urine correlate with clinical symptoms in male patients, it is unclear whether inflammasome is involved in cytokine production in the epithelial cells of the FRT. This study used both human cell line and cervix explants culture to analyze the relationship of epithelial cytokine production and inflammasomes and TLRs in gonococcal infection.

This study shows that gonococci can activate the inflammasome in epithelial cells in culture and in human cervical tissue explants. Gonococcal interactions also induced production of inflammasome-associated cytokines IL-18 and IL-1β.

4.2 Materials and Methods

4.2.1 Fluorescent inhibitor of Caspase 1 Assay (FLICA)

This assay is based on using a probe a caspase 1-specific amino acid sequence tagged to fluoromethylketone (FMK), a fluorescent moiety (220). The cell permeable probe enters cells and binds only to activated caspase 1 and inhibits it. Unbound probes are washed. Briefly, 10^5 cells per 96 well plate are seeded three days before the experiment. Cells are washed with fresh media without antibiotic on the day before the experiment and treated with bacteria at an MOI of 10 for 6 hours. After 6 hours, the media was removed, the fluorescent probe suspended in PBS is added to the cells and incubated for an hour at 37º C. The cells were then washed with PBS for 5 times and fixed with 4% paraformaldehyde before fixing on a glass slide. Slides were visualized within 24 hours as specified by the manufacturer of the kit (Immunochemistry Technologies, USA).

4.2.2 Measuring cytokines produced by epithelial cells

For measuring cytokine produced by epithelial cells, 10e5 cells were seeded per well on a 96 well plate three days before the experiment. Fresh media without antibiotics was added the day before the experiment. Bacteria were added to each well at an MOI of 10. The cells were treated with bacteria for 24 hours, supernatant collected and debris spun down. The supernatant was stored at -80º C until needed, after adding protease inhibitor (Roche). For ELISA, the supernatant was thawed out and appropriate volume used. IL-8 and IL-18 were measured using Ebioscience

Ready-SET-Go kit and IL-1β was measured using R&D Systems' kit. Instructions by the kit manufacturer were used for all the experiments.

4.2.3 Measuring cytokines produced by cervical explant tissue

Cervical tissue was obtained with consent from volunteer donors undergoing hysterectomy. The cervical chunks were cut into cylindrical blocks of tissue using a 6.5mm diameter biopsy punch. The cervical tissue chunks were separated as endocervix and ectocervis and placed inside a transwell insert in 24 well tissue culture plates. 24 hours later bacteria was added to the cervix chunks and incubated for 24 hours. After 24 hours, the supernatant was removed, debris spun out and stored the same way as above. The supernatant was used for ELISA as described above.

4.3 Results

4.3.1 GC activates inflammasome in epithelial cells

In order to determine if gonococcal interactions with epithelial cells will activate the inflammasome, epithelial cell lines, HEC-1B and T84, were incubated with *N. gonorrhoeae* MS11 (Opa-expressing and piliated strains were used) for 6 hours. The activation of inflammasomes was detected by the activation of caspase 1. Activated caspase 1 was detected using a fluorescent-labeled peptide YVAD that binds to activated caspase 1 (FLICA). The detection probe contains the YVAD peptide moiety that irreversibly binds with the activated caspase enzyme. A non-fluorescent probe with the WEHD peptide sequence which is also recognized by activated caspase 1 was used to competitively prevent YVAD probe from binding.

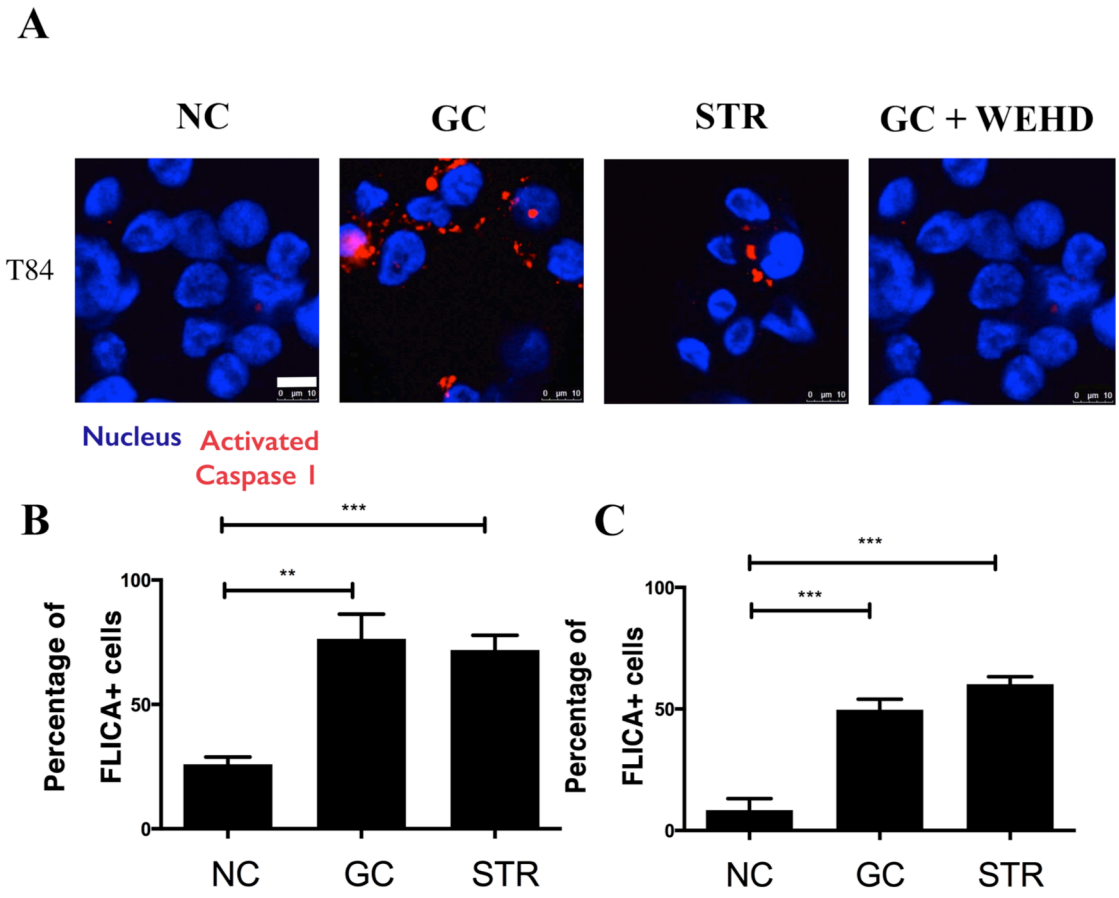


Figure 4.1 Gonococci activate caspase 1 in epithelial cells Figure 4.1 Gonococci activate caspase 1 in epithelial cells (A) Representative confocal image of non-polarized T84 epithelial cells either untreated (NC), or treated with gonococci (GC)(MOI:10), or staurosporine (STR)(10μM) or gonococci plus non-fluorescent inhibitor of caspase 1 (GC + WEHD)(10μM). Cells were then stained for activated caspase 1(Red) and nucleus (Blue), fixed and visualized by confocal microscopy. Scale bar corresponds to 10 μm. (B and C) The percentage of cells that were positive for activated caspase 1 (FLICA+) after no treatment or treatment with GC or STR in T84 cells (panel B) and HEC-1-B cells (panel C) from three independent experiments (** $p < 0.01$, *** $p < 0.001$).

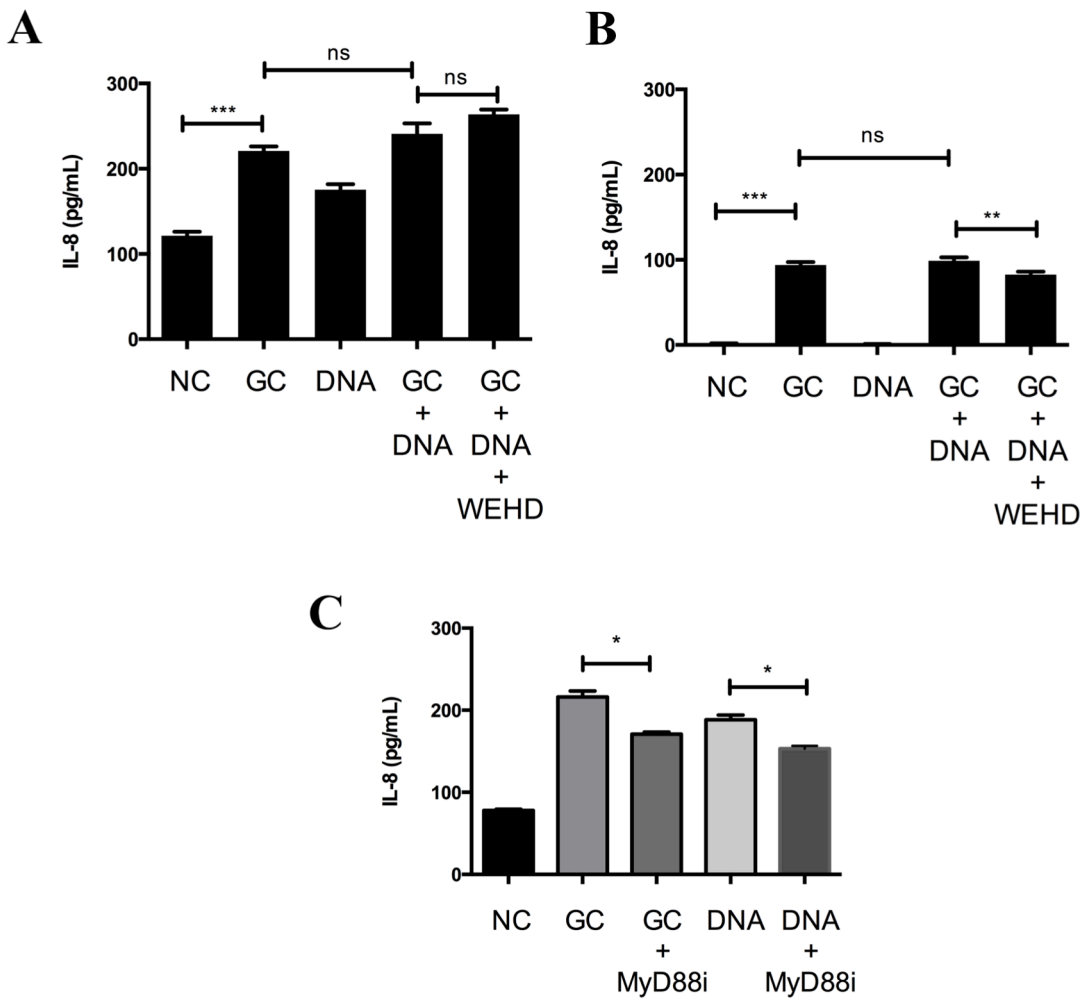


Figure 4.2 GC induce IL-8 production in epithelial cells. Non-polarized T84 cells (panel A) and HEC-1-B cells (panel B) were treated with GC (MOI: 10) alone or GC DNA (50 μg/mL) or GC + GC DNA or GC + GC DNA + WEHD (10 μM) and supernatant assayed after 24 hours for IL-8. (C) IL-8 levels in the presence and absence of MyD88 inhibitor (200 μM) after T84 cells were treated with either GC alone or GC DNA alone. Results shown are the mean (± SEM) of three independent experiments (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns not significant).

FLICA staining, indicating activation of caspase 1, was detected as fluorescent spots in the cytoplasm of epithelial cells incubated with either GC or staurosporine but not untreated cells (Figure 4.1A). The FLICA staining was abolished in epithelial cells pretreated with competing non-fluorescent caspase 1-specific probe, confirming that the specificity of FLICA to activated caspase 1. The relative level of caspase 1 activation was evaluated by the percentage of FLICA-positive cells (Fig. 4.1B). Compared to untreated epithelial cells, GC infection significantly increased the percentages of both T84 (~75%) and HEC-1-B cells (~50%) showing caspase 1 activation. The percentages of GC-infected epithelial cells with activated caspase 1 were similar to that of staurosporine-treated cells. These results indicate that GC interactions induce inflammasome activation in epithelial cells. This is consistent with previous studies on THP-1 cells, which showed that GC was able to activate caspase 1 (174).

4.3.2 GC infection induces IL-8 production in epithelial cells

Symptomatic gonorrhoeae is characterized by neutrophil recruitment and severe inflammation. However, patients suffering from asymptomatic disease show a lack of neutrophil recruitment. IL-8 functions as a chemokine for neutrophils and other immune cells. To determine whether GC infection induce IL-8 production in epithelial cells, epithelial cell lines, HEC-1-B and T84, were treated with *N. gonorrhoeae* MS11 for 24 hours at a starting MOI of 10. IL-8 secreted into culture media was measured using ELISA. After incubation with GC, the concentrations of IL-8 were significantly higher in both cell lines, compared to their no infection controls (Fig. 4.2). I determined if GC DNA could induce IL-8 production. While I

detected no significant effect of GC chromosomal DNA on IL-8 production in HEC-1-B cells, it modestly increased IL-8 production in T84 cells. To determine whether GC-induced IL-8 production in epithelial cells is mediated by Toll-like receptors (TLRs), I inhibited the common adaptor proteins of TLRs MyD88, using a peptide inhibitor. The treatment of MyD88 inhibitor significantly reduced GC-induced IL-8 production in T84 cells (Figure 4.2C). Our results indicate that GC interactions induce the production of IL-8 by epithelial cells in a TLR-dependent manner.

4.3.3 GC-induced IL-8 production is independent of inflammasome activation

We have shown that GC was able to induce both the activation of inflammasome and TLR-dependent production of IL-8 in epithelial cells. I examined if there was any interaction between the two pathways by analyzing the effect of the caspase 1 inhibitor WEHD on GC-induced IL-8 production. The treatment of epithelial cells with caspase 1 inhibitor did not significantly change IL-8 induction levels in GC infected cells. This result suggests that inflammasome activation is not responsible for and does not affect IL-8 production.

4.3.4 GC interactions induce the production of IL18 in epithelial cells in an inflammasome-dependent manner

Activation of inflammasome results in downstream activation of caspase 1, which produces activated IL-18 and IL-1β by cleavage of their proforms. In order to examine whether epithelial cells upregulate IL-18 and IL-1β production in response

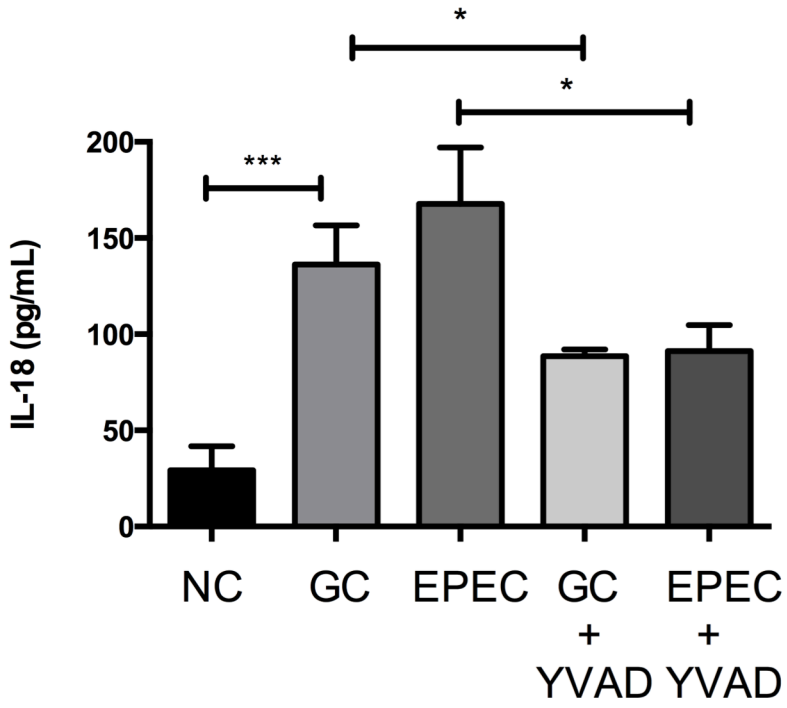


Figure 4.3 IL-18 production by non-polarized HEC-1-B cells. HEC-1B were untreated or treated with gonococci (GC) or enteropathogenic *E. coli* (EPEC) at an MOI 10 for 24 hrs and supernatant assayed for IL-18 by ELISA. EPEC was used as positive control and NC is untreated sample. GC-treated and EPEC-treated HEC-1-B cells were also co-incubated with caspase 1 inhibitor zYVAD-FMK (10 μM) and IL-18 measured. Results are mean (± SE) of two independent experiments (* $p < 0.05$, *** $p < 0.001$).

to *N. gonorrhoeae*, I measured their levels in the supernatant of GC-epithelial co-cultures using ELISA. While no IL-1β production was detected in both epithelial cell lines, the level of IL-18 in GC inoculated HEC-1-B cells was significantly higher than those cells without bacterial inoculation. No IL-18 production was detected in in T84 cells either inoculated with GC or Enteropathogenic *E. coli* (EPEC) (Figure 4.3). EPEC was used as a positive control since it was shown to activate inflammasomes (221). In order to determine if GC-induced IL-18 was inflammasome activated, I treated HEC-1-B cells with both GC and the caspase 1 inhibitor. The treatment of the caspase inhibitor significantly reduced the IL-18 level (Figure 4.3B), suggesting that GC-induced IL-18 production by HEC-1B cells requires inflammasome-activation.

4.3.5. GC-induced cytokine production by cervical explant tissue: In order to study if the results from cell lines can be replicated in human tissues, I established a human cervix tissue explant model. The cervix tissue explants were generated using a biopsy punch (6 mm diameter) and grown on transwell inserts (6.5 mm diameter). After incubation with GC at MOI 10 for 24 hours, supernatant was collected to measure IL-8 and IL-1β. Similar to the cell lines, GC inoculation induced significant increases in IL-8 production in the culture medium of endocervical tissue explants. However, I did not observe any IL-8 production by ectocervical tissue explants. Similar to epithelial cell lines, IL-8 production by endocervical tissue explants was unaffected by the caspase 1 inhibitor YVAD (Figure 4.4A). Our results show that GC inoculation results in the production of IL-8 from endo-, but not ecto-cervical tissue explants, which is independent of inflammasomes. The levels of IL-1β in the media of both endocervical and ectocervical tissue explants were significantly increased

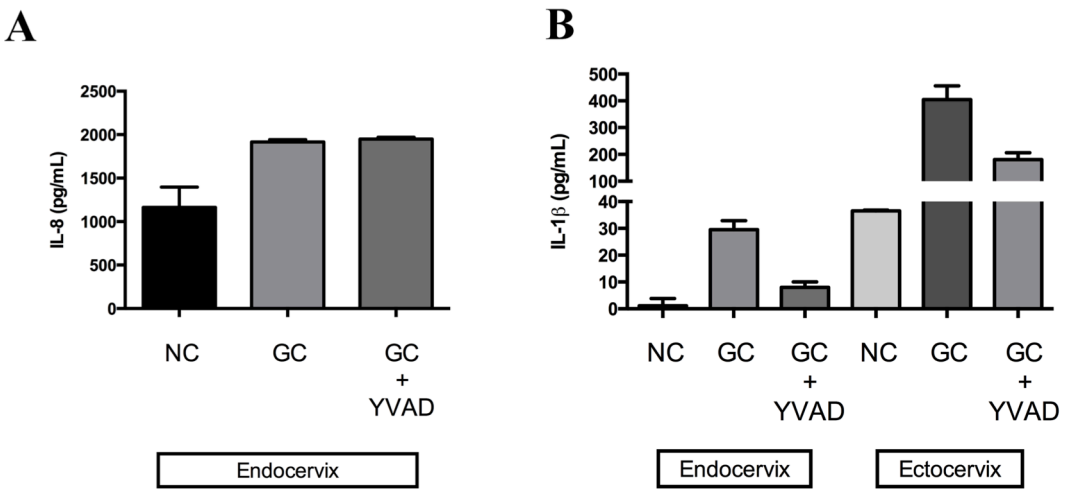


Figure 4.4 Cytokine production by cervical explant tissue. Cervical explant tissue (endocervix and ectocervix) were either untreated or treated with gonococci (GC) alone or GC + caspase 1 inhibitor (YVAD)(10 μM) for 24 hours and supernatants assayed for IL-8 (Panel A) and IL-1β (Panel B) by ELISA. Ectocervix did not produce detectable levels of IL-8 on stimulation with GC (data not shown).

produce in response to GC inoculation, compared to the tissue explants without GC inoculation. Furthermore, the IL-1β level was significantly reduced when the tissue explants were treated with the caspase 1 inhibitor (Figure 4.4B)**.** These results indicate that GC inoculation induces inflammasome-dependent production of IL-1β in both ecto- and endo-cervical tissues.

4.4 Discussion

To our knowledge, this study is the first to show inflammasome activation in epithelial cells in response to gonococcal infection. Activated caspase 1 was detected using a fluorescent probe-based assay in both the epithelial cell lines used, HEC-1-B and T84. In addition, I show that HEC-1-B cells were able to produce the inflammasome-dependent cytokine IL-18 following stimulation by GC. I did not detect any IL-1β production by either cell line. While IL-8 production was unaffected by inhibiting caspase 1, IL-18 levels were significantly reduced by treating the cells with caspase 1 inhibitor. I also show that GC-induced IL-8 production by epithelial cells is MyD88-dependent as IL-8 level was reduced by MyD88 peptide inhibitor. Experiments performed using human cervical explant tissue showed similar findings in terms of IL-1β, which was reduced by caspase 1 inhibitor, which suggests that HEC-1-B and T84 epithelial cell lines are a biologically relevant models to study gonococcal infection of epithelial cells. While GC infection increased IL-8 production by endocervix, which was unaffected by caspase 1 inhibition, ectocervical explant did not show any IL-8 production on infection with GC. Experiments in the Song lab have shown that endocervix and ectocervix have polarized and non-polarized epithelial cells respectively. Future experiments are needed to verify the

difference between endocervix and ectocervix in terms of cytokine production and inflammasome activation.

While the current study is preliminary and limited, the data needs to be confirmed with more replications. This study shows that GC-induced IL-8 production by epithelial cells was caspase 1-independent and MyD88-dependent. While it is unclear yet how GC trigger TLR activation, multiple molecules expressed by GC are potential agonists of TLR, such as LOS for TLR4, Porin for TLR2, PG fragments for TLR2, and CpG DNA for TLR9. While our cell line experiments show that caspase 1 activation does not affect GC-induced IL-8 production, I also observe similar results under *ex vivo* conditions using cervix tissue, where Il-8 production is unaffected by caspase 1 inhibition but IL-1β levels are reduced by using caspase 1 inhibitor. Caspase 1 activation and inflammasome activation need to be verified by western blot and immunofluorescence microscopy, using cell lines as well as cervical explant model. To further characterize the cytokine profiles of GC infected human epithelial cells and cervical tissue explants, I propose to use a cytokine array to screen for cytokines, including IL-10, IL-17, IL-23, IL-6 and TGF-β in the presence and absence of GC inoculation, the MyD88 inhibitor, and the caspase 1 inhibitor. This will help us understand the interaction between bacteria and host tissue in regulating the levels of proinflammatory and anti-inflammatory cytokines. Cytokine production by both models can be assayed using membrane-based cytokine arrays as well as bead-based cytokine assays. While I identified some preliminary differences between ectocervix and endocervix in terms of IL-8 production, similar experiments comparing polarized and non-polarized epithelial cells need to be carried out.

The mechanism of inflammasome activation in epithelial cells is unknown. Previous work has suggested that GC LOS is responsible for inflammasome activation in THP-1 cells by causing activation of cathepsin B. Another possible mechanism could be porin-mediated calcium influx, since porin- mediated apoptosis of epithelial cells has been shown to be inhibited by a caspase 1-specific inhibitor (18). It is possible that gonococcal-induced epithelial cell death involves both apoptosis and pyroptosis, the latter of which is the result of inflammasome activation. However, the difference between apoptosis and pyroptosis has not been fully defined (185, 186). Our future studies will investigate the role of GC LOS and porin in the induction of inflammasome activation.

As nearly half of gonorrhea patients are asymptomatic according to CDC reports, it is important to study the mechanism behind asymptomatic infections. It is paradoxical that gonococci can activate inflammasome and result in production of inflammatory cytokine IL-1β. It may be resolved by examine the balance between the inflammatory cytokines with the anti-inflammatory cytokines such as IL-10 and TGF-β, and their relationship with TLR and inflammasome activation. Activation of Rig-like Receptors (RLRs) has been shown to inhibit TLR-mediated production of inflammatory cytokines in CD4+ T cells (187). The crosstalk between TLRs and inflammasome activation pathway may be critical for gonococci to manipulate host immune responses.

The function of epithelial cells and the local immune system in the female reproductive tract is regulated by sex hormones, which is an addition factor that can modulate host cytokine responses to gonococcal infection. In an earlier chapter of

this thesis, our SEM analysis shows epithelial exfoliation in GC inoculated cervical tissue. IL-1β has been shown to play an important role in epithelial exfoliation during *Salmonella* sp. infection (188–190). While exfoliation is inflammasome mediated and considered protective by reducing infection load, exfoliation of cells in FRT have been associated with infertility (11, 222, 223). Our tissue model is ideal to understand the role of inflammasome in epithelial exfoliation. This work is important in the context of understanding how bacterial interaction with epithelial cells and cervix tissue *ex vivo* play a role in modulating mucosal immune response. The role of epithelial inflammasome activation needs to be further investigated using our *ex vivo* model.

Chapter 5: General discussion and future work

5.1 General summary

Carbohydrates are one of the most important biological macromolecules. LOS is a major surface glycoconjugate in *Neisseriaceae.* LOS is also a crucial immunostimulatory agent, which interacts with TLR4 on host cell surface. In this study, I took a bioinformatics approach to identify the genes involved in LOS biosynthesis in the various species of *Neisseriaceae.* I used the results to predict the structure of LOS, based on the distribution of the various genes. My work correlates with experimental evidence available for some of the commensal neisserial species. I observed that LOS core structure fundamentally differs between neisserial pathogens and commensals. While the commensal strains have three heptose moieties in the core region, neisserial pathogens have two heptose units and a GlcNAc molecule. This observation based on genomic data correlates with experimental observations published earlier. While the reasons for the difference are not clearly understood, I hypothesize that selection pressure in the form of cationic antimicrobial peptides is driving the pathogens to reduce the overall negative charge in the core region by incorporating a GlcNAc instead of another heptose. This change, I hypothesize, incidentally makes the LOS easy to extract from the outer membrane and hence more immunostimulatory. Experiments, as described earlier using BODIPY-LOS, need to be performed to understand differences in LOS extraction by host LPS-binding proteins.

My preliminary data suggests that loss of the third heptose increases polymyxin

MIC and loss of GlcNAc decreases polymyxin MIC by fourfold in neisserial commensals and pathogens respectively. The overall surface charge can be estimated in future experiments in the two mutant strains. I also propose to introduce the putative *waaQ* gene, which encodes the third heptosyltransferase, into *N. gonorrhoeae* and *rfaK*, which adds GlcNAc, into *N. sicca* 4320. Testing of TLR4 stimulation using these strains will further help us test our hypothesis.

Epiproteomic modification with carbohydrates has been well-studied in eukaryotes. The significance of protein glycosylation is recognized and the incredible complexity in its regulation and modulation has led its regulation to be dubbed 'the quantum mechanics of biology' (224). In contrast, bacterial protein glycosylation is only now being studied. Bacterial glycosylation, similar to prokaryotes, involves both O-glycosylation and N- glycosylation (225). A significant point of departure is that bacterial glycosylation appears to have a more diverse glycan structure, aided in part by promiscuity in donor- and acceptor-specificity of glycosyltransferases (225). While N-glycosylation has been studied in *Campylobacter jejuni, N. gonorrhoeae* has been the candidate bacteria to understand O-glycosylation. Gonococci has been shown to add diacetamido-2,4,6- trideoxyhexose (DATDH), followed by one to two galactose to its glycosylated proteins. The glycosylation mechanism has also been shown to glycosylate nearly 20 different proteins on the surface of *N. gonorrhoeae* (121, 211). Adding to the surface diversity is phase variation of genes involved in the glycosylation pathway. In this study, I show how this promiscuous protein glycosylation is modulated by NagZ, which has previously only been shown to be involved in peptidoglycan recovery pathway inside the cell. NagZ's novel function

shows that proteins with known function intracellularly or in periplasm could potentially have a novel function extracellularly. I show that while absence of NagZ results in increased biofilm formation, possibly to due to lack of ability to deglycosylate the surface proteins, this increase is in biofilm formation disappears when both NagZ and PglC are knocked out. NagZ deletion does not change alter bacterial growth rate, survival or blebbing. I also show that gonococci are able to form biofilm on the surface of polarized epithelial cells by using scanning electron microscope. While others have demonstrated gonococcal biofilm formation on epithelial cell models and glass surfaces, this is the first study to show biofilm formation on polarized epithelial cells (88, 118, 202).

In the final part of the study, I show that gonococci induce production of inflammatory cytokines such as IL-8, IL-18 and IL-1β in cell culture models as well as cervical explant tissue model. While IL-8 production appears to be affected by MyD88 inhibition, the caspase 1-dependent cytokines IL-18 and IL-1β unaffected by MyD88 inhibition. While gonococci activate the inflammasome by an unknown mechanism involving LOS-mediated cathepsin B activation in human monocytes, I demonstrate that gonococci are able to activate inflammasomes in epithelial cells (174). This study establishes inflammasome activation in epithelial cells and production of inflammasome-mediated cytokines IL-18 and/or IL-1β in epithelial cells and cervical explant tissue.

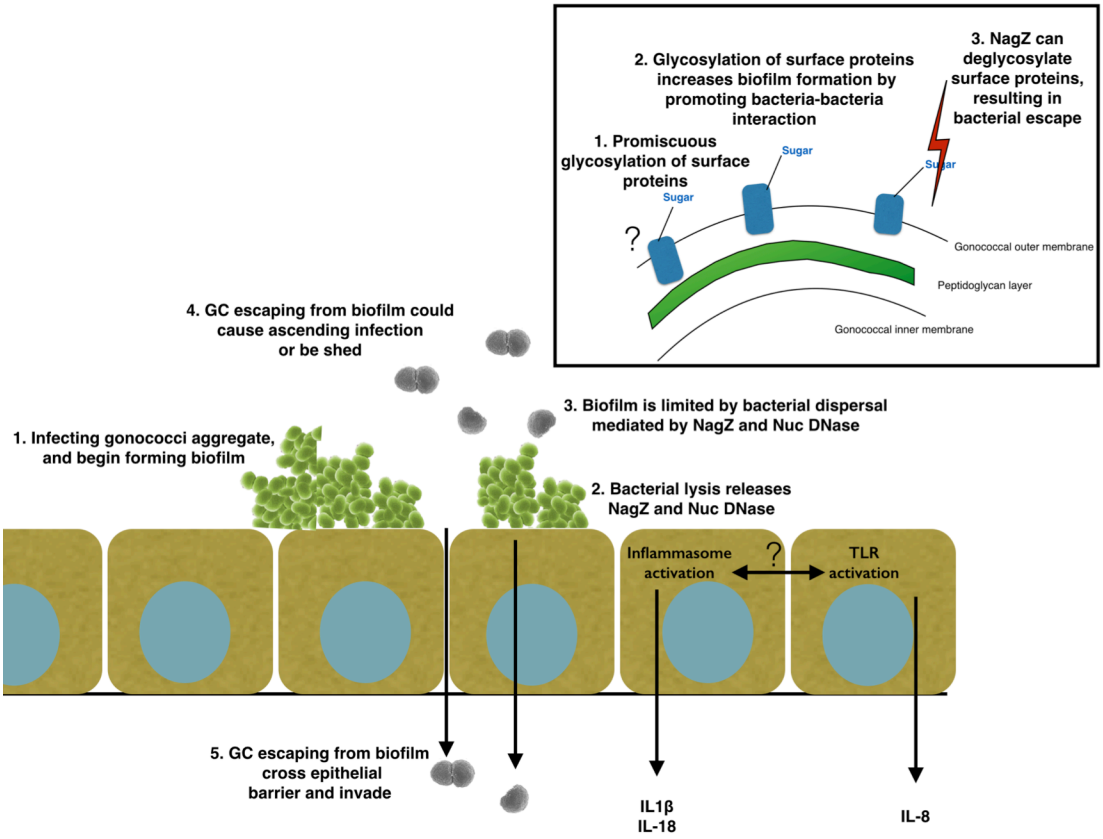


Figure 5.1 Working model of biofilm modulation and its role in gonorrhea. Biofilm formation on epithelial surface is regulated by NagZ and Nuc. Inset image shows working model for NagZ mediated bacterial escape from biofilm.

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5.2 Working Model

Based on the findings from my experiments, I propose the following working model. Gonococcal infection begins in the cervical region of FRT following sexual transmission. Initially the bacteria aggregate and form microcolonies on the surface of epithelial cells. These aggregates were reported by Apicella *et al,* on the cervical biopsy samples (87). However, as bacteria begin forming biofilm there is significant sloughing of epithelial cells as I observed on SEM experiments using cervical explant tissue (Figure 3.14). Other workers have also reported GC-mediated sloughing of epithelial cells using fallopian tube explants (176, 216). In order to limit sloughing and to continue colonization, bacteria use proteins such NagZ and Nuc nuclease to limit biofilm formation. The role of Nuc protein in limiting gonococcal biofilm has earlier been shown (118). In addition, prevention of sloughing of epithelial cells also means that the bacteria that are already attached to epithelial cells are not lost. Our lab has already established that gonococcal strains that do not aggregate are able to cross the epithelial monolayer in a more efficient fashion compared to strains that form large microcolonies.

My work has shown that NagZ-deficient strains form a thicker and denser biofilm, suggesting that NagZ plays a role in limiting formation of large microcolonies and promote bacterial escape from biofilm. These escaping bacteria are now free to either cross the epithelial layer and result in locally invasive disease or ascend into the FRT and cause PID. The free bacteria are also now available to cause disseminated gonococcal infection (DGI), since they are not held down by the biofilm. In support of this model, experiments comparing DGI strains isolated from

cervix and blood of the same patient have shown that the cervical isolates form a thicker biofilm than blood isolates (Nadia Kadry, unpublished observations). However, there is no evidence yet of the proteins that are modulated by NagZ, although evidence exists that NagZ can remove GlcNAc from not just PG monomers but also glycosylated proteins in *E. coli* (207). Further experiments will be carried out using lectin-binding studies to identify the glycosylated proteins that are involved. The evidence suggesting that surface glycoproteins (and not LOS) comes from the observation that loss of NagZ in the absence of PglC did not increase biofilm formation (Figure 3.23) and that NagZ had no effect on LOS (Figure 3.21). It is also possible that released peptidoglycan monomers could as cement and promote bacterial aggregation and subsequent biofilm formation.

Gonococci are in intimate contact with epithelial cells during infection, resulting in activation of TLR and other PRRs in epithelial cells (173, 182). I have shown that GC can also activate inflammasomes in epithelial cells, although the mechanism behind inflammasome activation is not known yet. It has been suggested that gonococcal LOS could activate cathepsin B by an unknown mechanism resulting in inflammasome activation. However, it is still not known if LOS structure affects inflammasome activation since gonococci have significant diversity in LOS structure. In addition, the inflammasome protein that is activated also needs to be identified. While preliminary work suggests that inflammasome activation has no effect on TLR-mediated cytokine production, such as IL-8, the possible interaction between these two pathways needs to be characterized. The role of biofilm formation also needs to be understood in the context of stimulation of innate immunity as well as

escape from immune response. Further studies need to be carried to answer these questions.

Taken together, my thesis demonstrates the diversity of surface lipooligosaccharides in *Neisseriaceae,* the role of glycoconjugates and their modulation by NagZ in gonococcal biofilm formation and finally activation of inflammasomes in epithelial cells by GC. This underlines the significant role played by glycosylated moieties in host-pathogen interaction.

5.3 Future Direction

The general focus of my research has been on the role of surface glycosylated moieties on the pathogenesis of gonorrhea. In the first part, I showed the diversity of LOS structure using *in silico* techniques among the various species in *Neisseriaceae* and identified a key difference in LOS core region between commensals and pathogens of *Neisseriaceae* (22). The second part involves characterizing a protein previously thought to be involved in peptidoglycan recovery and identifying its role in modulation of biofilm formation. While the mechanism of action of this protein is not completely understood, evidence suggests that NagZ is acting on surface glycosylated proteins and NagZ's action is dependent on PglC-mediated surface protein glycosylation. In the final part, I demonstrate preliminary evidence for inflammasome-mediated activation of caspase 1 by gonococci in epithelial cells and show production of caspase 1-dependent cytokines following gonococcal infection. These findings while helping us understand gonococcal pathogenesis have also raised many questions that need to be explored in more detail.

5.3.1 Question 1: How does LOS inner core difference modulate LOS-TLR4 interaction?

My study shows that there is conservation of LOS structure in neisserial commensals and pathogens. Neisserial pathogens and closely related species had the potential to decorate their LOS with phosphoethanolamine, sialic acid and acetic acid. In addition, neisserial pathogens and commensals had major differences in the way their LOS core region is synthesized. Jarvis *et al* have shown that neisserial pathogens and commensals differ in their ability to induce TLR4-mediated cytokine in THP1 cells. Taken together, these suggest that the differences I have identified could explain the mechanism behind the difference observed by other researchers. However, the exact molecular mechanism behind the difference and how the difference in core structure would affect TLR4 activation is still unknown. I hypothesize that the changes in core structure will alter the overall charge of LOS core and affect extraction of LOS from outer membrane by host proteins such as LBP. This hypothesis can be tested by using making mutants in commensal strains such that their LOS structure now resembles that seen in pathogenic strains, and looking at their ability to activate TLR4. Preliminary work by me has shown that knocking out *waaQ* gene increases the ability of *N. sicca* 4320 to activate TLR4 and knocking out *rfaK* reduces TLR4 activation by *N. gonorrhoeae* F62 strain. Further biochemical analysis needs to be done with BODIPY-tagged LOS molecules that will aggregate in solution and not fluoresce until extracted by LBP. Understanding the role of LOS inner core region in LOS extraction would help us develop better vaccine candidates such as vesicles with LOS embedded on the surface, and further our

understanding of structure-function relationship in neisserial LOS.

5.3.2 Question 2: Does surface glycosylation affect biofilm formation by modulating the behavior of surface adhesins or does it also affect eDNA-mediated biofilm formation?

My research work has shown that glycosylation of surface proteins is necessary for promoting bacterial interaction and biofilm formation. Bacterial glycosylation has not been studied as much as glycosylation in eukaryotic systems. The role of surface glycosylation in modulating bacteria behavior has been observed workers as well. Iwashkiw *et al* show that loss of pglL in *Acinetobacter baumanii* results in inability to form a thick biofilm as well loss of virulence in infection models (226). Similarly, glycosylation of adhesins has been shown to enhance their function in bacteria-bacteria interaction as well as bacteria-host cell interaction. Defects in glycosylation of the fimbrial protein Fap1 resulted in reduced biofilm formation in *Streptococcus parasanguinis* (124, 227). Similar effects on biofilm formation were observed in *Aeromonas hydrophila* and *Porphyromonas gingivalis* (228, 229). While I have characterized the effect of glycosylation, modulation of surface glycosylation by NagZ and its effect on biofilm formation, the surface target or targets that play a role in this interaction are yet to be characterized. Future work will identify the surface glycoconjugates that regulate initial aggregation and subsequent biofilm formation. It also needs to be understood if it is the same glycosylated proteins that promote initial aggregation as well as subsequent biofilm formation or if there are multiple surface molecules that affect the various stages of biofilm formation differentially. In addition, it needs to be understood if surface glycosylation affects of extracellular

DNA attachment to gonococci, since eDNA has been shown to play an important role in gonococcal biofilm formation (118).

5.3.3 Question 3: Does NagZ alter gonococcal interaction with epithelial cells?

My work has demonstrated that the intracellular protein NagZ is available extracellularly in stationary-phase grown gonococci. This has many implications in terms of novel functions for many intracellular enzymes. Based on these findings, I hypothesize that NagZ could also deglycosylate glycoconjugates on the surface of epithelial cells, and this deglycosylation could potentially modulate GC interaction with host cells in multiple ways. *E. coli* OmpA has been shown to interact with GlcNAc β 1–4 GlcNAc epitope on endothelial cell surface resulting in enhanced bacterial invasion (230). The Opa proteins in *N. meningitidis* and *N. gonorrhoeae* promote invasion of cells and act as lectins as well, suggesting that NagZ could not only promote bacterial dispersal but also invasion into epithelial cells (38, 44). This can be tested by using wheat germ agglutinin (which binds to terminal GlcNAc) to pretreat epithelial cells and perform and epithelial invasion assay using gonococci. GC has also been shown to release highly inflammatory peptidoglycan fragments, which activate TLR2 and NOD1(176, 231, 232). I have shown that purified NagZ was able to remove the terminal GlcNAc from immunostimulatory PG monomer fragments. I hypothesize that NagZ would be able to detoxify PG monomers and could contribute to reducing inflammation, which could explain the significant number of patients suffering from subclinical gonorrhea. Further, the role of GlcNAc released as a result of NagZ action should be investigated as N-acetyl glucosamine

has also been reported to curtail inflammatory response (233). Studying the role of free GlcNAc and the role of NagZ modulation of epithelial cell surface glycosylation would help us uncover novel mechanisms used by bacteria in surviving immune response.

5.3.4 Question 4: How does GC not elicit a potent inflammatory response in FRT even though it is able to activate inflammasomes?

In the final part of thesis, I show activation of inflammasomes in epithelial cells by gonococci. Previously Duncan *et al* have shown inflammasome activation in monocytic cell line by GC (174). Inflammasome activation has been shown to result in production of significant amount of the highly pro-inflammatory cytokines IL-1β and IL-18, which (along with IL-8) recruit neutrophils and initial a cascade of inflammatory responses. Almost half the number of patients with gonorrhea escape clinical diagnosis, according to CDC estimates, which suggests that gonococci do not elicit a strong inflammatory response (177). The mechanism behind this paradoxical observation needs to be resolved to understand the host response and develop vaccines against gonorrhea. It is possible that inflammasome activation is also resulting in pyroptosis of epithelial cells and local immune cells (174). However, this could only explain the lack of protective long-term immunity and does not explain the lack of a local acute inflammatory response in a significant patient population. NagZ-mediated cleavage of PG monomers and release of free GlcNAc could play a role as GlcNAc has been shown to diminish IL-1β-mediated activation of chondrocytes (233). In addition, GC induced production of anti-inflammatory cytokines such as IL-10 and TGF-β should also be investigated (175). GC also produces other pro-

inflammatory cytokines such as IL-8 and TNF-α, mediated by TLR activation (35, 182). The interaction between the different pathways involved in production of NF-κB-dependent cytokines and caspase 1-dependent cytokines has to be investigated to understand host response. My preliminary experiments have established that epithelial cell lines can be used along with human cervical explant model to study host immune response. Future experiments need to performed with polarized epithelial cells to understand if polarity affects immune response since TLR distribution is affected by polarity of epithelial cells. In addition, polarity has also been shown to be associated with mucin production in other cell lines, which could further hamper access to TLR (234). Future studies on our *ex vivo* model using cervix tissue will further our understanding of host response in gonococcal infection, which could result in development of better diagnostic techniques and vaccines.

Appendices

1. List of strains prepared

***N. gonorrhoeae* F62 ΔrfaK** rfaK is deleted, resulting in LOS truncation and absence of GlcNAc in LOS core region.

***N. gonorrhoeae* F62 ΔlgtF** lgtF gene is deleted, resulting in truncation of LOS

***N. sicca* 4320 ΔwaaQ** *waaQ* gene, which adds a third heptosyltransferase to the LOS core in *N. sicca* 4320 is deleted.

***N. gonorrhoeae* 1291 Δlpt3** Inability to add to PEA to the second heptose in the LOS core at 3rd position

***N. gonorrhoeae* 1291 Δlpt6** Inability to add to PEA to the second heptose in the LOS core at the 6th position

***N. gonorrhoeae* 1291 Δlpt3Δlpt6** Both *lpt3* and *lpt6* are deleted, resulting in complete lack of PEA in LOS core region

***N. gonorrhoeae* MS11 ΔnagZ** Deletion of *nagZ* gene

N. ***gonorrhoeae* F62 ΔnagZ** Deletion of *nagZ* gene

***N. gonorrhoeae* F62 ΔpglC** *pglC* has been knocked out resulting in inability to glycosylate surface proteins

***N. gonorrhoeae* FA1090 ΔpglCΔrfaK** *pglC* has been knocked out resulting in inability to glycosylate surface proteins and *rfaK* gene has been deleted resulting in a truncated LOS without the oligosaccharide unit and GlcNAc in the core region.

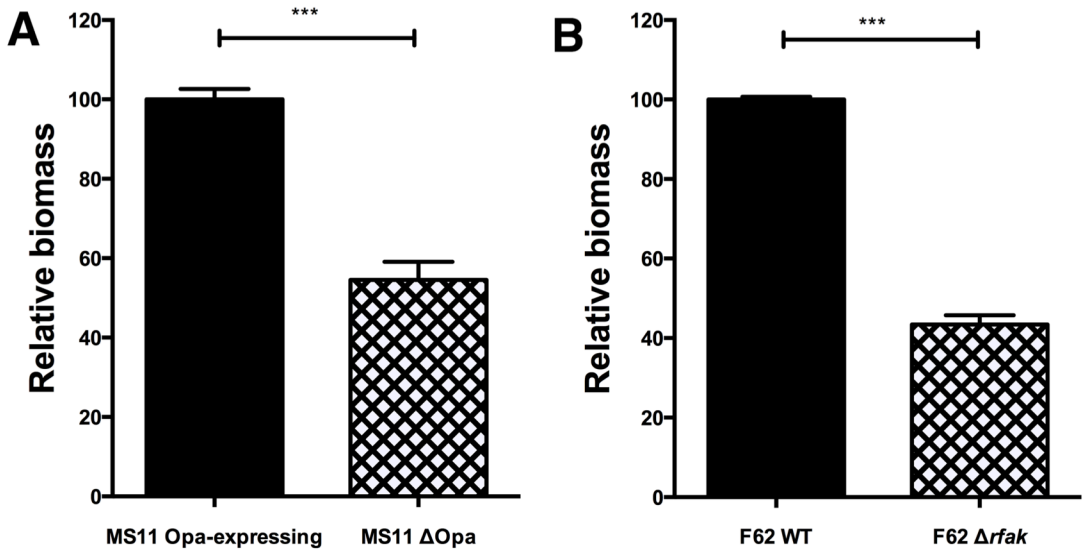

Figure 1. Biofilm formation by Opa mutant and Δ*rfaK* strains. Relative biomass formed by 48 hour static biofilm formed by (A) *N. gonorrhoeae* MS11 Opa-expressing strain and *N. gonorrhoeae* MS11 ΔOpa strain, and (B) *N. gonorrhoeae* F62 wildtype and *N. gonorrhoeae* F62 Δ*rfaK* strains. Data is mean (with SEM) of three independent experiments and analyzed by Student's t-test. (*** $p < 0.001$)

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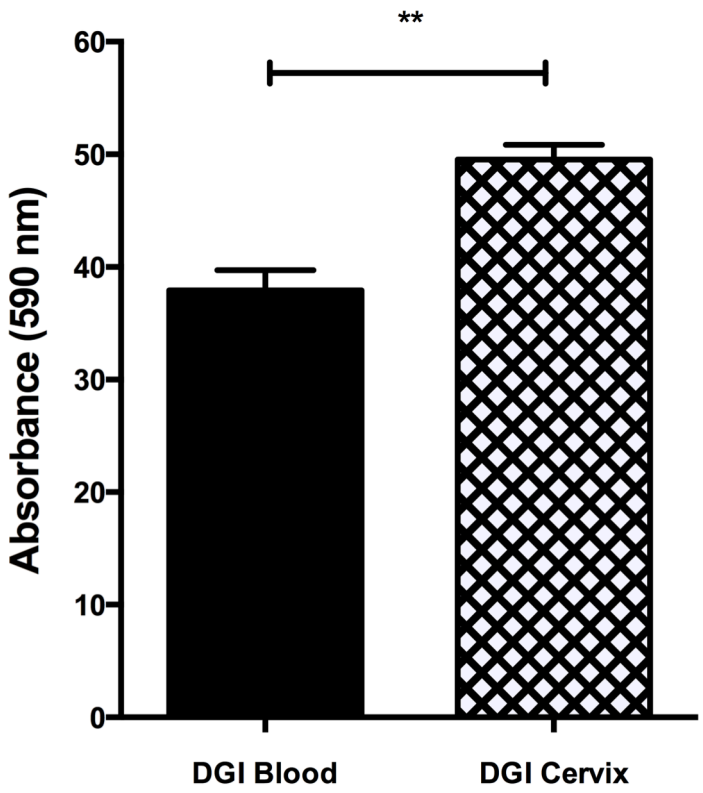

Figure 2. Biofilm formation by DGI isolates. Absorbance measured at 590 nm of 48 hour static biofilm formed by DGI blood and DGI cervical isolates from the same patient, after staining with crystal violet. (Work done by Nadia Kadry)

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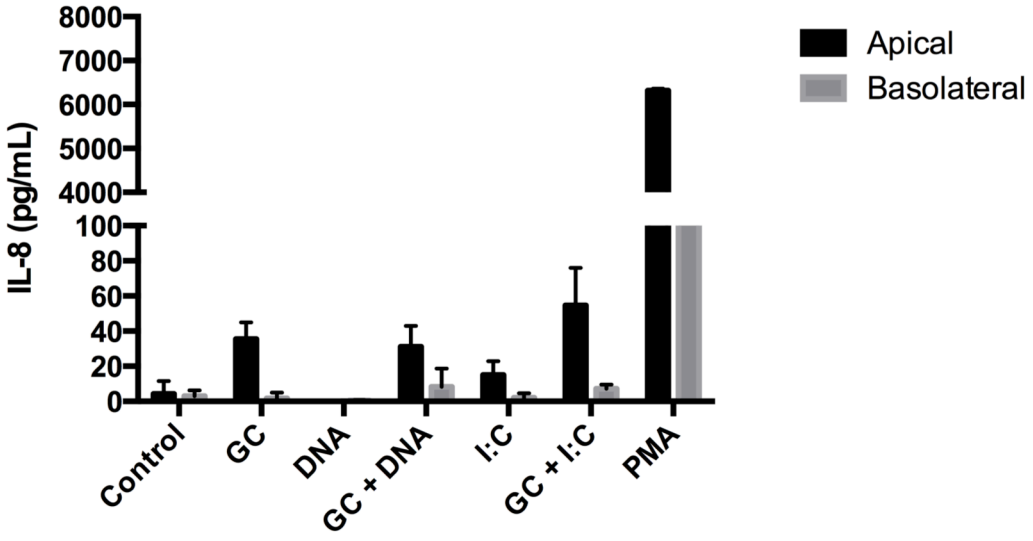

Figure 3. IL-8 production in polarized HEC-1-B cells. Polarized HEC-1-B cells were treated with GC alone or GC DNA or GC + GC DNA or poly (I:C)(25 μg/mL) or poly (I:C) + GC. Supernatant media was collected (after 24 hours of incubation) from apical and basolateral sides and assayed for IL-8. Phorbolmyristate (PMA) was used as a positive control. Results shown are from one experiment.

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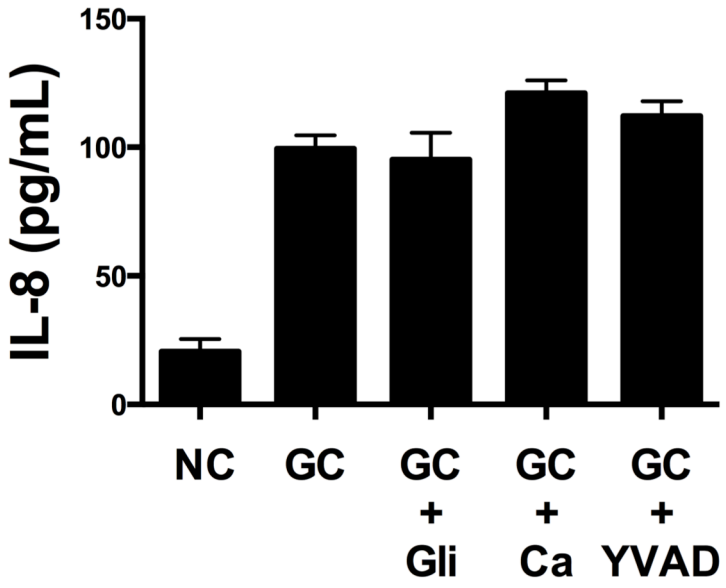

Figure 4. IL-8 production in non-polarized T84 cells. T84 cells were left untreated or treated with GC alone or GC and NLRP3 inhibitor Glibenclamide (25 μg/mL) or GC and Cathepsin B-inhibitor Ca-074 me (10 μM) or GC and YVAD (10 μM). Supernatant media was collected (after 24 hours of incubation) and assayed for IL-8. Results shown are from one experiment.

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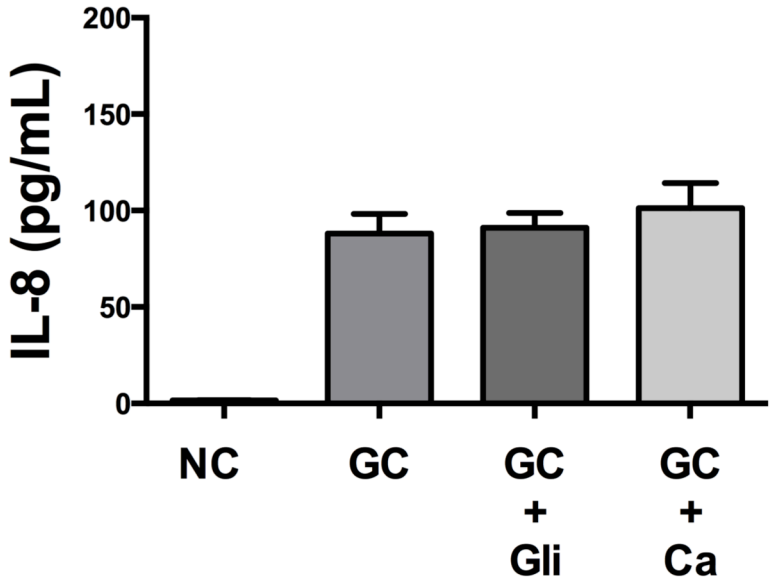

Figure 5. IL-8 production in non-polarized HEC-1-B cells. HEC-1-B cells were left untreated or treated with GC alone or GC and NLRP3 inhibitor Glibenclamide (25 μg/mL) or GC and Cathepsin B-inhibitor Ca-074 me (10 μM). Supernatant media was collected (after 24 hours of incubation) and assayed for IL-8. Results shown are from one experiment.

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