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Nazia Kamal Date

Functions of the *Neisseria meningitidis* TolC Protein

By

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Graduate Division of Biological and Biomedical Sciences
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B.A. History, University of California at Riverside, 2002
B.S. Biology, University of California at Riverside, 2002

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Abstract

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By Nazia Kamal

In *Escherichia coli*, TolC serves as the ubiquitous outer membrane protein (OMP) for many tripartite efflux pumps and participates in the export of bacterial proteins and exogenously added antimicrobials. In the first instance, it has the capacity to remove toxins, such as the repeat-in-toxin (RTX) toxin HlyA, also known as α-hemolysin, via the *hlyABCD* encoded type I secretion system. In the second instance, it participates in the efflux of antimicrobials by the AcrA-AcrB efflux pump amongst others. There are two *tolC* homologs in *Neisseria meningitidis*, *mtrE* and *tolC*. In both *N. meningitidis* and closely related pathogen *N. gonorrhoeae*, MtrE is the OMP for the *mtrCDE* efflux pump, which exports hydrophobic agents such as drugs (i.e. erythromycin), detergents (i.e. TX-100), dyes (i.e. crystal violet), and antimicrobial peptides (i.e. LL37). The TolC-like protein is present only in *N. meningitidis* and is absent from *N. gonorrhoeae*.

In this body of work, I report that unlike the genetic organization in *E. coli*, the meningococcal *tolC* gene is co-transcribed with *hlyD*, whereby *hlyD* is the first gene in the operon. Expression of this operon is growth phase-dependent, with maximal expression at the late logarithmic phase of growth. I documented that MtrE is the sole OMP required for drug export in *N. meningitidis* by the MtrC-MtrD-MtrE efflux system. Although TolC does not have a role in drug export in meningococci it is, however, required for secretion of FrpC, an RTX toxin. When the wildtype meningococcal *tolC* was introduced into RAM1129, an *E. coli tolC* deletion strain, it could functionally replace the *E. coli* protein as the OMP channel in drug export. Furthermore, the meningococcal TolC protein could restore extracellular α-hemolytic activity in strain RAM1129 mediated by the *hlyABCD* encoded type I secretion system. This work documented the ability of the meningococcal TolC protein to participate in drug export in *E. coli* and toxin secretion in both *E. coli* and *N. meningitidis*.

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Dedication

This dissertation is dedicated to my mother, Halida Kamal for her tenacity and is in loving memory of my father, Mustafa Kamal.

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Chapter 1: Introduction

I. *Neisseria meningitidis*

Neisseria meningitidis is a Gram-negative diplococcus and strict human pathogen. It can be transiently carried in the nasopharynx as a commensal, but in certain instances it can enter the bloodstream and either cause fulminant septicemia or cross the blood-brain barrier leading to meningitis. Unlike the other member of the pathogenic *Neisseria*, *N. gonorrhoeae*, that has caused the sexually transmitted disease gonorrhea for thousands of years, the meningococcus may be a relatively new pathogen as meningococcal disease was first described as a clinical entity in 1805 by Vieusseux (10).

A. Infection and Pathogenesis

Initiation of most cases of meningococcal meningitis begins with host acquisition of the bacterium by nasopharyngeal colonization through large respiratory droplets and spreads from person to person. Approximately 10% of healthy individuals harbor the bacteria in the upper airway (106). Carriage is affected by age, crowding (i.e. bars, dormitories, military barracks, Hajj pilgrims), inhalation of dry dusty air, and smoking (39, 45, 59, 88). In addition, damage to the upper respiratory tract by viral co-infections predisposes the individual to carriage and meningococcal disease (88). In a small percentage of colonized people, the bacteria gain access to the bloodstream resulting in meningococcemia and/or progress to the cerebrospinal fluid (51).

Following infection of human nasopharyngeal cells, meningococci penetrate the mucus barrier of the upper respiratory tract and attach to human epithelial cells resulting in microcolony formation (88). After attachment, viable meningococci may be

phagocytosed and transported within a phagocytic vacuole across nonciliated nasopharyngeal columnar epithelial cells and then escape into the bloodstream (93). In most instances, meningococcal colonization of mucosal surfaces leads to subclinical infection or mild symptoms, such as headache, photophobia, stiff neck, and lethargy. However, in 10-20% of cases, meningococci can enter the bloodstream and if not cleared, cause systemic infection in the form of bacteremia, metastic infection involving the meninges, or severe systemic infection with circulatory collapse and disseminated intravascular coagulation (45).

On entry of the meningococci into the bloodstream, the specialized properties of the microvasculature, such as regulating vascular permeability and providing a thrombo-resistant surface to circulating blood cells, are lost resulting in shock and multiorgan failure (59, 88, 93). A dramatic feature of severe meningococcal sepsis is the occurrence of widespread purpura fulminans, the classic hemorrhagic rash that often starts small and rapidly progresses (45, 59).

B. Epidemiology

The major impact of meningococcal disease is among children, resulting from the waning of protective maternal antibodies, where the infection rate and case-fatality ratio can be twenty times that of the adult population (59). In epidemic outbreaks, there is a shift in disease toward older children, adolescents, and adults (68, 94).

More than 500,000 cases of meningococcal disease are reported annually worldwide, with an estimated death toll of 135,000 (109). The prevalence of meningococcal disease varies with geographical location, with endemic disease occurring in developed countries and epidemic disease predominating in developing countries, such

as sub-Saharan Africa, the so-called “meningitis belt” (63, 88, 109). Epidemics of meningococcal disease occur in periodic waves in Africa; the last major epidemic in 1996-1997 affected more than 220,000 people in 10 countries (104). Studies have shown that environmental factors, such as absolute humidity and dust concentrations play a role in the cyclical nature of the epidemics (59, 68, 88). The 2006 epidemic cycle saw a three-fold increase in the number of cases reported from the previous year (104). Presently, Nigeria and Niger are suffering of one of the worst meningococcal meningitis epidemics in years. Thus far, over 25,000 people have fallen ill, more than 17,100 in Nigeria alone, and over 1500 individuals have died (74).

C. Treatment and Prevention

Antibiotic therapy is the only definitive treatment for meningococcal disease with penicillin G, ceftriaxone, or cefotaxime being effective (88). To prevent disease, prophylactic antibiotics are administered to close contacts of patients. These prophylactics include rifampin and ciprofloxacin (45). Resistance to antibiotics has emerged in the meningococcus. In India, meningococcal resistance to penicillin has been reported (40, 73) as has decreased susceptibility to ciprofloxacin (8) and the third generation cephalosporin ceftriaxone (50).

A major advancement in the prevention of meningococcal disease has been the introduction of the quadrivalent Menactra® vaccine, which recognizes serogroups A, C, Y, and W-135 and is conjugated to the diphtheria toxin (45). Menactra® allows generation of T-cell dependent responses and can be used in people aged 11 to 55 (68, 109). Menomune® is another meningococcal vaccine given to patients outside the Menactra® age group and recognizes the same serogroups as Menactra®. However, as it

is unconjugated, drawbacks of Menomune® are that it does not provide T-cell memory, provides no herd immunity, and the duration of its action is short - less than three years for children under the age of three (45). The development of vaccines for *N. meningitidis* serogroup B, however, is hampered by immunologic tolerance to serogroup B capsular sialic acid residues in fetal neural tissue (88, 94). Nonetheless, research groups using a "reverse vaccinology" approach, which is an *in silico* genome based approach (26), has led to a development of a recombinant 5-component vaccine against meningococcal serogroup B strains (5CVMB), which forms the basis of a MenB vaccine entering phase III clinical trials. Initial results indicate that this vaccine is well tolerated and induces bactericidal antibodies against several genetically diverse serogroup B *N. meningitidis* strains (83).

The rapid onset of the disease, the high rate of mortality in the absence of antibiotic treatment (51), and the persistence of serogroups in specific locations show that the meningococcus remains a worldwide threat (88). The onset of the disease is due to important virulence determinants, which are discussed below.

II. Meningococcal Virulence Factors

Several virulence factors have been identified in *N. meningitidis*: capsule, type IV pili, porins, Opa proteins, endotoxin, iron uptake systems, factor H binding protein, and efflux pumps. Of these, capsule, pili, Opa proteins, and endotoxin undergo both phase variation and antigenic variation (4, 94). Phase variation refers to a reversible switch of "on" or "off" in the level of expression of one or more proteins. This occurs among individual cells of a clonal population via slipped-strand mispairing. Antigenic variation,

on the other hand, is the modification of the bacterial cell surface proteins to evade host immune response (98).

1. Capsule: One of the most important virulence factors is the polysaccharide capsule that surrounds the diplococcus. Meningococcal strains have been classified by serological typing based on antigenic variation of the capsular polysaccharide. Of the thirteen serogroups identified, five serogroups (A, B, C, W135, and Y) cause more than 90% of the invasive disease worldwide (106). Capsules provide a selective advantage to the pathogen by enabling the meningococci to evade killing from host defenses like complement (27), phagocytes(13), and antimicrobial peptides (AMP) (86). In addition, the capsule enables the bacteria to avoid opsonization or neutralization by preexisting anticapsular antibody (93). The capsule also has anti-adherent properties that promote meningococcal transmission, spread, and survival externally and within intracellular compartments such as phagocytic vacuoles (94). Furthermore, Swartley *et al*. demonstrated that certain strains can switch capsules as the result of transformation and horizontal DNA exchange *in vivo,* indicating that closely related virulent meningococcal clones may not be recognized by traditional serogroup-based surveillance and can escape vaccine-induced or natural protective immunity (89).

2. Type IV Pili: Type IV pili are long hair-like structures extending from the bacterial surface that facilitate primary adherence to epithelial cell surfaces (106). Pili play a major role in the ability to interact with non-phagocytic cells and are the means by which capsulated bacteria may initially adhere to epithelial cells. The neisserial pilus is a polymer composed of the glycoprotein pilin. Alternative glycoforms of pilus are also produced due to phase variation of pilin glycosylation genes (2). Pilus-mediated

adhesion is a two-step process that requires the expression of the adhesin PilC1 and the expression of an appropriate pilin variant (94). Some pilin variants have the ability to modify the degree of adhesiveness through the formation of bundles of pili that increases bacteria-bacteria interactions (51). Rayer *et al.* have shown that meningococcal mutants lacking type IV pili were defective in survival in adenoid organ culture system (72). Type IV pili are likely to contribute to survival during interactions with epithelial cells, either acting as an adhesin or by promoting aggregation (14).

3. Porins: Porins are the most represented outer membrane proteins in the pathogenic *Neisseria* species. Porins function as pores for the exchange of ions between the bacterium and the surrounding environment. It has been suggested that neisserial porins bind or insert into the membrane of epithelial cells at the sites of close contact between the bacteria and host cells during infection, which might contribute to bacterial pathogenicity (43, 94). The phase variable PorA and PorB outer membrane proteins are two major porins in *N. meningitidis*. PorA is phase variable in a variety of ways including slipped-strand mispairing, point mutations in the coding region, or insertion of an IS element in the coding region (1, 97). Gene switching and expression of phase-variable proteins is a central aspect of the population response to changing conditions and contributes to the pathogenesis of the bacterium (106).

Both PorA and PorB inhibit human neutrophil actin polymerization, degranulation, and phagocytosis (5). The inhibition of actin nucleation may influence the invasive ability of the meningococcus and impair the protective capacity of neutrophils. Recently, Orihuela *et al.* have shown that the meningococcus binds to the endothelium of the blood-brain-barrier (BBB) via the host laminin receptor. The adhesins that are

required for this binding are PilQ (see section II-2) and PorA. The authors suggest the binding brings circulating bacteria into intimate contact with the BBB cells and that the contact may facilitate bacterial uptake into human cells (55).

4. Opa Proteins: Neisserial opacity-associated adhesin (Opa) proteins were originally identified because their expression leads to a change in colony opacity and color due to an increase in bacterial aggregation (11). Opa proteins are located on the meningococcal surface, promote intimate interaction with the host, and modulate host immunological responses (94). Opa proteins undergo phase variation and this variation serves the purpose of facilitating the interaction of the bacterium with different host cell types (22). Sequence variation can influence the specificity of Opa variants for different members of the human carcinoembryonic antigen cell adhesion molecule (CEACAM) family of proteins that harbor the CD66 epitope, and their specificity to cell surface saccharides. In addition, Opa proteins modulate the host immune response and stimulate secretion of proinflammatory cytokines from human macrophages, the levels of which correlate with severity of meningococcal disease (7). Opa proteins are not required for initial colonization of the host, as neisserial pili are (22), but colonies re-isolated from human volunteer challenge experiments with *N. gonorrhoeae* express Opa proteins, suggesting the importance of Opa expression for neisserial infections (28).

5. Endotoxin: *N. meningitidis* endotoxin induces the production of pro-inflammatory mediators in the host leading to septic shock in meningococcal disease (106). Unlike the endotoxin of enteric bacteria, the meningococcus lacks the repeating polysaccharide O-antigens, and so is referred to as lipooligosaccharide (LOS). LOS contributes to serum resistance, the ability of the bacteria to resist the killing action of the membrane attack

complex of complement. Meningococcal LOS is a critical virulence factor in *N. meningitidis* infections and is involved in many aspects of pathogenesis, including the colonization of the human nasopharynx, survival after bloodstream invasion, and the inflammation associated with the morbidity and mortality of meningococcemia and meningitis (29, 59). Additionally, the structure of meningococcal LOS is subject to antigenic variation due to phase-variable expression of certain genes encoding LOS biosynthesis enzymes. These changes result in switching between immunotypes, which is proposed to have functional significance in disease (4).

6. Iron uptake systems: Acquisition of iron and iron complexes is another major determinant in the pathogenesis of *N. meningitidis*. The dependence on iron has been well established for the meningococcus. For example, virulence of the bacterium is enhanced in experimental infections by the injection of iron compounds, such as iron dextran or human ferritransferrin in the animal host (63). As a result, *N. meningitidis* possess several iron uptake systems that rely on high-affinity receptors for iron bound host proteins including transferrin-binding proteins, lactoferrin-binding protein, haemoglobin-binding protein, and haemoglobin-haptoglobin binding protein (106). The above proteins bind to human iron-carrying protein and facilitate the release of the bound iron, thereby allowing the bacterium to internalize the newly freed iron.

Additionally, when the bacteria encounter iron-depleted conditions, such as those in the blood and mucosal secretions, the expression of a number of virulence factors are increased, including those called iron regulated proteins (Frp), which were shown to be related to the RTX (repeat-in-toxin) family of bacterial cytotoxins (91, 92). The RTX family of cytotoxins are a group of related protein toxins found in many Gram-negative

bacteria, including hemolysin of *E. coli* (HlyA), *Bordetella pertussis* adenylate cyclase-hemolysin (CyaA), and leukotoxin of *Pasteurella haemolytica* (LktA). More on RTX toxins are described below in section III-B.

7. Factor H binding protein: The genome of *N. meningitidis* strain MC58 was screened for vaccine candidates and one of the most promising antigens found was the factor H binding protein (fHbp), a 27kDa lipoprotein that binds human factor H (fH)(67). fH is a negative regulator of the alternative complement activation pathway (18) and is responsible for recognition of host cells and tissues and mediates discrimination among microbial pathogens (58).

Human fH binds to sialic acid of microbial surfaces through fHbp. fHbp allows meningococci to coat its surface with fH thereby mimicking human tissue and avoid complement mediated lysis (67). fHbp is important for survival in normal human blood and serum, and can even protect meningococci against killing by the cationic antimicrobial peptide LL37 (83). However, the high affinity interaction between fH and fHbp may contribute to the haemorrhagic rash seen in meningococcal sepsis since the meningococci sequester fH in the plasma, thereby depleting the circulating levels and de-regulating complement, thus rendering host cells in the vascular compartment more susceptible to complement-mediated vascular damage (81).

Schneider and colleagues have identified complement control protein domains 6 and 7 on fH as sites recognized by fHbp. Moreover, they have shown that sucrose octasulphate, a sulphated analogue of glycosaminoglycans, prevented the interaction between fH and fHbp. This led them to suggest that inhibitors specifically designed to block the interaction between fH and fHbp may both promote both complement-mediated

clearance of bacteria and prevent vascular damage by inhibiting fH sequestration in plasma (81).

8. Efflux Pumps: In addition to the above virulence factors, there are also other mechanisms that aid the in the pathogenesis of *N. meningitidis*. One of these mechanisms is the use of efflux pumps, or secretion systems, which aid the bacteria to resist the action of antibiotics. The efflux pumps possessed by the meningococcus, and other pathogens, contribute to their pathogenic mechanisms by providing a means to escape the effects of a number of antimicrobial compounds that bathe mucosal surfaces (85).

III. Secretion systems

A. Overview on efflux pumps

In Gram-negative bacteria, the majority of multidrug efflux pumps share a three-component organization: a transporter located in the inner membrane which functions with an outer membrane protein to form a channel and a periplasmic accessory protein. In this arrangement, efflux complexes traverse both the inner and outer membranes, and thus facilitate direct passage of the substrate from the inside the bacterium to the external medium (107).

So far, six cytoplasmic membrane transport protein families have been described: ATP binding cassette (ABC), the major facilitator super family (MF), resistance/nodulation/cell division (RND), small multidrug resistance (SMR), multidrug and toxic compound extrusion (MATE), and the multidrug endosomal transporter (MET) family (61), however only the first five are associated with multidrug resistance in prokaryotes (65). Figure 1 illustrates the four main drug efflux pumps possessed by the pathogenic *Neisseria*.

The ABC super family includes uptake or efflux systems for a range of substrates, including drugs, sugars, amino acids, carboxylates, metal ions, and peptides. They are inner membrane proteins comprised of four domains that are fused into a single polypeptide. There are two transmembrane spanning domains that are hydrophobic and two that are hydrophilic nucleotide binding domains which couple the energy of ATP hydrolysis to the transport process (99). The transport of the substrate further requires a periplasmic accessory protein and an outer membrane channel. The MacAB system of *N. gonorrhoeae* belongs to this family and recognizes macrolides such as azithromycin and erythromycin (77). MacA belongs to the membrane fusion protein family, whereas MacB is the ABC transporter.

The MF super family includes proton, sodium ion, and solute driven transporters. It includes uptake or efflux systems for a range of substrates, including sugars, drugs, neurotransmitters, carboxylates, amino acids, osmolites, iron-siderophores, and nucleosides (60). Proteins of this family and subfamily are found in membranes of archeae, eubacteria, and even the mammalian central nervous system (79). The FarAB efflux pump of *N. gonorrhoeae* belongs to this family and recognizes antibacterial long chain fatty acids (34). The inner membrane protein FarB exports long chain fatty acids in conjunction with membrane fusion protein FarA and the outer membrane protein channel MtrE(33).

The RND super family consists of large proteins with twelve transmembrane segments. These families include efflux systems for drugs and metal ions. Examples of RND families include MtrD of the MtrC-MtrD-MtrE family in *N. meningitidis* and *N.*

gonorrhoeae (85) and AcrB of the AcrA-AcrB system of *E. coli* (108); a more detailed overview of these two systems are described below in section III-C.

The SMR family consists of small bacterial proteins of about one hundred amino acids with four transmembrane segments. It is related to a larger group of transporters that is involved in transport of sugars, purines, and other metabolites (62). The first SMR transporter identified was in *Staphylococcus aureus* where it confers resistance to ethidium bromide and quaternary ammonium compounds (6, 65). The energy source for transport for MFS, RND, and SMR families is the proton motive force, an electrochemical gradient in which the movement of hydrogen ions drives the transport of the substrate (61, 65, 79).

The MATE family includes proteins from bacteria, yeast and plants, which have twelve transmembrane domains (61).This family includes the NorM transporter which confers resistance to norfloxacin, ciprofloxacin, ethidium bromide, kanamycin, and streptomycin. It appears to function via a drug:sodium ion antiporter. NorM has been studied extensively in *Vibrio parahaemolyticus* (47), but it also has homologs in *N. gonorrhoeae* and *N. meningitidis.* Mutations in either the gonococcal or meningococcal *norM* gene resulted in increased bacterial sensitivity to compounds harboring a quaternary ammonium on an aromatic ring (75).

B. ABC Transporters and Secretion of RTX toxins

Export by ABC transporters is referred to as type I secretion. This involves the transport of polypeptides directly from the cytoplasm of the cell into the extracellular environment using ATP Binding Cassette transporters. ABC transporters are ubiquitous secretion systems identified in virtually every form of life including higher eukaryotes

(99). In bacteria, ABC transporters are involved in secretion of toxins and degradative enzymes. One of the most characterized ABC systems is the secretion of hemolysin (HlyA) from pathogenic *Escherichia coli* (101, 102, 103).

HlyA, an RTX toxin, has both cytolytic and cytotoxic effects on erythrocytes, monocytes, and endotheilial cells (15) (96). RTX, repeat-in-toxins, are pore forming hemolysins. They are secreted as inactive precursors and are activated by acylation, usually at a specific lysine residue. They are characterized by glycine and aspartate rich repeats at the C-terminal secretion signal domain (15, 56). HlyA stimulates the respiratory burst and secretion of vesicular constituents thereby affecting cytokine release (87). In addition, HlyA alters the membrane permeability of host cells, causing lysis and death. HlyA causes target cell lysis by forming pores which display cation selectivity and voltage and pH dependence (44). Lysis of erythrocytes might provide bacteria with iron, while killing nucleated cells may prevent phagocytosis (87).

The type I secretion system for HlyA is composed of three proteins: HlyB, HlyD, and TolC. HlyB is the inner membrane protein with ATPase activity. It has an N-terminal membrane-spanning domain and a C-terminal ABC domain. The binding of ATP and its subsequent hydrolysis induces a conformational change in HlyB and provides the energy for the transport of HlyA (66). Figure 2 depicts transport of HlyA via a type I secretion system (35). TolC is a homotrimeric outer membrane protein that forms the transport pore. With the crystal structure solved to high resolution, it has been noted that the channel tunnel is 140 Å in length and has an internal diameter of 35Å (32). There is also a 100 Å α-helical barrel, or tunnel, anchored in the outer membrane by a 12-stranded β-barrel (107). HlyD forms the membrane fusion protein. It is anchored in the cytoplasmic

membrane by a single transmembrane domain. It functions to form a homo-trimeric complex that spans both the inner and outer membranes and binds directly to TolC and HlyB (66, 102).

HlyA secretion is believed to occur after the mature HlyA interacts with the preformed HlyB/D complex in the cytoplasm. After the HlyA secretion signal, which is about 50-60 amino acids on the C terminus, binds to the HlyB/D complex, HlyD induces contact with TolC. The periplasmic end of the TolC tunnel is sealed by coiled coils and untwists only when in contact with HlyD. The hemolysin then passes into TolC from HlyD via a continuous tunnel made by HlyD-TolC complex. The binding and hydrolysis of ATP via HlyB is directly coupled to the secretion of hemolysin (56, 85, 94).

There are RTX homologs in other bacteria, such as adenylate cyclase toxin CyaA from *Bordetella* (100) or leukotoxin LktA from *Pasteurella* (16), among others (44). Sparling's laboratory observed that when *N. meningitidis* is grown under iron limited conditions, it expresses a number of outer membrane proteins in increased quantities. One of these proteins was designated as iron-regulated protein FrpC (92).

FrpC possesses the characteristic RTX motif found in *E. coli* α-hemolysin, but lacks hemolytic or cytotoxic activity (15, 56). However, in the meningococcus, the biological role of the product encoded by the *frpC* gene remains unknown (15, 91). Nonetheless, FrpC is believed to be involved in virulence because convalescent-phase sera of a number of patients after invasive meningococcal disease contained high levels of antibodies recognizing the FrpC protein (56). Studies have yet to show the biological activity of FrpC in models involving various cultured cell types or the activity of purified recombinant FrpC on phagocytic cells (69). Although Forman *et al.* showed that a strain

of *N. meningitidis* lacking RTX toxin was still as virulent as a wildtype strain in an infant rat model of infection, it does not exclude the possibility that FrpC might pay a role in other steps essential for meningococcal virulence, such as colonization in the nasopharynx or escape from endocytic vacuoles (15).

Proteins that are secreted by *N. meningitidis* are thought to play a role in its pathogenesis. Further studies are needed to elucidate the mechanism of protein transport via the type I secretion apparatus and how the system is regulated. The secretion of FrpC presents a useful model for studying the mechanism of recognition and transport.

C. RND Transporters

Efflux pumps of the RND family are expressed by Gram-negative bacteria and are associated with clinically significant multi-drug resistance (60, 65). In general, the substrate is exported directly to the external medium, rather than into the periplasm. This gives bacteria a great advantage because once exported into the external space, drug molecules must traverse the outer membrane barrier to reenter the cell (53).

i. MtrCDE efflux system:

The *mtr* (multiple transferable resistance) system was first observed by Maness and Sparling in 1973. They observed that a mutation in *N. gonorrhoeae* expressed increased resistance to multiple hydrophobic agents that was transferable via bacterial transformation (41). The *mtr* efflux system is composed of a three gene operon (*mtrCDE*) that forms a tripartite pump that exports substrates from the cytoplasmic membrane directly to the extracellular environment, thereby bypassing the periplasm. MtrD is the RND transporter with twelve transmembrane domains and is located in the inner membrane (20). MtrD interacts with MtrC, a 44kDa membrane fusion protein found in

the periplasm that brings the inner membrane transporter into contact with an outer membrane channel (38). In turn, MtrC makes contact with the outer membrane protein MtrE, which serves as the export channel to the extracellular environment (12). The MtrCDE efflux pump has a broad range of antimicrobials it can export: dyes (e.g. crystal violet and ethidium bromide), detergents (e.g.Triton X-100 and SDS), antibiotics (e.g. azithromycin and rifampin) (20, 85), and certain antimicrobial peptides produced by mammalian cells or other cationic antimicrobial peptides (e.g. LL37) (84). The *mtrCDE* gene complex is similar to the pump possessed by *E.coli* AcrA-AcrB-TolC (52, 57, 85) and MtrE and TolC share a 22.6% identity over a 186 amino acid stretch (12).

ii. AcrAB efflux system:

The AcrA-AcrB pump is responsible for what has been known since the 1960s as the "Acr phenotype"(36). A mutation in the pump leads to sensitivity to acriflavine and other hydrophobic cations, tetracycline, beta-lactams, and detergents like bile acids and SDS. The original mutant was hypersusceptible to drugs and defective in cell division, which is why the family of such pumps is named the RND family (38).

The AcrA-AcrB efflux system of *E. coli* is composed of the RND transporter AcrB, located in the inner membrane of the bacterium, and a periplasmic accessory protein AcrA (54, 65). AcrAB together with the outer membrane protein TolC form a transenvelope structure (36, 52, 54, 108). The structure of AcrB was solved by Murakami *et al*. who showed the protein to be a trimer with three-fold rotational symmetry, the first crystal structure for a proton motive force driven transport protein (49). Each protomer is composed of a transmembrane region 50 Å wide and a 70 Å protruding headpiece. The top of the headpiece opens like a funnel, where TolC might directly dock into AcrB. The

protomers are called binding, access, and extrusion protomers. Based on three different conformations observed for the protomers of AcrB, it is thought that AcrB transports drugs by undergoing a cyclic conformational change from the access, binding, to finally extrusion conformation (48, 53).

Crystallographic observations suggest that in the access state, the vestibule, an area near the entrance of the uptake channel, is open to the periplasm, but the binding site is still shrunken in size. In this state, potential substrates have access to the vestibule. In the binding state, the vestibule is kept open and the binding pocket is expanded to accommodate the substrate. Therefore, drugs enter into the vestibule from the surface of the cytoplasmic membrane, move through the uptake channel, and bind to the different locations in the voluminous aromatic pocket. At this stage, the exit from the binding site is blocked by the central helix inclined from the extrusion protomer. Then, in the extrusion state, the vestibule is closed, and the exit is opened because the central helix is inclined away. The bound drug is pushed out into the top funnel by shrinking of the binding pocket (48).

IV. Transcriptional Regulation of Efflux Pumps

Transcriptional control is an important level of gene regulation, with mechanisms ranging from complete negative regulation to complete positive regulation. Most genes are subject to multiple, overlapping regulatory mechanisms. In general, the confirmed regulators of bacterial drug transporter genes belong to one of four regulatory protein families: AraC, MarR, MerR, and TetR (19, 82). The known transcriptional activators belong to the AraC family, whereas the known repressors fall into the MarR, MerR, or TetR families.

The TetR protein controls the expression of the *tet* genes, whose products confer resistance to tetracycline (23). MtrR, a member of the TetR family of repressors, is present in the pathogenic *Neisseria* (21, 57, 70). In *N. gonorrhoeae*, the *mtrR* gene is divergently transcribed with respect to the *mtrCDE* operon. MtrR binds to its target site upstream of the *mtrCDE* operon preventing expression from the efflux pump (37). However, in *N. meningitidis*, the MtrR repressor is non-functional (76). Rather, the meningococcal *mtrCDE* efflux pump is negatively regulated by IHF (integration host factor) (76). IHF is a histone-like DNA binding protein that induces a significant bend in the DNA upon interaction with its recognition site. IHF functions as an accessory factor in a wide variety of processes including replication, site-specific recombination and transcription in *E. coli* (17). In *Neisseria meningitidis*, an IHF recognition site can be carried in a 155-159 base pair insertion sequence element known as the Correia element. Correia elements are small insertion sequences that have long terminal inverted repeats and TA nucleotide target site duplication, resembling a compound transposable element (9). One such element is upstream of *mtrCDE* (76). Furthermore, Rouquette-Loughlin *et al.* have shown that *mtrCDE* is post-transcriptionally regulated by cleavage of sites located in the inverted repeats (76).

In *E. coli*, *tolC* is regulated at the transcriptional level by MarA, an AraC-like protein (3, 19). AraC was the first transcriptional activator described regulating the arabinose operon. AraC-like proteins, including MarA and MtrA, are primarily transcriptional activators that regulate diverse bacterial functions including sugar catabolism, responses to stress, and virulence (42, 78). The defining characteristic of the AraC family of protein is a carboxy terminal helix-turn-helix DNA binding domain (3).

The mar (*m*ultiple *a*ntibiotic *r*esistance) regulatory locus consists of the *marRAB* operon and the divergently transcribed *marC. marC and marB* both encode proteins of unknown function. The *mar* regulon is responsible for the Mar phenotype manifested as resistance to a variety of structurally diverse and medically important antibiotics (71, 82). MarA is the global activator whose intracellular levels are controlled by the product of the first gene of the *marRAB* operon, MarR. Both proteins bind to *marO*, a region of DNA that separates the two transcriptional units and contains a large number of regulatory protein binding sites, within and around the *marRAB* promoter (19, 82).

MarA activates its own transcription and that of a large number of *mar* regulon genes by binding to 20 bp DNA sequences known as marboxes that are located near the promoters for the target genes (19). In addition, over-expression of MarA or its homologs SoxS and Rob increase the transcription of *tolC* (3). MarA binds DNA as a monomer and its 20 bp marbox binding sites are asymmetric. It possesses two separate helix-turn-helix DNA binding domains linked by a long α-helix. In order for MarA to simultaneously contact two major grooves, the DNA in the MarA-marbox complex is bent by 35^{o} (19).

The MarR repressor controls the intracellular levels of MarA and hence plays a crucial role in the MarA-mediated activation of *mar* regulon promoters. It represses the synthesis of MarA by binding as a dimer to two distinct regions in *marO*, site I and site II, which are located downstream from the MarA binding site. By binding to site I, which is between the -35 and -10 regions of the *marRAB* promoter, MarR prevents access by RNA polymerase and thus prevents expression from the promoter. By binding to site II, located near the ribosome binding site, MarR interferes with open complex formation (3,

19, 82). The crystal structure for MarR contains a DNA binding domain belonging to the winged helix family (82).

Overall, MarA activates expression of the *mar* regulon, including *tolC* and *marRAB*, whereas MarR down-regulates this response by repressing the synthesis of MarA. Over-expressing MarA from a plasmid or adding the antibiotics tetracycline or chloramphenicol can induce the *mar* regulon. Additionally, adding weak aromatic acids, such as salicylate, an uncoupling agent, or a redox cycling compound, such as plumgagin, could block the binding of MarR to *marO* in vitro (82). These agents are ligands for MarR, and thus MarR dissociates from *marO* and *marA* transcription is increased (19).

IV. HlyB, HlyD, and TolC in *Neisseria meningitidis*

Klee *et al.* described pathogenicity islands in *Neisseria meningitidis* that were absent from the closely related pathogen *N. gonorrhoeae* (31). Generally, these islands are horizontally acquired, range from 20 to 200 kilobases, are characterized by inverted repeats or different G+C content, and are thought to cause pathogenesis(64). One particular pathogenicity island contained *hlyD-tolC*. As discussed earlier, HlyD is involved in secretion of the RTX toxin HlyA by serving as the membrane fusion protein in *E. coli* while TolC is the OMP that serves as the efflux channel. Hotopp and colleagues believe that the island that contains *hlyD* and *tolC* may have been transferred from the *Moraxella catarrhalis* plasmid, a respiratory colonizer which can cause otitis media, sinusitis, and pneumonia (25).

In addition to having *hlyD* and *tolC* homologs of *E. coli*, the meningococcus also has a *hlyB* homolog which is further upstream as annotated by the published genome of MC58 (90, 105) . I have shown that *hlyD-tolC* is co-transcribed in an operon (Chapter

2)(30). Both loci, *hlyB* and *hlyD-tolC*, are flanked by DNA that are thought to be insertion sequences, which are associated with horizontal gene transfer. Wooldridge *et al.* have demonstrated that the three proteins are unusually organized as a functional type I secretion system (105).

Tzeng *et al.* have shown that *hlyB* and *hlyD-tolC* regulation is directly and negatively controlled by the MisR-MisS two-component system in *N. meningitidis strain* NMB (Serogroup B) (80). Briefly, they carried out transcriptional profile analyses of the NMB *misR* mutant to identify MisR regulated genes. MisR and its partner MisS are part of a two component regulatory system (95). Two component regulatory systems are a major means for bacteria to recognize and respond to a variety of environmental stimuli (24). When the sensor kinase senses a signal it autophosphorylates and then transfers a phosphate to activate the response regulator. The active response regulator can now participate in DNA binding and transcriptional control, perform enzymatic activities, bind RNA, or engage in protein–protein interactions (46). Furthermore, Tzeng *et al.* have demonstrated that the MisR-MisS is the first two-component system that mediates secretion of the RTX cytotoxin family by controlling the expression of the type I secretion system (80).

Research Aims:

Recently, there has been an emergence of human microbial pathogens that are resistant to many of the currently available antibiotics making treatment of certain infections problematic. Antibiotic resistance is mediated not only by well-known mechanisms such as enzymatic inactivation and alteration of target sites, but also by modifying target accessibility through energy dependent efflux pumps (60).

The *tolC* gene of *Escherichia coli* encodes an outer membrane protein that serves as a channel for both efflux pumps and the secretion of α-hemolysin (66, 96, 101). Secretion of α-hemolysin also requires products of the *hlyBCD* operon (102, 103). *tolC* and *hlyD* homologues are also found in *Neisseria meningitidis*, but not the related pathogen *N. gonorrhoeae* (30, 31). In *E. coli*, MarA, which is a member of the AraC subfamily of transcriptional activators, transcriptionally regulates expression of *tolC* (19). Since the meningococcus uses multidrug efflux pumps similar to those in *E. coli* that function with TolC (12, 85) and produces a toxin (FrpC) that is similar to α-hemolysin (91), the focus of this work was to determine the function and regulation of *tolC* in *N. meningitidis*. Accordingly, the work in this dissertation addressed the following specific aims:

1. To ascertain the function of meningococcal TolC in drug efflux and toxin secretion in both meningococcal strain M7 and *E. coli tolC* deficient strain, RAM1129.
2. To determine whether *hlyD* and *tolC* are transcriptionally linked and if they are regulated by MarA homologs of *Neisseria*.
3. To clarify which amino acids of meningococcal TolC are important for drug efflux and toxin secretion in RAM1129.
4. To elucidate if meningococcal TolC has biological significance.

Figure 1: Efflux pumps possessed by *Neisseria*

The four main drug efflux pumps possessed by *N. gonorrhoeae* and *N. meningitidis*. Members of the RND, MATE, ABC, and MF superfamilies recognize hydrophobic agents (HAs-MtrCDE), quaternary compounds (QC-NorM), macrolides (MacAB), and long chain fatty acids (FA-FarABMtrE), respectively. OM indicates outer membrane and IM indicates inner membrane.

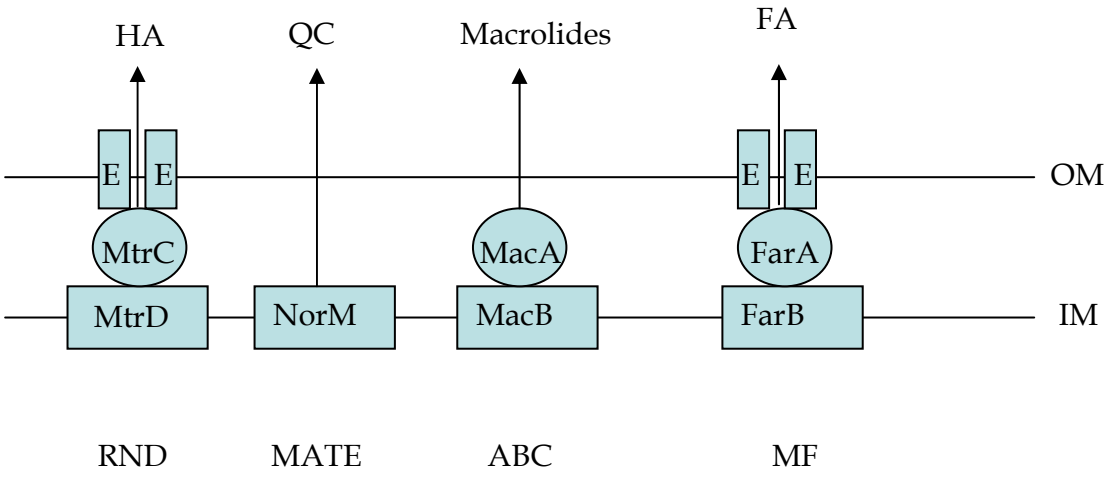
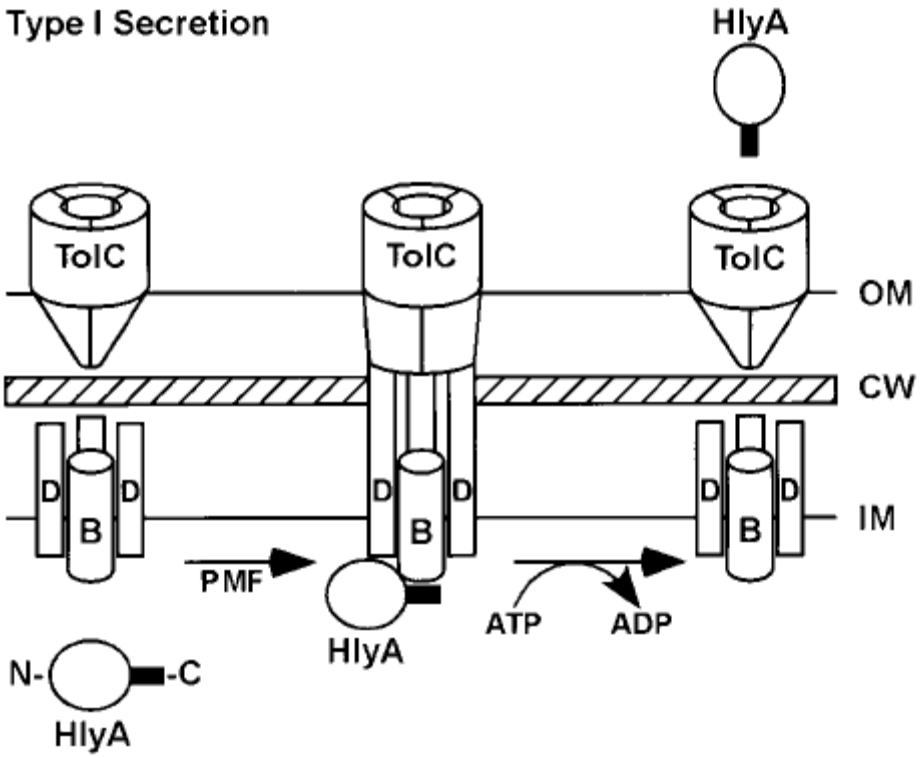


Figure 2: Schematic of type I secretion.

HlyA interacts with the ABC transporter HlyB and the membrane fusion protein HlyD. HlyA is secreted with energy from ATP hydrolysis and the interaction of HlyD with the outer membrane protein TolC by *E. coli*. Figure from Lee *et al*. 2001 (35).



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Chapter 2: The TolC-like protein of *Neisseria meningitidis* is required for extracellular production of the RTX toxin FrpC but not for resistance to antimicrobials recognized by the Mtr efflux pump system

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A 2.9 kilobase pair locus in *Neisseria meningitidis* was identified containing transcriptionally linked open reading frames encoding TolC- and HlyD-like proteins. Although the meningococcal TolC protein was required for extracellular production of the RTX FrpC toxin, it could not functionally replace the MtrE protein as the outer membrane protein channel in drug export by the MtrC-MtrD-MtrE efflux pump.

Neisseria meningitidis is a strict human pathogen that can cause the life-threatening diseases meningitis and/or septicemia (21). Although much remains to be learned about the virulence determinants expressed by this pathogen, it is clear that production of an anti-phagocytic capsule, lipooligosaccharide and surface exposed, cell-attachment structures (e.g., pili), are involved in pathogenicity (21). The availability of the genome sequence for two different capsular serogroup strains (MC58 and Z2491) (14, 23) has allowed for the identification of genes that may contribute to the capacity of the meningococcus to cause disease. The MC58 and Z2491 genome sequences contain an open reading frame (ORF) that would encode a protein bearing significant similarity and identity to the TolC outer membrane protein (OMP) of *Escherichia coli* and a second ORF, classified as a *hlyD* pseudogene (9), closely linked within a genetic island in the chromosome of strain Z2491.

TolC in *E. coli* and TolC homologues in other bacteria serve as the OMP channel for multi-drug efflux pumps (1, 4, 5, 10, 15, 27, 28). In *E. coli*, TolC is also essential for the secretion of the RTX toxin α-hemolysin HlyA (27, 29, 31) via a type I secretion system that consists of an inner membrane transport protein (HlyB) and a periplasmic protein (HlyD) that links the transporter with TolC. Meningococci, but not

gonococci, produce an RTX toxin termed FrpC and its production is reportedly stimulated by iron-limiting conditions (24-26). Wooldridge *et al*. (32) documented the presence of *hlyB*, *hlyD* and *tolC* genes in meningococci. Although *hlyB* and *hlyD* in *E. coli* are organized in a single operon with *hlyA and hlyC* (31), the tandemly linked *hlyD* and *tolC* in meningococci were found to be unlinked to *hlyB*. Moreover, Wooldridge *et al*. indicated that despite their close physical linkage, *hlyD* and *tolC* were not co-transcribed. Mutagenesis of either *hlyB* or *hlyD* in *N. meningitidis* revealed (32) that both were necessary for extracellular production of FrpC, supporting the notion that HlyB and HlyD (and presumably, TolC) form a type I secretion system in meningococci similar to that used by *E. coli* to secrete α-hemolysin (15,29,31).

With respect to a possible involvement of drug efflux, the amino acid sequence of the meningococcal TolC is similar (22.6% identity) over a 186 amino acid stretch to the MtrE OMP of *N. gonorrhoeae* strain FA19 (2) and *N. meningitidis* (16). Previous studies revealed that TolC and MtrE complex with their respective membrane fusion protein (AcrA in *E. coli* [5] and MtrC in gonococci [2] and meningococci [16]), which in turn interact with their cognate cytoplasmic membrane transporter protein (AcrB in *E. coli* [5,28] and MtrD in gonococci [8] and meningococci [16]), for energy-dependent export of antimicrobials. Functionally and structurally, the AcrA-AcrB-TolC and MtrC-MtrD-MtrE efflux pumps are highly similar (19). The MtrC-MtrD-MtrE efflux pump serves to export antimicrobial hydrophobic agents (HA) that enter gonococci or meningococci (2,6-8,11,16,19,30), including those that bathe mucosal surfaces and are part of the innate host defense system (e.g., antimicrobial peptides [18], bile salts [8] and progesterone [8]). The documented role of TolC in export of antimicrobials and HlyA from *E. coli* (4,5,27-

29,31) and the possession of a TolC homolog with similarity to MtrE in meningococci but not gonococci (9 and see below) led us to examine whether the meningococcal TolC protein could function in FrpC export and efflux of antimicrobials. (A preliminary account of this report was presented at the 15th International Pathogenic *Neisseria* Conference held in Cairns, Australia from September 10th-15th, 2006).

Transcription of the *hlyDtolC* locus in meningococci. In agreement with Klee *et al*. (9) and Wooldridge *et al*. (32), we identified a 1.403 kb locus in the annotated genome sequence of meningococcal strains MC58 and Z2491 that contains an ORF that would encode a TolC-like protein (data not presented). Analysis of the genome sequence flanking this ORF also revealed a closely linked upstream ORF that encodes a protein similar to the HlyD protein of *E. coli*. The *hlyDtolC* locus or the individual genes could not be identified in the on-line (www.genome.ou.edu) genome sequence of *N. gonorrhoeae* strain FA1090 and could not be amplified by PCR from genomic DNA prepared from this strain or eight other gonococcal strains (FA19, F62, RD5, DGI 4784, UU1, 3115, DGI 1918, and DGI 14804) (data not presented). In contrast, it could be amplified from genomic DNA prepared from meningococcal strain NMB (data not presented), its acapsular mutant derivative strain M7 (22) (Figure 1) and strain 0929 (data not presented). Thus, between the two pathogenic *Neisseria* species, the *hlyDtolC* locus is likely restricted to the meningococcus, a conclusion supported by the findings of Klee *et al*. (9). We determined the nucleotide sequence of the *hlyDtolC* region that was amplified by PCR of chromosomal DNA from *N. meningitidis* strain M7. We found that the nucleotide sequence of this 2.95 kB region was virtually identical to the on-line sequence for strain MC58 (23) with the exception of three single nucleotide missense mutations in

hlyD, which would cause amino acid replacements at positions 28 (K→E), 285 (N→S) and 356 (I→V) in the full-length protein, and two silent mutations in *hlyD* at nucleotide positions 1,017 and 1,167 (GenBank accession number 1015055). In agreement with the predicted HlyD and TolC proteins produced by strains MC58 and Z2491 (14, 23), we determined that the amino acid sequence of the HlyD protein that would be produced by strain M7 (475 amino acids) was 39% identical (62% similarity) to HlyD produced by *E. coli* over the entire protein, while the TolC proteins of *E. coli* and *N. meningitidis* strain M7 (467 amino acids) were 25% identical (45% similarity).

By sequence analysis, a putative promoter element between the meningococcal *hlyD* and *tolC* genes in strain M7 could not be identified, suggesting that they were transcriptionally-linked and that a promoter element upstream of *hlyD* is used for transcription of *hlyD* and *tolC*. Indeed, a putative promoter element containing near (5/6 matches) consensus –10 and –35 hexamer sequences, separated by 17 bp, was identified 56 bp upstream of *hlyD* (Figure 1). In order to test whether the ORFs were transcribed together, total RNA was extracted from early, mid-log, late-log and stationary phase GCB broth cultures (containing defined supplements I and II [6] and 4.3% [w/v] sodium bicarbonate) of *N. meningitidis* strain M7 as described previously (19). These RNA preparations were employed in reverse transcription (RT)-PCR reactions that used a primer (TolC5) annealing within the putative *tolC* gene (Figure 1A), while the subsequent PCR reaction used TolC5 and primer HlyD5 that annealed in the upstream *hlyD* gene. As is shown in Figure 1B, a specific band of 574 bp was observed most predominantly from the RNA prepared from the late-log culture. This result was contrary to an earlier report by Wooldridge *et al*. (32) that concluded that *hlyD* and *tolC* are not

transcriptionally linked. In order to ascertain the reason for the discrepant results, we performed RT-PCR experiments using the growth and RT-PCR conditions and primers described by Wooldridge *et al*. (32) but were unable to detect RT-PCR products (data not presented). In particular, we found that the operon was maximally expressed by late-log phase cultures and was poorly expressed by stationary-phase cultures (Figure 1C), such as that used by Wooldridge *et al*. (32) to prepare total RNA; as an RNA loading control, we used RT-PCR to monitor the transcript levels of *rnpB* (17). Taken together, we conclude that the procedures employed by Woolridge *et al*. (32) were not optimal to detect transcriptional linkage of *hlyD* and *tolC*.

The meningococcal TolC-like protein is required for extracellular production of FrpC but is not needed for resistance to hydrophobic antimicrobials. Because TolC in *E. coli* functions both in the export of HlyA through a type I secretion system and as the OMP channel for efflux pumps to remove antimicrobial agents, we asked if the meningococcal TolC-like protein had similar dual properties. For this purpose, we examined a panel of antimicrobials recognized by the AcrA-AcrB-TolC efflux system of *E. coli* and the MtrC-MtrD-MtrE efflux system of meningococci (16) and gonococci (2,5,8,18). The MtrC-MtrD-MtrE efflux system was chosen for analysis because of the similarity between TolC and MtrE (see above) and its similarity to the AcrA-AcrB-TolC efflux system.

We previously reported (2) the construction of a nonpolar insertional mutation in the *mtrE* gene (*mtrE*::kan) of *N. gonorrhoeae* strain FA19. DNA from this transformant mutant (RD1) was used to transform *N. meningitidis* strain M7 for resistance to kanamycin (100 μg/ml) as described previously (2,16). A similar nonpolar mutation was

constructed in the meningococcal *tolC* gene. Briefly, PCR was used to amplify a 1.6 kb sequence from genomic DNA of strain M7 and the purified product was cloned into pBAD. The resulting plasmid construct, pBAD::*tolC*, was then purified from an *E. coli* transformant. The promoterless, nonpolar kanamycin resistance (Kan^R) cassette (*aphA-3*) from pUC18K (12) was cloned into a unique PstI site located at position 443 bp within the *tolC* sequence, resulting in pBAD::*tolC*::*kan*. The insertion of the cassette was verified by PCR and by DNA sequence analysis (data not presented) of a plasmid construct prepared from an *E. coli* DH-5α transformant. A subsequent transformant of meningococcal strain M7 was prepared with this plasmid and the replacement of the wild type chromosomal copy of *tolC* with the inactivated gene in a representative transformant of strain M7 was confirmed by PCR and DNA sequence analysis (data not presented). M7*tolC*::*kan*, M7*mtrE*::*kan,* and parental strain M7 were then analyzed for their levels of susceptibility to a panel of diverse antimicrobial agents (antibiotics [azithromycin, penicillin, and streptomycin], dyes [crystal violet and ethidium bromide], a nonionic detergent [Triton X-100] and an antimicrobial peptide [LL-37]) recognized by the MtrC-MtrD-MtrE efflux pump produced by gonococci (6,8,18) and meningococci (16). As is shown in Table 1, insertional-inactivation of *mtrE* significantly enhanced meningococcal susceptibility to the test hydrophobic agents but did not impact bacterial susceptibility to the hydrophilic antibiotic streptomycin. In contrast, inactivation of *tolC* did not impact levels of meningococcal susceptibility to the hydrophobic antimicrobials or streptomycin (Table 1). Thus, the meningococcal TolC-like protein is not essential for export of the antimicrobial agents examined, a function that is instead served by MtrE.

Since the 198 kDa RTX toxin FrpC (3,13,25) produced by meningococci is similar to HlyA of *E. coli* (23, 24), we next asked whether *tolC* is needed for its extracellular appearance as is the case for HlyA production by *E. coli* (31). In preliminary experiments that used sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting to detect FrpC with a rabbit anti-FrpC antiserum (13), we were able to detect FrpC in whole cell lysates of strains M7 and M7*tolC::kan* grown under iron-replete or iron-restricted conditions (data not presented). However, while extracellular FrpC could be detected in culture supernatants of strain M7 under iron-replete (data not presented and lane 1, Figure 2) and iron-deficient conditions (data not presented), it could not be detected in supernatants from strain M7 *tolC::kan* grown under iron-replete (data not presented and lane 2, Figure 2) or iron-deficient conditions (data not presented). In other experiments, we were able to detect extracellular FrpC in culture supernatants of strain M7*mtrE::kan* (data not presented).

In order to verify that loss of TolC production and not a polar effect of the mutation was responsible for loss of extracellular FrpC production in the *tolC* mutant strain, we performed a complementation test that utilized the Neisserial Insertional Complementation System (20). This system allows for expression of a gene at a second site (between *lctP* and *aspC*) on the meningococcal chromosome. Briefly, primers with PacI and PmeI restriction sites were designed to amplify *tolC* such that the PCR product contained 45 bp of DNA sequence upstream of *tolC* (including the ribosome binding site), the full coding sequence and 250 bp of downstream DNA. The primer used in the forward direction was tolCF-Pac1 (5′-CGTTAATTAAATCCGCCGGTTCGGACATG-3′) and the reverse primer was tolCR3PmeI (5′-

CGGTTTAAACAGCTCAACCCGATTGAGAAG-3′). The PCR product was then cloned into pGCC4 (20) that had been digested with PacI and PmeI (20), which contains an isopropylthio-β-D-galactoside (IPTG)-regulated *lac* promoter, and *E. coli* DH-5α transformants were obtained by selection for resistance to kanamycin that was included in LB agar at 50 μg/ml. Plasmid DNA from a representative transformant was prepared and digested with NotI to liberate an 8.1 kB fragment containing the wild type *tolC* gene from strain M7 and the flanking plasmid-borne *ermC*, *lac* promoter, $lacI^{Q}$, *aspC* and *lctP* sequences (20). An agarose gel-purified NotI fragment was then used to transform strain M7*tolC*::*kan* for resistance to erythromycin (1 μg /ml). The insertion of a wild type copy of *tolC* between *lctP* and *aspC* in a representative transformant was verified by PCR and DNA sequencing (data not presented). In order to determine whether the presence of the copy of *tolC* could restore extracellular production of FrpC in M7*tolC::kan*, we analyzed culture supernatants from strains M7, M7*tolC::kan* and a representative M7*tolC::kan* $tolC^{+}$ transformant (complemented strain). For this purpose, meningococci were grown overnight on GCB agar (with or without 1 mM IPTG) that lacked the normally added glucose in defined supplement I (6); standard iron-replete conditions were employed. After overnight growth, bacteria were scraped from the agar and resuspended in 1 ml of GC broth, incubated at 37 C for 30 minutes, vortexed and culture supernatants were collected by centrifugation. The culture supernatants from the test strains were examined for FrpC levels by SDS-PAGE and immunoblotting. As is shown in Figure 2, the *tolC* complemented strain, but not M7*tolC::kan* (lane 2, Panels A and B), produced extracellular FrpC (Panel A, lane3) in the presence of IPTG and this extracellular production was IPTG-dependent (Panel B, lane 3). In the presence of IPTG, the level of

FrpC produced by the complemented strain was similar to that of wild type strain M7 (Panel A, lane 1 compared to lane 3). Thus, we conclude from this data that extracellular FrpC production by meningococci requires the TolC-like protein.

Our results indicate that the meningococcal TolC-like protein is not required for efflux of HA recognized by the *mtr* system in meningococci (16) but is needed for extracellular production of the RTX-like toxin FrpC (Figure 2). In contrast to an earlier report (32), we determined that the tandemly linked *hlyD* and *tolC* genes are co-expressed (Figure 1), a finding that supports the notion that they are organized as an operon. We also found that the *hlyDtolC* operon in meningococci is maximally expressed during the late-log phase of growth (Figure 1) when levels of free iron would be depleted. However, the significance of this observation is unclear as it is not yet known if conditions of iron-restriction, which would be expected *in vivo*, is important in controlling *hlyDtolC* expression or how iron-limitation impacts FrpC production as has been reported by others (24-26).

While FrpC production in an infant rat model for septicemia could not be correlated with virulence (3) and it lacks cytotoxic activity, there is reason to believe, as emphasized earlier by Wooldridge *et al.* (32), that its production may be of importance during meningococcal carriage, infection and/or disease. First, FrpC is expressed during human meningococcal disease and is highly antigenic (13) as determined by the presence of high levels of immunoglobulins IgG and IgA in convalescent sera from patients recovering from meningococcal infection. Second, *frpC* alleles are present in numerous capsular serogroup B and C strains. Its absence in other *Neisseria*, particularly the gonococcus, has raised the question as to whether its function is unique for

meningococcal disseminated disease (32). While the *hlyD* and *tolC* genes are present in numerous clinical isolates of the major clonal lineages of meningococci (9,13) and together with *hlyB* and *hlyD* (32) are required for FrpC export, it is not yet clear if this type I secretion system secretes other proteins important for meningococcal pathogenesis. Accordingly, it is important to define the physiologic and genetic control mechanisms that regulate this export system in meningococci.

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Table 1. MIC values of HA against meningococci

	MIC values						
	Az[A]	Pen	Cv	Eb	TX-100	LL-37	Str
Strain							
M7	2	0.5	1	24	25	16	25
M7 *mtrE*::Kan	0.25	<0.12	<0.25	<3	<12.5	4	25
M7 *tolC*::Kan	2	0.5	1	24	25	16	25

[A]Expressed in micrograms per ml as determined by the method of Hagman *et al*. (6) for Az (azithromycin), Pen (penicillin), Cv (crystal violet), Eb (ethidium bromide), and Str (streptomycin) and TX-100 (Triton X-100) or Shafer *et al.* (19) for antimicrobial peptide LL-37.

Figure Legends

Figure 1: Organization and transcription of the *hlyDtolC* operon in *N. meningitidis* strain M7. **Panel A:** The *hlyD* and *tolC* genes are separated by 68 bp in strain M7. A putative promoter element located 56 bp upstream of *hlyD* is shown. The near consensus –10 and –35 hexamers are separated by 17 bp; nucleotides that differ from the consensus sequence are underlined. **Panel B:** Shown is the agarose gel profile of RT-PCR products generated using total RNA extracted from exponentially growing *N. meningitidis* strain M7. The site of annealing of the HlyD5 (5′-ATGCGGTGGTGAAGATTGAG-3′) and TolC5 (5′-AATCAGCCGAATGTTGCTGC-3′) oligonucleotide primers are shown. Lane 1: RT-PCR product of the *hlyDtolC* sequence obtained when the reaction included RT; Lane 2: same as Lane 1 but RT was omitted; Lane 3: RT-PCR product of the control *rnpB* transcript that was generated using oligonucleotide primers RnpB1F (5′-CGGGACGGGCAGACAGTCGC-3′) and RnpB1R (5′-GGACAGGCGGTAAGCCGGGTTC-3′). **Panel C:** Agarose gel profile of RT-PCR products generated is shown. RNA was prepared from a broth culture of strain M7 at different phases of growth and subjected to RT-PCR using primers HlyD5 and TolC5. Lane 1: early log; Lane 2: mid-log; Lane 3: late log; Lane 4: stationary phase. **Panel D:** Agarose gel profile of RT-PCR product of the control *rnpB* transcript (17). Samples in each lane are the same as that described for Panel C.

Figure 2: Complementation of the *tolC* mutation restores extracellular production of FrpC. Supernatants from meningococcal strains M7 (lane 1), M7*tolC*::kan (lane 2) and M7*tolC*::kan *tolC*+ (lane 3) strains grown in the presence (panel A) or absence of 1 mM IPTG (panel B) were solubilized and subjected to SDS-PAGE that employed a 6 % (w/v) SDS-PAGE gel. FrpC (198 kDa [3, 13, 24, 25, 32]) was detected by immunoblotting using rabbit anti-FrpC and goat anti-rabbit IgG conjugated to horseradish peroxidase. As the levels of total protein in these culture supernatants were frequently < 1 μg/ml, an equal volume (10 μl) of culture supernatant from each of the test strains was examined by immunoblotting. In other experiments (data not presented), extracellular FrpC could not be detected even in 10-fold concentrated supernatants from strain M7*tolC*::kan.

Figure 1:

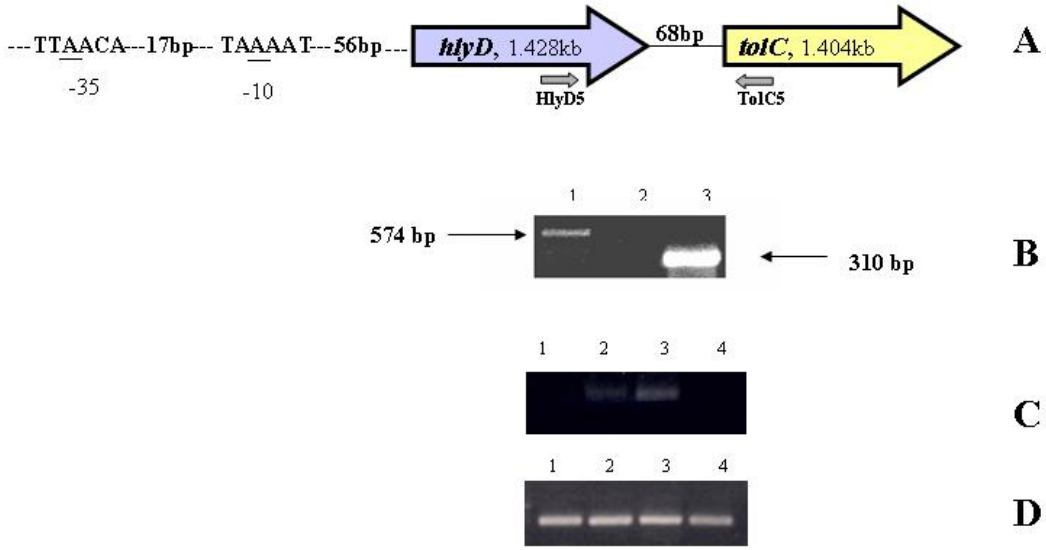
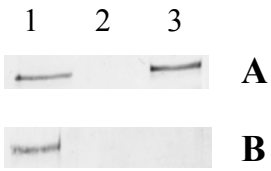

---TTAACA---17bp--- TAAAAT---56bp---
-35
-10
hlyD, 1.428kb
68bp
tolC, 1.404kb
HlyD5
TolC5
A
1 2 3
574 bp
310 bp
B
1 2 3 4
C
1 2 3 4
D

Figure 2:



Chapter 3: Biologic Activities of the TolC-like Protein of *Neisseria meningitidis* as Assessed by Functional Complementation in *Escherichia coli*

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Neisseria meningitidis can produce a TolC-like protein needed for secretion of FrpC, but not efflux of antimicrobials. We now report that expression of the meningococcal _tolC_ gene in a TolC-deficient strain of _Escherichia coli_ can restore properties of α-hemolysis and antimicrobial resistance known to involve efflux pumps.

Despite considerable progress in understanding the pathophysiology of invasive meningococcal disease and the contribution of bacterial virulence factors, much remains to be learned. In particular, little definitive information is available regarding virulence factors that *Neisseria meningitidis* (Nm) secretes to the extracellular fluid during infection and how they might damage host cells and tissues or modulate inflammatory responses that are protective or damaging. Certain strains of Nm (8), but not the related pathogen *N. gonorrhoeae* (Ng), produce a type I secretion system similar to the HlyB-HlyD-TolC efflux pump of *Escherichia coli* (25) known to be important in the extracellular production of α-hemolysin (HlyA) (11, 23, 24). Klee *et al*. (8) described a DNA island in certain strains of Nm that contains tandemly linked *hylD* and *tolC* genes that we showed (7) are co-transcribed. Wooldridge *et al.* (25) demonstrated that Nm contain an unlinked *hlyB* gene and predicted the presence of a functional HlyB-HlyD-TolC efflux system in Nm. We (7) recently showed that meningococcal FrpC, which bears amino acid similarity to HlyA (20, 21), but has unknown biological activity (4), is secreted by the HlyB-HlyD-TolC efflux pump.

Although Nm use TolC in conjunction with HlyB-HlyD for FrpC export (7, 24), it is not required for efflux of antimicrobials as shown previously for its function with the

AcrA-AcrB system in *E. coli* (5). Rather, the MtrE protein of Nm and Ng works with the MtrC-MtrD efflux proteins as the outer membrane protein (OMP) channel for export of antimicrobials (3,7,14,18). The AcrA-AcrB-TolC and MtrC-MtrD-MtrE efflux pumps are structurally and functionally similar (18). The amino acid similarity between the TolC OMPs possessed by *E. coli* and Nm, their similarity to MtrE (3) and the fact that Nm has two similar OMPs (MtrE and TolC) led us to further investigate the biologic activity of meningococcal TolC. Accordingly, we asked if it could functionally replace TolC used by *E. coli* in its secretion of HlyA and efflux of antimicrobials. Using a complementation strategy, we found that TolC produced by Nm can participate in both export functions when expressed in *E. coli*, suggesting that it has the intrinsic ability to interact with components of bacterial efflux pumps that are involved in antimicrobial resistance and toxin secretion.

Bacterial strains, culture conditions, antimicrobial susceptibility testing and detection of α-hemolysis. Nm strain M7 (7) was the primary meningococcal strain used in this investigation. M7 and genetic derivatives bearing mutations in *mtrE* or *tolC* (Table 1) were grown on gonococcal medium base (GC) agar (Difco Laboratories, Detroit, MI) or GCB broth containing defined supplements I and II (2) at 37^{o}C under 3.8% (v/v) CO_2 as described previously (6). The construction of the M7*tolC*::kan strain was previously described (7). Isogenic *E. coli* strains MC4100 and RAM1129 were kindly provided by R. Misra (1) and were grown on Luria-Bertani (LB) agar plates at 37^{o}C. The susceptibility of bacterial strains to a panel of antimicrobials (azithromycin, ciprofloxacin, crystal violet, ethidium bromide, novobiocin, palmitic acid and Triton-X 100) was determined by the agar dilution method (6); all chemicals were purchased from

Sigma Biochemical (St. Louis, MO). Bacterial susceptibility to the human cathelicidin LL-37 was determined as described by Shafer *et al.* (17); LL-37 was kindly provided by J. Pohl (Microchemical Facility of Emory University). Results from antimicrobial susceptibility tests are reported as minimal inhibitory concentrations (MIC) and all experiments were repeated at least three times. α-hemolytic activity of *E. coli* strains due to extracellular production of HlyA (1, 22, 24) was determined using sheep blood agar plates, which were purchased from Becton, Dickinson and Company (Franklin Lakes, NJ).

Expression of meningococcal *tolC* and *mtrE* in *E. coli* and mutagenesis of *mtrE*. pNK1 bearing a cloned Nm *tolC* gene behind an isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible *lacZ* promoter in pGCC4 (19) has been described previously (7). pGCC4 and pNK1 were introduced into *E. coli* strains MC4100 and RAM1129 by electroporation and transformants were selected on LB agar containing erythromycin (300 μg/ml for MC4100 and 5 μg/ml for RAM1129). The *mtrE* gene was PCR-amplified from chromosomal DNA of Nm strain MC58 using primers mtrE2 (5'-CCTTTGCATTGTCTGCCTGCAC-3') and mtrE5 (5'-AAAGGCGGATTGGACGGCGG-3'). The 1082 bp PCR product was purified and cloned into pBAD following the manufacturer's protocol (Invitrogen, Carlsbad, CA). Transformants of *E. coli* strain TOP10 were selected on LB agar containing 100 μg/ml of ampicillin. The resulting plasmid construct (pNK2) was then purified from a transformant, and digested with NaeI that recognizes a unique site within *mtrE*. The omega cassette, encoding resistance to both spectinomycin and streptomycin (9), was digested from plasmid pHP45 (12) and ligated into pBAD::*mtrE* that had been digested

with NaeI to give pNK3. pNK3 was used to transform strain M7 and transformants were selected on GC agar plates containing 60 μg/ml of spectinomycin (Spc). Spc-resistant transformants containing *mtrE::*Spc were verified by PCR. DNA from strain M7*mtrE*::Spc was then used to transform strain M7*tolC*::Kan (7) to create strain M7*tolC*::Kan *mtrE*::Spc.

Functional activities of the Nm TolC. We previously reported that the meningococcal TolC-like OMP, which is similar (26% identity) to the MtrE OMP used by the *mtrCDE*-encoded efflux pump in Ng (6) and Nm (14) to export hydrophobic antimicrobials, is required for secretion of FrpC, but is not required for antimicrobial resistance mediated by the MtrC-MtrD-MtrE efflux pump (7). In order to determine if meningococcal TolC participates in antimicrobial resistance that is mediated by other efflux pumps produced by Nm or Ng, we examined the susceptibility of transformants of strain M7 that contained insertional mutations in *mtrE, tolC,* or both to substrates of the FarA-FarB (10), MacA-MacB (15) or NorM (13) or MtrC-MtrD-MtrE efflux pumps (6). We found (Table 1) that loss of *mtrE*, but not *tolC*, increased Nm susceptibility to substrates of the FarA-FarB (palmitic acid) or MacA-MacB (azithromycin) or MtrC-MtrD-MtrE (azithromycin, ethidum bromide, and LL-37) efflux pumps; neither mutation impacted Nm susceptibility to a substrate (ciprofloxacin) of the NorM pump. Thus, in agreement with our earlier study (7), the TolC OMP produced by Nm is not required for meningococcal resistance to antimicrobials mediated by the repertoire of efflux pumps possessed by this pathogen (16, 18).

Since the TolC proteins of *E. coli* and Nm are similar (22.4% identity), we next asked if the Nm TolC protein could functionally replace the *E. coli* protein with respect to

resistance to antimicrobials known to be recognized by the AcrA-AcrB-TolC efflux pump (5) and extracellular production of α-hemolysin by the HlyB-HlyD-TolC type I secretion system (23). For this purpose, we introduced pGCC4 and pNK1 into *E. coli* strains MC4100 and RAM1129 (as MC4100 but TolC-deficient). Using a rabbit anti-TolC antiserum (kindly provided by H. Zgurskaya, University of Oklahoma, Norman, OK) that cross-reacts with the meningococcal protein to identify TolC in Western immunoblots, we were able to detect the meningococcal TolC in whole cell lysates from transformants of RAM 1129 bearing pNK1 but not pGCC4 (data not presented). Importantly, we found that expression of the Nm *tolC* gene from pNK1 could endow strain RAM1129 with an antibiotic resistance phenotype similar to parental strain MC4100; however, RAM1129 with the pGCC4 vector remained hypersusceptible to the tested antimicrobials (Table 2). Unlike Nm *tolC*, expression of the *mtrE* gene in *E. coli* RAM1129 failed to restore antimicrobial resistance (data not presented).

As a further test of the ability of the Nm TolC OMP to functionally replace the TolC produced by *E. coli*, we examined the α-hemolytic activity of the test strains because secretion of HlyA requires a functional HlyB-HlyD-TolC efflux pump (24). As is shown in Figure 1, we found that expression of the Nm *tolC* gene from pNK1, but not *mtrE* (data not presented), in non-hemolytic strain RAM1129 endowed it with an α-hemolytic phenotype similar to wild type *E. coli* strain MC4100; RAM1129 with empty vector pGCC4 remained non-hemolytic (Figure 1).

We conclude that the meningococcal TolC protein can functionally replace TolC of *E. coli* in two major export activities: efflux of antimicrobials and HlyA secretion. In contrast, when expressed in Nm the same protein seems to only function in secretion of

the FrpC protein, which has unknown biologic activity (4). Thus, although the MtrC-MtrD-MtrE efflux system possessed by Ng and Nm is structurally and functionally similar to AcrA-AcrB-TolC of *E. coli,* the pumps have a preference for their respective OMPs, MtrE or TolC, used as a channel for extracellular export of antimicrobials. Our previous work with expression of the Nm *tolC* in strain M7 suggested that TolC produced by this pathogen was not involved in antimicrobial efflux, but our current work indicates that it has the intrinsic ability to work with AcrA-AcrB in drug efflux in an *E. coli* background. We suggest that this is due to differences between the AcrA-AcrB vs. MtrC-MtrD proteins that are involved in interacting with the OMP component (TolC vs. MtrE) of tripartite efflux systems.

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Table 1. Susceptibility of Nm strains to antimicrobials recognized by efflux pumps

	MIC values				
Strain	Az[A]	Eb	LL-37	Cip	PA
M7	2	12	7.8	0.02	400
M7*tolC*::kan	2	12	7.8	0.02	400
M7*mtrE*::spc	0.125	0.75	1.98	0.02	50
M7*tolC*::kan *mtrE*::spc	0.125	0.75	1.98	0.02	50

[A]Expressed in micrograms per ml as determined by the method of Hagman *et al*. (6) for Az (azithromycin), Eb (ethidium bromide), Cip (ciprofloxacin) and PA (palmitic acid) or Shafer *et al*. (17) for antimicrobial peptide LL-37.

Table 2. Expression of Nm *tolC* in RAM1129 increases resistance to antimicrobials

	MIC values			
Strain	Nov[A]	Eb	CV	TX-100
MC4100	500	> 200	> 40	> 1600
RAM1129	40	5	1.5	200
RAM1129 pGCC4	40	5	1.5	200
RAM1129 pNK1	500	> 200	> 40	> 1600

[A]Expressed in micrograms per ml as determined by the method of Hagman *et al.* (6) for Nov (novobiocin), Eb (ethidium bromide), CV (crystal violet) and TX-100 (Triton-X 100).

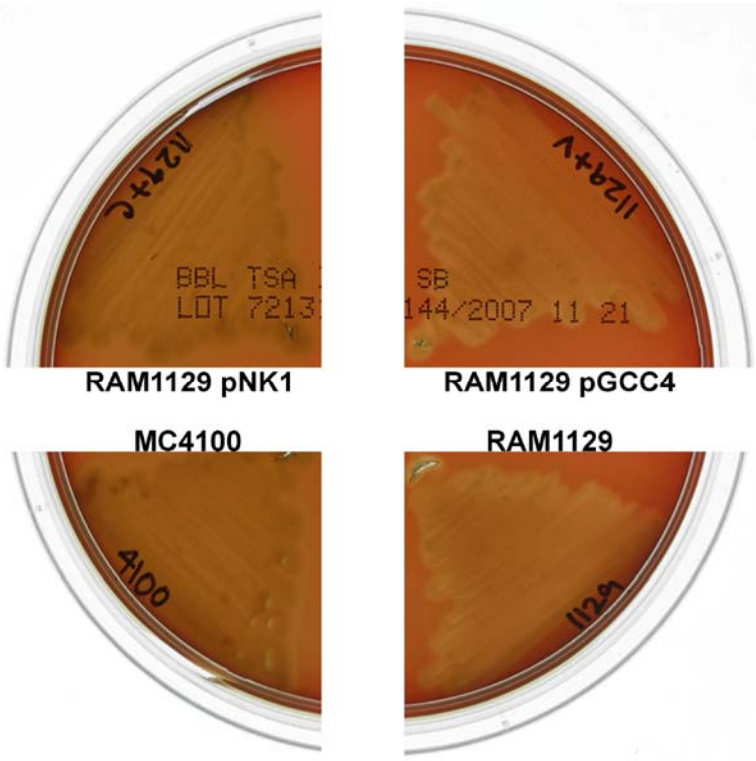
Figure Legend

Figure 1. **Extracellular α-hemolysin production in RAM1129 expressing Nm *tolC***

E. coli strains were grown overnight on sheep blood agar plates containing

IPTG. Only strains MC4100 and RAM1129 pNKI showed an α-hemolytic phenotype.

Figure 1:



Chapter 4: Additional Observations

1. Regulation of *hlyD-tolC* in *Neisseria meningitidis*
2. Biofilm Formation
3. Cytotoxic Studies

The role of TolC was assayed for other phenotypic properties.

I. Regulation of *hlyD-tolC* in *Neisseria meningitidis*

As described in Chapter 1, the *mtrCDE* efflux pump of *Neisseria meningitidis* is regulated by the association of IHF (integration host factor) and the Correia element and not the DNA binding proteins MtrR or MtrA as is the case with *Neisseria gonorrhoeae* (15). However, the regulation of *tolC* in *E. coli* is controlled by MarA and its homologs SoxS and RobA (1, 6). Previous experiments performed by Lee and Shafer have shown that the FarAB efflux pump of *N. gonorrhoeae* has strong homology to EmrAB of *E. coli*, which uses TolC as its outer membrane protein (11). The authors have also shown that the FarAB efflux system is negatively regulated by FarR and this regulation requires IHF (10, 11). There are two MarR homologs in *N. meningitidis* strain MC58, NMB 1585 (MarR-Multiple Antibiotic Resistance) and NMB 1843 (FarR-Fatty Acid Resistance). MC58 is a serogroup B strain whose sequence was annotated and published by TIGR (The Institute for Genomic Research) on their website (http://www.tigr.org/db.shtml).

We wanted to know whether the meningococcal MarR homologs would have an effect on regulation of *tolC*, *mtrE,* or *hlyD* expression in *N. meningitidis* strain M7. For this purpose, I first made non-polar insertions into the coding region of each target regulatory gene, *farR* and *marR*. An *aphA-3* cassette, bearing resistance to kanamycin (13), was inserted into each gene in the same manner for *tolC* as described previously (8).

After confirming the insertional mutations, M7, M7*marR::kan*, and M7*farR::kan* were grown in gonococcal base (GC) broth cultures containing defined supplements I and II (4)and 4.3% (wt/vol) sodium bicarbonate to extract total RNA. As shown in Figure 1, M7*marR::kan* has a growth defect as compared to the parental strain M7 and its isogenic strain M7*farR::kan*. Total RNA was extracted from late-log phase cultures as described (8) because late log phase of growth was shown to correlate with the maximal expression of the *hlyD-tolC* operon in M7. These RNA preparations were employed in reverse transcription PCR (RT-PCR) using 1 μg of RNA from each strain to assess transcript levels of *tolC*, *mtrE*, *hlyD*, and *rnpB*, a housekeeping gene, respectively at late log phase. As shown in Figure 2, the transcript levels of *tolC*, *mtrE*, *hlyD*, and *rnpB* using 1μg of RNA are consistent for each regulator when compared to the parental strain, implying that FarR and MarR do not seem to regulate the genes of interest.

II. Biofilm Formation

A biofilm is a structured community of sessile bacterial cells enclosed in a polysaccharide matrix that adhere to each other and either abiotic or biotic surfaces (17). Common examples are found throughout nature, such as the slime on the surface of a rock in a stream or the film inside a flower vase. However, there are pathogenic bacteria growing as biofilms that can lead to chronic infections. According to the Centers for Disease Control and Prevention, 65% of all infections in developed countries are caused by biofilms (12).

Neisseria meningitidis, along with *Neisseria gonorrhoeae*, are among the pathogens that can form bacterial biofilms (9, 16, 18). Apicella's group has shown that wild-type encapsulated *N. meningitidis* can form biofilms on human tissue culture cells in a flow chamber and that the organisms within the biofilm remain encapsulated (3).

Imuta *et al*. showed that in enteroaggregative *E.coli* (EAEC), an emerging enteric diarrheal pathogen, a *tolC* mutant had less aggregative adherence to an abiotic surface and thus showed decreased biofilm formation compared to the parental strain (7). As stated in earlier chapters, there is a *tolC* homolog in *Neisseria meningitidis*. The possibility of a *tolC* mutant altering biofilm formation in EAEC led us to examine whether the *N. meningitidis tolC* mutant was altered in biofilm formation using strains M7, M7*tolC*::*kan*, and M7*tolC*::*kan tolC*$^{+}$ (Chapter 2).

To visualize biofilms, bacteria were inoculated at a 1:100 dilution from an overnight liquid culture in GC broth and supplements (Chapter 2) in 24-well polystyrene plates containing 500 μl of GC broth. The wells containing bacterial culture were stained with 300 μl of 0.4% crystal violet per well for four minutes. The stained wells were subsequently washed with distilled water twice to remove residual crystal violet and placed in a 37^{o}C incubator for ten minutes. The stained biofilms were dissolved in 33% acetic acid and quantitated by measuring optical density at 580 nm (OD_{580}) at either 24 or 48 hours after inoculation. As shown in Figure 3, there was no difference in biofilm formation between the parental strain M7 and its *tolC* mutant. Furthermore, no difference in biofilm formation was detected at the different time intervals (data not presented). Thus, unlike EAEC, the TolC protein of meningococci does not appear to be involved in biofilm formation.

In addition to assessing the role of *tolC* in meningococcal biofilm formation, we also wanted to know whether the meningococcal *tolC* could substitute for *E. coli tolC*. We performed the biofilm assay as described above, substituting LB broth for GC broth. We assayed biofilm formation in wildtype *E. coli* strain MC4100, an *E. coli tolC*-deficient strain RAM1129, and a strain with meningococcal *tolC* present as a plasmid, RAM1129 pNK1. However, there was no change in biofilm formation when the meningococcal *tolC* was present in an *E. coli tolC*-deficient strain (data not shown).

III. Cytotoxic Studies

We wanted to know whether the meningococcal TolC protein is involved in the secretion of toxins or other virulence factors that may kill human macrophage or epithelial cells. There have been data to show that bacteria can produce certain proteins capable of secreting factors that destroy host cells. One example of that is that of LasA and LasB of *Pseudomonas aeruginosa* (5). It is also known that meningococcal LOS (endotoxin) contributes to cytotoxicity (19). We used strains M7, M7*tolC::kan*, and M7*tolC::kan tolC*$^{+}$ to infect THP-1 (macrophage like human cells) and A549 (lung carcinoma epithelial cells) in a dose-response manner. THP-1 cells were grown in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum, 50 micrograms of penicillin, 50 micrograms of streptomycin, 1% non-essential amino acids and 1% sodium pyruvate. Cells were split and fed every 3 days with RPMI and the above supplements. A549 cells were grown in Dulbecco's MEM (Invitrogen), supplemented as above and split every 3 days. Culture flasks were incubated at 37^{o}C with humidity under 5% CO_2.

Bacterial supernatants were prepared in GC broth at an optical density at 550nm (OD_{550}) of 0.4, which corresponded to approximately 2 x 10^8 CFU per mL. Toxicity was determined by assessing cellular viability and proliferation using the trypan blue exclusion method (14). 1 x 10^6 THP-1 cells were seeded in a 24 well tissue culture plate with 10^8, 10^6, or 10^4 CFU per mL of bacterial supernatants and incubated for five days. Cells were diluted 1:1 with the vital dye trypan blue 0.4% solution in PBS from Cellgro, Mediatech (Herndon, VA) and viable cells were counted using a hemacytometer. As shown in Table 1, there was no difference in cell viability as measured with a hemacytometer between the wildtype and TolC-deficient strains, implying that TolC does not secrete virulence factors that kill THP-1 cells.

Although TolC does not play an apparent role in cell viability, we wanted to know whether membrane damage was associated with TolC. Necrotic cell death is accompanied by membrane damage and the release of cytoplasmic components like LDH into the medium. LDH (lactate dehydrogenase) is an oxidoreductase which catalyzes the interconversion of lactate and pyruvate. LDH is most often measured to evaluate the presence of tissue or cell damage (2). LDH levels were determined using a colorimetic assay by Roche according to manufacturer's instructions. A549 cells were grown to 70 to 80% confluence in 24-well microtiter plates as described above. The cells were washed three times with Dulbecco's MEM. A549 cells were mock infected with medium or infected with supernatants from M7, M7*tolC::kan*, or M7*tolC::kan tolC+* at various concentrations for 5.5 hours. Culture supernatants were collected, centrifuged, and prepared according to manufacturer's instructions and absorbance at an optical density of 490 nm was determined. We found no difference in LDH release between the three

strains tested (data not shown). These results, along with the results obtained from the trypan blue exclusion method, indicate that there is no identifiable role for meningococcal *tolC* to kill host cells.

Figure 1. Growth curve of M7, M7*farR::kan*, and M7*marR::kan*

Strains were grown with agitation in GC broth with supplements as described by Kamal *et al*. (8). The *mar* mutant was grown in duplicate to verify growth deficiency. Arrows depict time at which cells were harvested for RNA extraction for each strain. M7 and M7*farR::kan* were harvested at the same time point whereas M7*marR::kan* was harvested at a later time point.

Figure 1:

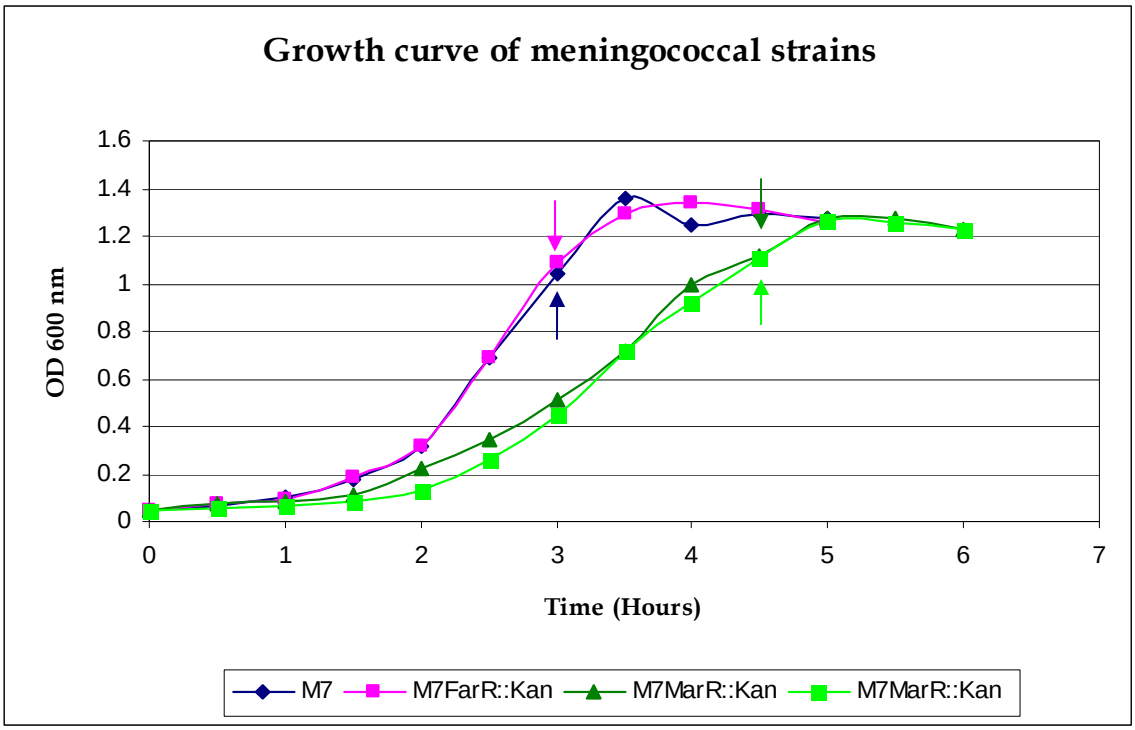

Growth curve of meningococcal strains
OD 600 nm
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
1
2
3
4
5
6
7
Time (Hours)
M7
M7FarR::Kan
M7MarR::Kan
M7MarR::Kan

Figure 2. Transcription of *tolC*, *mtrE*, *hlyD*, and *rnpB*.

RNA prepared from: Lane 1: M7, Lane 2: M7*farR::kan*, or Lane 3: M7*marR::kan*. Oligonucleotide primers used for the *tolC* transcript were tolC2 (5'-GCGTCCCATTACCAGCGTG- 3') and tolC3 (5'-GTTCTTGCGCCATGATTTGG -3'), for the *mtrE* transcript were mtrE2 (5'-CCTTTGCATTGTCTGCCTGCAC-3') and mtrE5 (5'-AAAGGCGGATTGGACGGCGG-3'), for hlyD were hlyDF (5'-TTATGAAGCGGTATTGGCGG-3') and hlyDR (5'-GCAGGCGACTGTATTGTCAT-3'), and for the *rnpB* transcript were RnpBF1 (5'-CGGGACGGGCAGACAGTCGC-3') and RnpB1R (5'-GGACAGGCGGTAAGCCGGGTTC-3').

Figure 2:

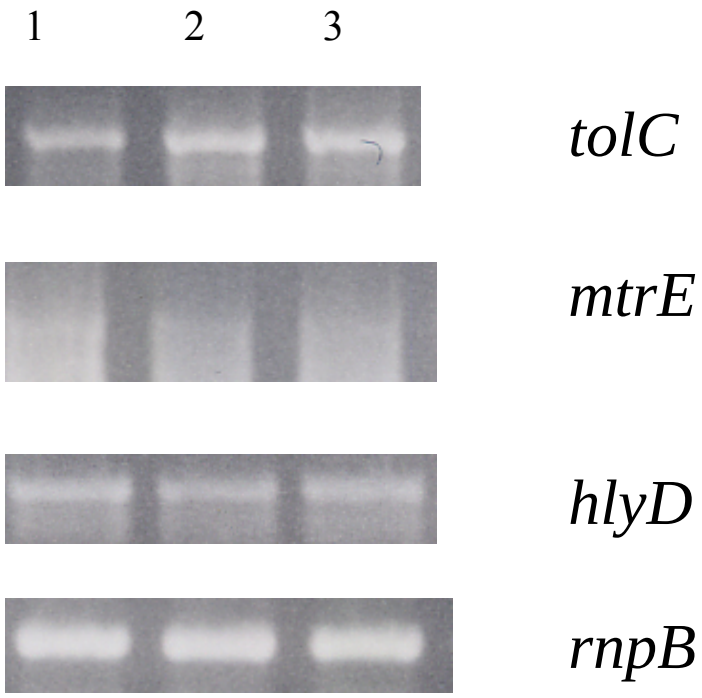


Figure 3. Crystal violet staining of biofilms.

Biofilms formed by meningococcal strains M7*tolC::kan* and M7*tolC::kan tolC+* were compared to those formed by the parental strain M7 by visualization with crystal violet staining on a polystyrene surface.

Figure 3:

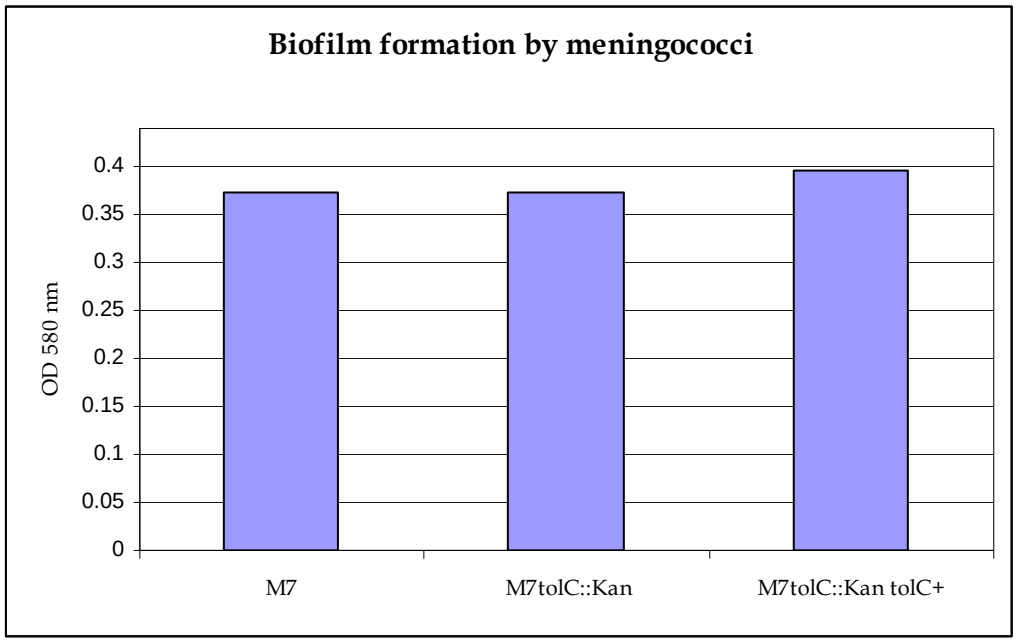

Biofilm formation by meningococci
OD 580 nm
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
M7
M7tolC::Kan
M7tolC::Kan tolC+

Table 1. Cell viability measured by Trypan Blue Exclusion

Strain	% Viable
M7	43.5 ± 3.2
M7*tolC::kan*	48.3 ± 5.3
M7*tolC::kan tolC+*	39.5 ±3.3

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Chapter 5: Summary and Discussion

Although the meningococcus and gonococcus are both strict human pathogens and are very similar at the genetic level (23), they cause very different diseases. Moreover, invasive meningococcal disease can often be fatal while gonococcal infections rarely cause death. Certainly, while the possession of a capsule by meningococci, but not gonococci, helps to explain some differences in virulence, other reasons likely exist. In this respect, Klee *et al*. discovered a set of DNA islands in certain strains of meningococci that were uniformly absent in gonococci. A subset of these genes encode putative virulence factors (9). Among the genes within one DNA island were *hlyD* and *tolC*, which have been characterized in *E. coli*: HlyD is part of a type I secretion system that functions in the extracellular export of the repeat-in-toxin (RTX) α-hemolysin (HlyA) (12, 17). TolC is the outer membrane protein (OMP) channel for a variety of multidrug efflux pumps (13, 14) and also works with HlyB-HlyD in secretion of α-hemolysin. Meningococci also produce a putative RTX toxin termed FrpC, but its biological activity remains unknown (4).

We sought to define the function of the meningococcal TolC-like protein and examined its possible role in antibiotic resistance and FrpC secretion. Unlike *E. coli*, meningococccci produce a second TolC-like protein, MtrE, which functions as the outer membrane protein channel for export of antimicrobials by the MtrC-MtrD-MtrE efflux system (2, 18). *N. meningitidis* is not unique in having more than one TolC protein. For instance, *Francisella tularensis* (5) and *Pasteurella multocida* (7) have two TolC-like proteins, while *Helicobacter pylori* has four TolC homologs encoded by genes dispersed through its chromosome (25). What is unique in the meningococcus is that just one of the

TolC-like proteins (MtrE) is solely responsible for antimicrobial mediated efflux, whereas in other Gram-negatives with more than one TolC, they are all involved to some degree in antimicrobial efflux. For example, of the two *tolC*-like genes (*pm0527* and *pm1980*) present in *Pasteurella*, *pm0527* appears to be the predominant TolC conferring higher levels of resistance and recognizing a greater range of substrates than *pm1980*. Nonetheless, a *pm1980* mutant showed increased susceptibility to a small number of antimicrobials (7). Gil *et al.* (5) observed that although both the *tolC* homologs in *F. tularensis*, *tolC* and *ftlC*, were involved in multidrug efflux, they were not required for replication in macrophages. However, in *F. tularensis*, only TolC was critical for virulence in mice by the intradermal route (5). This is an example of the different properties possessed by each of the TolC homologs.

In *E. coli*, *hlyD* and *tolC* map to different sites in the chromosome, but in meningococci they are tandemly linked and co-transcribed as an operon. The expression of the *hlyD-tolC* operon was found to be growth phase-dependent, with maximal expression at late log phase (Chapter 2, Figure 1). We hypothesized that this was due to the presence of regulatory factors that modulate *hlyD-tolC* expression. In *E. coli*, *tolC* is regulated by the Mar (multiple antibiotic resistance) system. Regulation of expression of the *marRAB* operon in *E. coli* involves both autorepression and autoactivation (11). MarR is the repressor encoded by the first gene of the operon and binds as a dimer to two operator sites to repress its own expression and that of two other genes in the operon. However, binding of an inducing compound to MarR transforms MarR into a non-DNA binding conformation, thereby permitting *marRAB* transcription to proceed resulting in *marA* expression (6). As MarA production increases, it is able to activate transcription of

tolC (1). The meningococcus has two MarR homologs: NMB1585 (*marR*) and NMB 1843 (*farR*; *f*atty *a*cid *r*esistance). FarR regulates *farAB* (10), which is similar to *emrAB* regulation in *E. coli.* The *EmrA-EmrB* efflux pump uses TolC as its outer membrane secretion channel (16). Using RNA extracted from isogenic strains that differ in the presence of *farR* or *marR,* we were unable to show that these DNA-binding proteins control *hlyD-tolC* expression in meningococci (Chapter 4). However, during the course of this work, Tzeng's group showed that MisR, the response regulator of the MisR-MisS two component system (24), is the major transcriptional repressor *in vivo* of *hlyD-tolC* and *hlyB* (19). This is of particular interest because MisR-MisS expression is similar to the PhoP-PhoQ of *Salmonella enterica* (24), which controls levels of bacterial susceptibility to cationic antimicrobial peptides and modulates salmonella virulence (3). Unpublished observations from the Shafer laboratory have revealed that in gonococci, expression of *misR-misS* is increased during growth in the presence of a sub-lethal level of the human antimicrobial peptide termed LL-37 and that MisR-MisS controls certain gonococcal genes known or presumed to be involved in virulence.

In that the TolC protein produced by meningococci has the ability to function in type I secretion of an RTX toxin (FrpC), we presumed that it is important for meningococcal pathogenesis. Heretofore, however, several studies have failed to show a function for the meningococcal RTX toxins (20-22) secreted by HlyB-HlyD-TolC. Even though these iron-regulated proteins are similar at the amino acid level to HlyA of *E. coli*, they lack hemolytic activity and are not required for virulence of meningococci in an animal model (4). They are, however, antigenic during natural infection as convalescent sera from patients with invasive meningococcal disease have anti-FrpC antibodies (15). It has been

argued that because these proteins are frequently produced by meningococci (15, 20), are antigenic during natural infection and their secretion apparatus is, in part, encoded by a DNA island (9) FrpC must be important. In my opinion, this is a circular argument and unless an animal model can be developed that more fully mimics all aspects of meningococcal disease in humans (including colonization, meningitis, and/or sepsis), it is impossible to rigorously test this hypothesis.

Due to the known function of TolC in determining levels of antibiotic resistance expressed by *E. coli* due to efflux pump systems, we also asked if expression of *tolC* influences levels of bacterial susceptibility to antimicrobials. Although a null mutation in *tolC* did not impact the antimicrobial susceptibility of meningococci (Chapter 2), expression of the meningococcal *tolC* gene in an *E. coli* TolC-deficient strain increased bacterial resistance to a panel of structurally diverse antimicrobials known to be recognized by the AcrA-AcrB-TolC efflux system (14). Why might this be? I propose that the meningococcal TolC provides the correct interactions to facilitate contacts with the cognate inner membrane drug transporter protein (AcrB) or membrane fusion protein (AcrA) in *E. coli*, but is unable to do so with the MtrD or MtrC proteins in meningococci due to amino acid sequence differences between these proteins. An alternative hypothesis is that *tolC* is poorly expressed in meningococci compared to *mtrE* and that insufficient levels of TolC naturally exist in meningococci to satisfy the needs of the Mtr pump in strains lacking MtrE. Results from preliminary experiments support this latter hypothesis. Briefly, I have performed β-galactosidase assays to test the strength of the *hlyD* promoter and surmised that it is very weak compared to that of the *mtrCDE* promoter (data not presented). The lack of strength of the *hlyD* promoter has also been

documented by Tzeng's group (19). Since this is a weak promoter, less TolC is being produced and perhaps this is why it does not function in efflux of antimicrobials in the meningococcus. In contrast, when *tolC* is over-expressed in *E. coli* from an inducible *lac* promoter, sufficient levels of TolC accumulate for its participation in drug efflux by AcrA-AcrB (Chapter 3).

This work also identified the meningococcal TolC protein as being important for RTX toxin secretion. It is clearly necessary for FrpC extracellular secretion in meningococci by the HlyB-HlyD type I secretion system (Chapter 2). Moreover, it can functionally replace TolC of *E. coli* for HlyA secretion (Chapter 3). Presumably, this is an indication that its level of expression is sufficient to satisfy the needs of the HlyB-HlyD system in both of these Gram-negative bacteria and/or its structure permits proper contact with the two exports systems in *E. coli* but not meningococci.

Continued work on the meningococcal TolC protein should test a number of hypotheses that have been developed as a consequence of the results that have been obtained during the course of this study. First, does TolC contribute to virulence in an animal model? Herein, one would examine whether loss of *tolC* due to mutation impacts the ability of meningococci to infect and survive. While there is clear evidence that TolC participates in FrpC secretion, there is no evidence that FrpC has biologic activity. The proposed study would address whether other extracellular components of meningococci could be secreted through a TolC-dependent process. In this vein, it will be necessary to detect and define the substrates that exit meningococci via TolC. This could be done by proteomic and/or mass spectrometry analyses of extracellular components. The present infant rat model measures bacteremia and the development of a model system for

nasopharyngeal infection would be needed. Second, it is important to define why TolC does not function in antibiotic resistance in meningococci, but when it is expressed in an *E. coli* background it can functionally replace the endogenous protein. Is this simply due to levels of expression or is it tied to amino acid differences in efflux pump proteins that are important in protein-protein interactions during formation of the pump? Accordingly, mutations can be made in the periplasmic end of TolC modeled after the *E. coli* homolog to test which interactions are important for substrate recognition and subsequence efflux. Third, why has the DNA island containing *hlyD-tolC* not appeared in the gonococcus despite the known ability of meningococci to transfer DNA to gonococci? Horizontal transfer of DNA between the pathogenic *Neisseria* can occur in mixed culture or during infection. For instance, the Correia element present in the intergenic region between *mtrR* and *mtrCDE* in the meningococci is absent from this site in gonococci (18). However, in the late 1990s the Correria element was found at this site in a group of azithromycin-resistant gonococcal isolates from Kansas City (8), suggesting horizontal gene transfer from meningococci to gonococci. There is also evidence of DNA transfer from the gonococcus to the meningococcus whereby a meningococcal isolate had its *porB* gene replaced with that from a gonococcus (26). Can this DNA island be transferred to and stably maintained in gonococci?

Studies addressing the above hypotheses should provide further insights regarding the actual role of TolC in meningococci and its importance in toxin secretion and antimicrobial resistance.

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