STUDIES OF *NEISSERIA MUSCULI* TYPE IV PILUS AND CAPSULE USING THE NATURAL MOUSE MODEL OF COLONIZATION AND PERSISTENCE

by

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Abstract

The *Neisseriaceae* family is a group of Gram-negative bacteria that forms part of the microbiota of humans and animals. Within the genus *Neisseria*, there are only two pathogens, *N. gonorrhoeae* and *N. meningitidis* (Nme) which infect only humans and cause disease. Others are either commensals of humans or animals including wild mice, dogs, cats, cows, and non-human primates. Pathogenic *Neisseria* also behave like commensals in that they are able to colonize the mucosal surfaces asymptomatically. Commensal *Neisseria* are little studied and there is no small animal model for studying commensal *Neisseria*-host interactions in a natural setting. There are small animal models for studying pathogenic *Neisseria* infection, but due to their strict tropism for humans, they are heterologous systems which require treatment of hormones, antibiotics, and invasive procedures. Recent work in the So laboratory has circumvented the tropism issue. Former postdoctoral researcher Nate Weyand isolated *Neisseria musculi* (Nmus) from wild mice, and showed Nmus is genetically related to human *Neisseria* – including genes encoding many host interaction factors such as Type IV pilus and capsule. I expanded this study and my investigations showed that Nmus colonize laboratory mice. This work, described in chapter 2, led to a natural mouse model that now allows studies on asymptomatic colonization from the standpoint of the bacterium and the host. Using the natural mouse model of commensal *Neisseria* colorization and persistence, I showed that host genetics, host immune status, and bacterial host interaction factors are essential determinants for *Neisseria* colonization and persistence. Type IV pilus fiber and Type IV pilus retraction null mutants are defective in establishing colonization. This natural mouse model of commensal *Neisseria* colonization and persistence will allow us to identify new neisserial and host genes that are important for asymptomatic colonization and/ or persistence. I also showed that Nmus expresses capsule, which was a unique trait of invasive Nme, and capsule influences biofilm formation. Analyzing the capsule biosynthesis locus of Nmus, I identified additional

transcriptional regulatory motifs suggesting that the mechanisms in controlling capsule production between Nmus and Nme might be different. With the recent discovery of capsule biosynthesis genes among human and animal commensal *Neisseria*, the dogma of capsule being a unique trait of invasive Nme is no longer valid. The expression of capsule locus by commensal *Neisseria* allows investigators to study the transcriptional and functional similarities and/or differences between commensal and pathogenic capsules as well as the potential impact(s) of capsules shared by the pathogenic and commensal *Neisseria*.

Chapter 1- Introduction

Chapter 1.1
Concepts of microbe-host interactions

Before 1880, the Miasma theory, the configuration of the stars, and curses from gods were thought to be the causes of diseases. In 1840, the German anatomist Friedrich Henle published a paper describing the requirements for defining the causative relationship between microbes and their associated infectious diseases (1). In the late 19th century, Robert Koch's Postulates, established a method to determine whether a disease was caused by a microbe. A number of other scientists, notably Thomas Rivers, Robert Huebner, Alfred Evans, Louis Pasteur, and Stanley Falkow also developed alternative paradigms to try to define the relationship between microbes and infectious diseases. Advances in molecular biology have broadened our understanding of microorganisms and led to the appreciation that host-microbes interactions do not always result in diseases. For example, trillions of microbes colonize the human body without causing overt disease. In addition, some microbes only cause disease in certain hosts. These findings led to a revision of the terminology that explains complex host-microbe interactions. Chapter 1.1 focuses on a review of concepts and terms that are used to describe players and outcomes of host-microbe interactions, namely, mutualism, commensalism, parasitism; and pathogenesis, asymptomatic colonization, persistence, and virulence factors. The meaning of these terms can vary in different eras and situations. Therefore, choices of definitions are made according to the context of this dissertation.

Microbial pathogenesis

At the Tenth International Congress of Medicine in Berlin, 1890, Robert Koch presented his Postulates to set out rules for proving the role of a microbe in causing a particular disease (Table 1.1). Microbes were considered pathogens if they met the criteria described in his Postulates. However, there are limitations to his Postulates, since not all microbes could be cultured *in vitro*. In 1979, Alfred Evans revised Koch's Postulates. His principles (now known as

Evan's Principles) refined the causation of disease by incorporating both the epidemiological principles and the immunological status of the host (2). With increasing cases of microbes causing diseases only in immunocompromised hosts, Stanley Falkow and colleagues refined Koch's Postulates and proposed the "Molecular" Koch's Postulates (Table 1.1) (3). Falkow defined microbes as pathogens based on the identification of virulence factors (genes responsible for causing disease). Deletion of virulence gene(s) should lead to the loss of pathogenicity. In the late 20th century, Arturo Casadevall and Liise-anne Pirofski revised the definitions of pathogens, pathogenicity, virulence, and virulence factor based on host damage (Table 1.2) (4, 5). They proposed classifying pathogens into six groups, with each groups having a unique profile of damage-response curves. This curve reflects the damage caused by either the host immune responses or intrinsic pathogen characteristics, or both (4). In summary, these concepts provide a framework for stringent testing of a microbe for its disease-causing ability.

Table 1.1. A comparison of Koch's Postulate and Falkow's Molecular Koch's Postulates (6)

Koch's Postulate	Falkow's Molecular Koch's Postulates
The parasite occurs in every case of the disease in question and under circumstances which can account for the pathological changes and clinical course of the disease.	The phenotype or property under investigation should be associated with pathogenic members of a genus or pathogenic strains of a species.
The parasite occurs in no other disease as a fortuitous and nonpathogenic parasite.	Specific inactivation of the gene(s) associated with the suspected virulence trait should lead to a measurable loss in pathogenicity or virulence, or the gene(s) associated with the supposed virulence trait should be isolated by molecular methods. Specific inactivation or deletion of the gene(s) should lead to loss of function in the clone.

After being fully isolated from the body and repeatedly grown in pure culture, the parasite can induce the disease anew.	Reversion or allelic replacement of the mutated gene should lead to a restoration of pathogenicity, or the replacement of the modified gene(s) for its allelic counterpart in the strain of origin should lead to loss of function and loss of pathogenicity or virulence. Restoration of pathogenicity should accompany the reintroduction of the wild-type gene(s).

Table 1.2 Definitions of microbial pathogenesis proposed by Casadevall and Pirofski (4)

Term	Proposed definition
Pathogen	A microbe capable of causing host damage; the definition can encompass classical pathogens and opportunistic pathogens; host damage can result from either direct microbial action or the host immune response
Pathogenicity	The capacity of a microbe to cause damage in a host
Virulence	The relative capacity of a microbe to cause damage in a host
Virulence factor (or determinant)	A component of a pathogen that damages the host; can include components essential for viability including modulins

Mutualism, Commensalism, and Parasitism

The human body is colonized by 10^{13} to 10^{15} bacteria, collectively referred as the microbiota (7). These microbes interact with each other and with the host. These interactions, referred as symbiosis, are classified into three categories, mutualism, commensalism, and parasitism.

A mutualistic relationship between two organisms is one where both organisms benefit (8, 9). There are two types of mutualistic relationships: obligate and facultative. In obligate mutualism, the survival of one or both organisms is dependent on the other. In facultative mutualism, the survival of both organisms is not dependent upon their relationship. Bacteria can colonize the human digestive system and live in a mutualistic relationship with the hosts. These bacteria facilitate the digestion of organic compounds and provide essential vitamins to the host. In return, bacteria receive nutrients from the host and a place to live.

In commensalism, one organism benefits from the relationship while the other does not gain any benefit or is unharmed by it (8, 10). Bacteria that colonize, replicate, and persist inside the host without causing any detectable damage or clinical symptoms are termed commensals. Commensalism is not always absolute. Commensals such as *Candida albicans* and coagulase-negative *Staphylococcus* spp. can sometimes lead to symptomatic infections under certain conditions, for example, when the immune system of their host is suppressed (11, 12). Pathogens such as *Neisseria meningiditis* (Nme) and *Neisseria gonorrhoeae* (Ngo) often colonize their host, man, asymptomatically for an extended period of time (13–17). In addition, there are often debates when defining whether a relationship is mutualistic or commensalistic, because it is not always an absolute situation when determining whether both organisms gain benefits or only one organism has it.

Finally, parasitism describes a relationship which involves one organism living on the host and deriving benefit from this interaction, possibly causing damage to the host (18). Ticks and fleas survive on the body of their hosts by consuming their blood for nutrients. Tapeworms burrow into the intestines of animals and consume the partially digested food, eventually depriving the host of vital nutrients.

Since microbe-host interactions are both variable and unpredictable, these concepts and terms are context dependent. Therefore, in this dissertation, I will use the term commensalism to refer to a state of microbial colonization that does not result in overt clinical outcomes (Table 1.3) (5) and use this term to describe any commensal-host interactions.

Table 1.3 Categories of microbe-host-interactions proposed by Casadevall and Pirofski based on the damage framework (5)

Term	Proposed definition
Infection	Acquisition of a microbe by the host; most infections are followed by multiplication of the microbe in the host, but this is not universal because some helminth infections can involve a single organism that does not replicate in the host
Colonization	A state of infection that results in a continuum of damage from none to great, with the latter leading to the induction of host responses that could eliminate or retain the microbe, or progress to chronicity or disease; for organisms that induce no damage during infection this state is indistinguishable from commensalism
Commensal	A microbe that induces either no damage or clinically inapparent damage after primary infection
Commensalism	A state of infection that results in either no damage or clinically inapparent damage to the host, though it can elicit an immune response
Persistence	A state of infection in which the host response does not eliminate the microbe, resulting in continued colonization over time; persistence may evolve into overt disease, depending on the balance of the host-microbe interaction
Mutualism	A state of colonization whereby both the host and the microbe benefit as a consequence of infection

Chapter 1.2
Effects of microbiota on human physiology and gut and immune system development

Trillions of microbes (including commensal bacteria, archaea, viruses, and eukaryotic microbes) live on and within mammals (7). Over the course of evolutionary time, the host has adapted to microbes and vice versa, and a mutualistic relationship has been established (19). This mutualistic relationship between the host and the gut microbiota is now heavily investigated. Although the majority of commensals are found within the gastrointestinal tract, there are also distinct microbiota colonizing other parts of the body, including the oral cavity (20, 21), respiratory tract (22), and urogenital tract (23, 24). A balanced microbiota helps to digest dietary fibers (25), provides essential metabolites (26), stimulates the gut and immune system development (27, 28), and protects the host from pathogen colonization (29–32). *Neisseria* spp.

is found to asymptomatically colonize various body locations including the gastrointestinal tract, oral cavity, respiratory tract, and urogenital tract. I will therefore focus on describing the microbiota of these organs in this Chapter.

The gastrointestinal tract microbiota

Extensive efforts have been made to identify and characterize the composition of the microbiota along the human gastrointestinal (GI) tract. Molecular techniques targeting the 16S ribosomal RNA gene and high-throughput sequencing have helped to identify the major bacterial (33–39), viral (40–42) and fungal (43–45) genera along the GI tract (Fig. 1.1.1). Large-scale projects such as the European Metagenomics of the Human Intestinal Tract (46) and the US Human Microbiome Project (47, 48) have also made tremendous progress towards understanding the symbiotic relationship between gut microbes and their host (49).

The GI tract consists of the oral cavity, esophagus, stomach, small intestine, caecum, and large intestine. Each compartment has its unique chemical and physical characteristics, and these characteristics affect the composition and diversity of the microbiome.

The oral cavity is the first location where food is processed into smaller pieces. It is a unique environment that is sub-divided into the non-keratinized buccal mucosa, the keratinized mucosa (tongue, gingiva, and the subgingival sulcus), and the abiotic surfaces such as the tooth enamel, dental restorations and implants. Since the oral cavity is an open system, the oral microbiome has a combination of transient visitors and persistent colonizers. These persistent colonizers often form biofilms on the mucosa.

Due to the structural, spatial, and functional differences within the oral cavity, the microbiome between the mucosa and the tooth-related niches are remarkably different (50–52). Based on

culture-independent analyses, the most common genus in the oral cavity is *Streptococcus*; minor genera at this location include *Neisseria, Gemella, Granulicatella*, and *Veillonella* (36, 47, 53, 54).*Streptococcus mitis*, *S. pneumoniae*, and *G. adiacens* occupy both dental and mucosal niches while *Rothia dentocariosa*, *Actinomyces* spp., *S. sanguinis*, *S. gordonii*, and *A. defectiva* preferentially colonize teeth, and *Simonsiella muelleri* only colonizes the hard palate (36). As in the oral cavity, the most common genus in the esophagus is *Streptococcus* (55).

Food is transported by peristalsis through the esophagus into the stomach. The stomach has the fewest microbes (10^1 - 10^3 CFU per gram), due to its acidity (pH<4). Analysis from 23 gastric endoscopic biopsy samples shows that the microbiome of the stomach is mainly comprised of Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, and Fusobacteria (34).

After processing in the stomach, the solid food becomes chyme and enters the intestines slowly. The lower GI tract has the highest variety of chemical, nutrients, and microbial composition. The small intestine is more acidic, has a higher level of oxygen and antimicrobials than the large intestine. Therefore, the small intestine is dominated by fast-growing facultative anaerobes such as *Lactobacillaceae* and *Enterobacteriaceae* (56). Analyses of human biopsy and ileostomy samples indicate the most commonly found bacterial genera are *Clostridium*, *Streptococcus*, and *Bactericides* (57–61). The caecum and large intestine have the highest biodiversity and numbers of bacteria. With lower concentrations of antimicrobials, slower transit time, and oxygen level, both the caecum and the large intestine are dominated by the fermentative polysaccharide-degrading anaerobes. Analysis of mouse biopsy samples shows that the mouse caecum is enriched in the *Ruminococcaceae* and *Lachnospiraceae* while the large intestine is mainly colonized by the *Bacteroidaceae* and *Prevotellaceae* (62).

Besides the microbial varieties along the GI tract, there are also variations across the cross-sectional axis of the gut. Laser capture micro-dissection analyses show that the central luminal compartment and the inter-fold cryptic region of the mouse gut have distinct microbial communities (63, 64). The Firmicutes families *Lachnospiraceae* and *Ruminococcaceae* are enriched between the inter-fold while the *Prevotellaceae*, *Bacteroidaceae*, and *Rikenelaceae* are dominant in the lumen (63). Caution is needed when generalizing these findings to the whole population, because diet, health, age, and method of sampling and analyses might be different between studies.

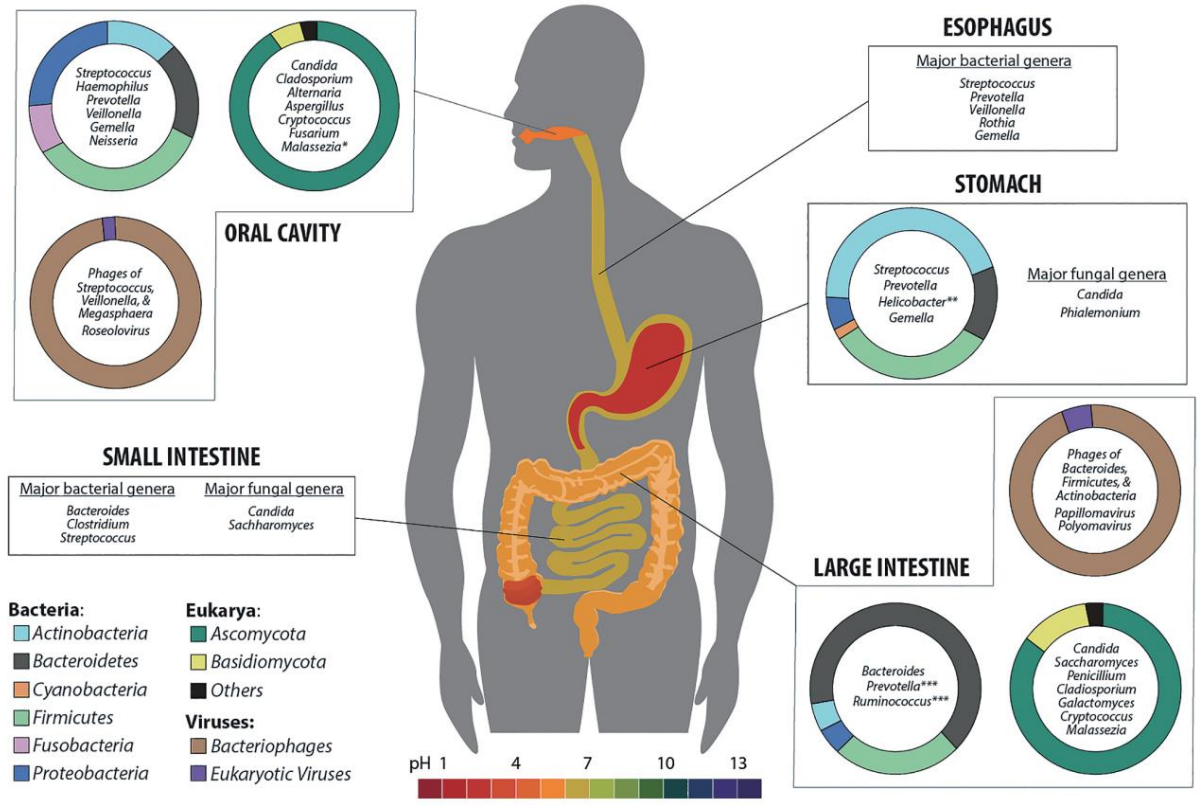

Figure 1.1.1 The microbiota composition of GI tract (65). Dominant genera in each GI tract location are presented. Colors on the doughnut plots correspond to the legend in the lower left corner; the GI tract is colored according to the pH scale at the bottom. (*Malassezia was very abundant in one study and was not detected in another study. ** The abundance of Helicobacter may vary greatly between individuals. *** Proportions of these and other colon genera vary with age, diet, & geographical location.) [Reproduced from ref (65)]

The upper respiratory tract microbiota

The respiratory tract is divided into the upper respiratory and lower respiratory tracts. The upper respiratory tract includes the anterior nares, nasal passages, paranasal sinuses, the nasopharynx and oropharynx, and the portion of the larynx above the vocal cords. The lower respiratory tract includes the portion of the larynx below the vocal cords, the trachea, bronchi, bronchiole, and alveoli. The highest bacterial density is observed in the upper respiratory tract (Fig. 1.1.2). For this dissertation, I will focus on describing the upper respiratory tract microbiota.

Similar to the GI tract, the upper respiratory tract also has distinct anatomical structures and is exposed to various environmental factors. These diverse niches are colonized by various bacteria. The anterior nares are mostly colonized with keratin-associated bacteria such as S*taphylococcus* spp., *Propionibacterium* spp., and *Corynebacterium* spp. (66–68). Around 20% of the population carry *S. aureus* in their nostrils asymptomatically (69). The nasopharynx is lined with a stratified squamous epithelium and respiratory epithelial cells. Besides overlapping with the anterior nares, the microbiota of the nasopharynx also contains nasopharyngeal bacteria such as *Dolosigranulum* spp., *Haemophilus* spp., and *Streptococcus* spp (70–73). Finally, the oropharynx, which is lined with non-keratinized stratified squamous epithelium, is colonized with *Neisseria* spp., *Rothia* spp. and anaerobes, including *Veillonella* spp., *Prevotella* spp., and *Leptotrichia* spp. (74–77).

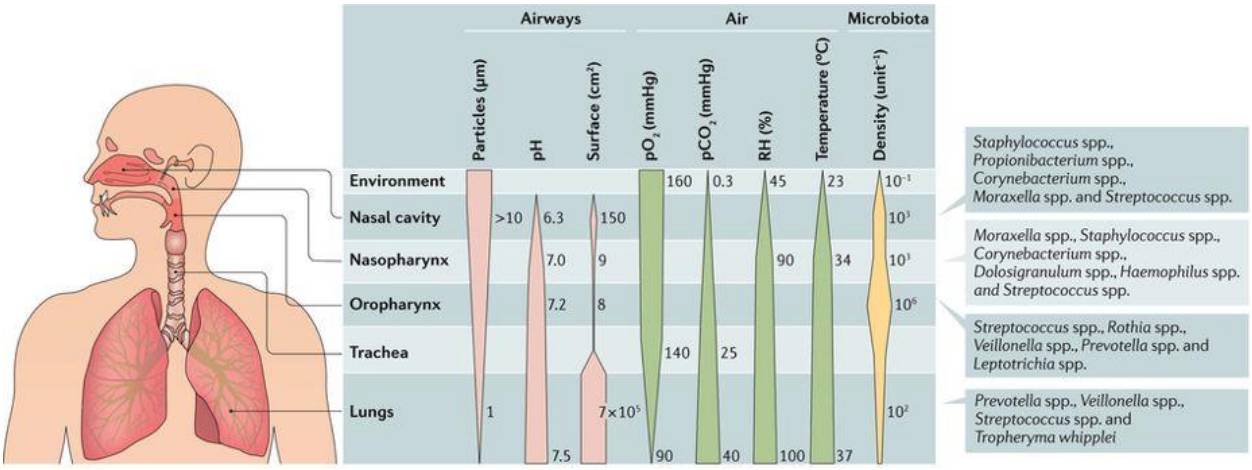

Figure 1.1.2 The physiological and microbial gradients along the respiratory tract (78)**.** The pH gradually increases along the respiratory tract, whereas most of the increases in relative humidity (RH) and temperature occur in the nasal cavity. The partial pressures of oxygen (pO_2) and carbon dioxide (pCO_2) have opposing gradients that are determined by environmental air conditions and gas exchange at the surface of the lungs. Inhaled particles that are more than 10 μm in diameter are deposited in the upper respiratory tract, whereas particles less than 1 μm in diameter can reach the lungs. These particles include bacteria-containing and virus-containing particles, which are usually larger than 0.4 μm in diameter. These physiological parameters determine the niche-specific selective growth conditions that ultimately shape the microbial communities along the respiratory tract. The unit by which bacterial density is measured varies per niche; the density in the environment is depicted as bacteria per cm^3 (indoor) air, density measures in the nasal cavity and nasopharynx are shown as an estimated number of bacteria per nasal swab, and the densities in the oropharynx and the lungs represent the estimated number of bacteria per ml of oral wash or bronchoalveolar lavage, respectively. [Reproduced from ref (78)]

The vaginal microbiota

The healthy vaginal microbiome is mainly colonized with *Lactobacillus* spp. (*Lactobacillus crispatus, Lactobacillus gasseri, Lactobacillus iners* and *Lactobacillus jenseni*) (23, 79). These *Lactobacillus* spp. play important roles by lowering the environmental pH through lactic acid production, bacteriostatic and bactericidal compound production, or through competitive exclusion (80–83). In addition, they prevent a number of urogenital diseases, such as bacterial vaginosis, yeast infections, sexually transmitted infections, urinary tract infections, and HIV infection (84, 85). In some cases, anaerobic bacteria, such as *Atopobium, Dialister, Gardnerella, Megasphaera, Prevotella,* and *Peptoniphilus* can also be isolated from healthy women (23).

Similar to other subdivisions of microbiota, the composition of the vaginal microbiota also changes dynamically over a woman's lifecycle. Before puberty, the vaginal microbiota is mostly dominated by Gram-negative anaerobe bacteria, including *Bacteroides, Fusobacterium, Veillonella*, some Gram-positive anaerobic bacteria, including *Actinomyces, Bifidobacterium, Peptococcus, Peptostreptococcus*, and *Propionibacterium*, as well as some aerobic bacteria such as *Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus viridans*, and *Enterococcus faecalis* (86, 87). During puberty and estrogenic fluctuation, the vaginal microbiota is mostly colonized by *Lactobacillus crispatus, Lactobacillus gasseri, Lactobacillus iners*, and *Lactobacillus jenseni* (88). During menopause, the microbiota is dominated by *L. crispatus, L. iners, G. vaginalis*, and *Prevotella* and a lower abundance of *Candida, Mobiluncus, Staphylococcus, Bifidobacterium*, and *Gemella* (89). Together, changes in hormonal and menstrual cycles as well as sexual activity can alter the composition of the vaginal microbiota (90).

The male genital tract microbiota

The male genital tract microbiota is understudied compared to other body sites. The lower male genital tract (urethra and coronal sulcus) microbiota is usually colonized with Firmicutes, Actinobacteria, Fusobacteria, Proteobacteria, and Bacteroidetes (91). Like the vaginal microbiota, the composition of the male genital microbiota is also affected by the differences in ethnicity, changes in hormones, sexual activities, and circumcision status. Using culture-independent molecular analyses methods (16S rRNA/Pyrosequencing), Price et al. assessed the impact of circumcision on the male genital microbiota. The anaerobic bacterial abundance detected in pre-circumcision swabs is higher than those in post-circumcision ones (92). In addition, the biodiversity of the pre-circumcision microbiota appears more heterogeneous than the post-circumcision microbiota. Before circumcision, the dominant bacterial families are Pseudomonadaceae, Clostridiales Family XI, and Prevotellaceae with minor population of

Fusobacteriaceae, Bifidobacteraciaceae, and Staphylococcaceae. After circumcision, the male genital microbiota is mainly colonized with , Pseudomonadaceae, Corynebactericeae, and Staphylococcaceae (92). Whether the change in microbiota based on circumcision status is associated with disease or healthy condition is yet to be tested.

The gut microbiota is essential for maintaining gut physiology

A balanced microbial flora is essential for proper gut function. Commensal bacteria help to digest fibers that the body cannot break down. For example, *Bacteroides ovatus*, a commensal of the human gut, encodes a polysaccharide utilization locus that is involved in metabolizing the indigestible dietary fibers xyloglucans (93). *Lactobacillus* and *Bifidobacterium* encode enzymes which help to digest fructooligosaccharides and oligosaccharides that are common in fibers (94).

The gut microbiota produces metabolites from anaerobic fermentation of undigested complex carbohydrates in the colon. One of the metabolic end-products are short-chain fatty acids (SCFAs), including acetic acid, butyric acid, and propionic acid. The gut microbiota produces 50-140 mM SCFAs daily (95) and they are absorbed by the intestinal epithelial cells as an energy source. SCFAs help in maintaining the normal gut physiology and mucosal immunity. Besides being an energy source, SCFAs also act as signaling molecules that regulate gene expression, immune cell differentiation, and Intestinal epithelial cells proliferation (96). The gradient of SCFAs between the lumen and crypt is thought to stimulate the turnover of Intestinal epithelial cells (97). SCFAs stimulate mucus production by Goblet cells (98, 99) and increase both the peripheral and colonic regulatory T cells population (100–102). Feeding allergic airway diseases mice (which serve as a model for asthma) with SCFA-supplemented diets can lower colonic inflammation and attenuate allergic airway disease (103, 104).

Polyamines (putrescine, spermidine, and spermine) are also microbial metabolites derived from the diet and produced by the host and microbial cells. They induce the production of mucus and secretory IgA in the small intestine (105, 106). Feeding rats with a polyamine-deficient diet led to intestinal mucosal hypoplasia (107). They also protect the integrity of intestinal epithelial cells by stimulating the production of tight junction proteins (occluding, ZO-1, and E-cadherin) (108, 109), which, in turn, regulate paracellular permeability and affect mucosal barrier function. These findings suggest that the production of polyamines through host-microbial interactions is essential for maintaining gut physiology.

Lastly, the gut microbiota also provide critical vitamins that the host cannot produce (110). Vitamin B12 is produced by lactic acid bacteria (111) while folate, a vitamin involved in DNA repair and synthesis, is produced by *Bifidobacteria* (112).

The gut microbiota directs the development of the gut and systemic immune system

Experiments with germ-free (GF) mice revealed the importance of the microbiota in influencing the development of the gut and systemic immune system. GF mice have an enlarged cecum (113), reduced gastrointestinal motility (114, 115), longer villi and shorter crypts (116), and a reduced amount of antimicrobial peptides and regenerating islet-derived protein 3 gamma (RegIIIγ) (117) *Lactobacillus rhamnosus* GG (118), *Akkermansia muciniphila* (119), and *Lactobacillus plantarum* (120) participate in regulating intestinal epithelial cells integrity and cell renewal. GF mice also have a thinner mucus layer than conventionally reared mice. These malformations of the gut in GF mice can be reversed upon reintroduction of the gut microbiota (121). Inoculation of GF mice with SCFA-producing *Bacteroides thetaiotaomicron* or *Faecalibacterium prausnitzii* induce goblet cell differentiation and mucus production (122). In the absence of gut commensals, GF mice have smaller Peyer's patches and mesenteric lymph

nodes (123). One of the major immune defects in GF mice is within the intestinal T cell subsets. The intestinal $CD4^+$ and $CD8^+$ T cell numbers are decreased significantly in GF mice (123). Reconstitution of GF mice with *B. fragilis* resets the balance between the T helper 1 and 2 cells (124). T helper 17 cells, which maintain mucosal barrier integrity and clearance of pathogens, are absent in the small intestine of GF mice (125). They can be restored by colonizing GF mice with either standard mouse microbiota or segmented filamentous bacteria (125, 126). Even though GF mice have normal number and maturation of B cells, they produce less IgA and IgG1 antibodies in both the mucosal and nonmucosal organs (127, 128). The production of IgA and IgG1 antibodies can be restored by conventionalizing the normal flora back to the adult GF mice (129).

The microbiota protects against pathogen colonization

The microbiota directly and indirectly inhibit pathogen. These can be mediated via direct interactions between commensals and pathogens, for example, competition for nutrients and niches, and killing of pathogens (Fig. 1.1.3A) 2) commensal-mediated improvement of host defense (Fig 1.1.3B).

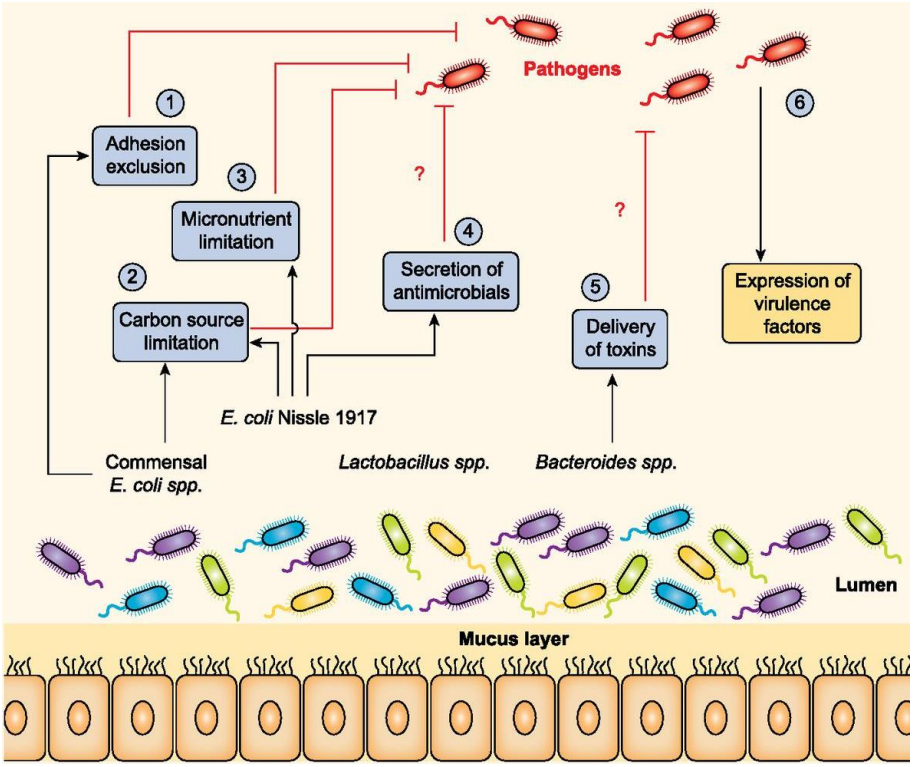

Figure 1.1.3A Mechanisms by which commensals directly inhibit infection by enteropathogens (29)**.** 1) Adhesion exclusion: Commensals compete for space with pathogens to initiate adherence. 2-3) Competition of space: commensals compete against pathogens for carbon and iron source limitation. For example, commensal *E. coli* limit availability of nutrients for pathogens. 4) Secretion of antimicrobials: commensals produce bacteriocins and microcins to kill pathogens. 5) Delivery of toxins: Commensals inject toxins directly into the cytosol of pathogens using their Type VI secretion system. 6) Pathogens also express a variety of virulence factors to promote colonization. [Reproduced from ref (29)]

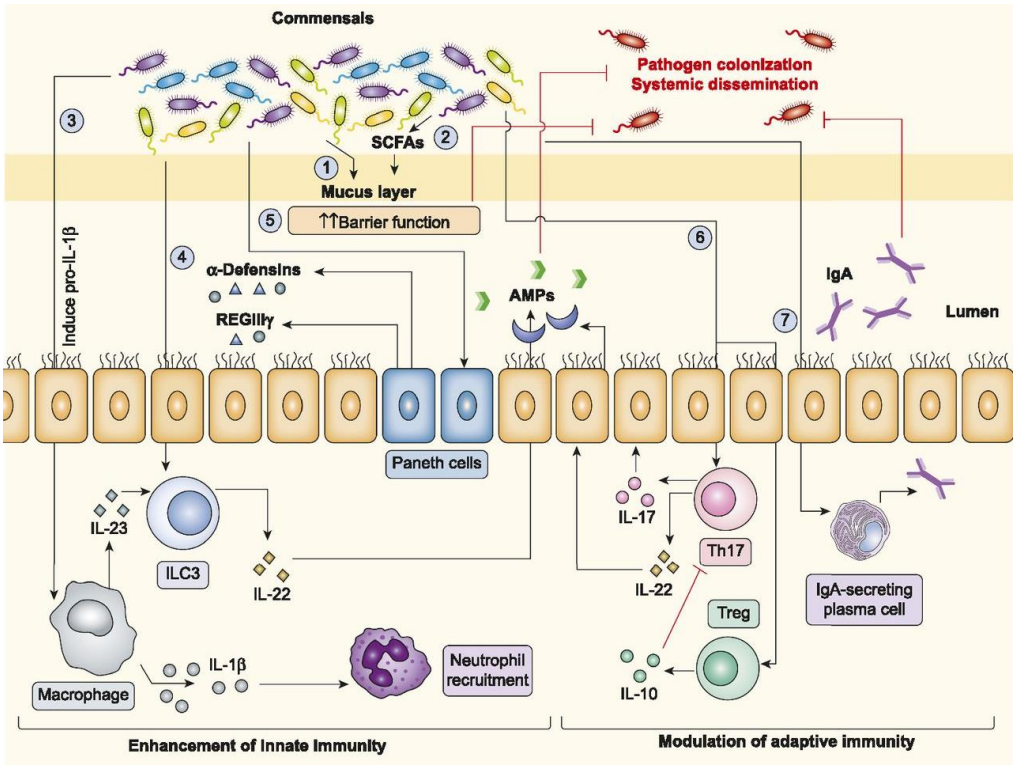

Fig. 1.1.3B Indirect mechanisms by which commensals inhibit infection by enteropathogens (29). 1) Barrier function: Commensals stimulate the production of mucus which forms a physical barrier between the gut epithelial cells and the microflora. 2) SCFA production: The gut commensal metabolize undigested fiber into SCFAs which are important in maintaining the normal gut physiology and mucosal immunity 3) Induction of pro-IL-1B cleavage: Commensals can stimulating IL-1B production which facilitates neutrophils migration. 4) IL-22 secretion by ILCs: Commensals can induce the secretion of IL-22 which, in turn, stimulate the production of antimicrobial peptides (AMPs). 5) Stimulate the production of REG3γ and α-defensins: Commensal-host interaction stimulates the secretion of AMPs by the Paneth cells. 6) T cell differentiation: SCFAs produced by the commensals can induce Th17 and Treg differentiation which produces anti-inflammatory cytokines (IL-10, IL-22, IL-17). 7) sIgA: commensals stimulate the production of sIgA by plasma B cell. [Reproduced from ref (29)]

Many pathogens and commensals share the same niches and/or nutrients for colonization and persistence inside the host. Commensal bacteria use different mechanisms to out-compete pathogens for limited resources and space. *B. fragilis* produces factors that allow penetration of the mucus layer and colonization of intestinal crypts (130). Commensal *Escherichia coli* strains HS and Nissle 1917 metabolize multiple sugars in the gut and limit their availability to pathogenic enterohemorrhagic *E. coli* (EHEC) and enteropathogenic *E. coli* (EPEC) (131–134). *E. coli* Nissle 1917 also inhibits the colonization of pathogenic *Salmonella Typhimurium* by

restricting the availability of carbohydrates and iron (135, 136). Commensal *Clostridium scindens* encodes the 7 α-hydroxysteroid dehydrogenase enzyme that converts primary bile acids into secondary bile acids; in turn, secondary bile acids inhibit the growth of pathogenic *C. difficile* (137). In addition, consumption of oxygen by commensal facultative anaerobes, such as the *Enterobacteriaceae*, reduce the pathogenicity of *Shigella flexneri,* which requires oxygen for expression of virulence factors (138). These and other findings underscore the importance of a balanced microflora in preventing the colonization of pathogens through nutrient limitation.

Commensals also inhibit the growth of pathogens by secreting toxins and antimicrobial peptides (139). *E. coli* Nissle 1917 produces small antimicrobial peptides, termed bacteriocins, which inhibit the growth of EHEC (140). Commensal *Staphylococcus lugdunensis* produces a peptide that inhibits the growth of pathogenic *S. aureus* (141). *S. epidermidis* produces a protease which prevents biofilm formation and nasal colonization of *S. aureus* (142). Bacteroidetes use their phage-like Type VI secretion system to inject toxins into pathogenic competitors (143, 144). Commensal bacteriocins inhibit the colonization of pathogens within and outside of its genus (145, 146).

The presence of commensal *Neisseria lactamica* in the upper respiratory tract reduces the risk of Nme infection (147–150). In a controlled human challenge study, the natural carriage rate of meningococci was reduced from 24.2% to 14.7% 2 weeks after *N. lactamica* inoculation (148). Moreover, a reduction in meningococci carriage rate was observed in people carrying *N. lactamica*, but not people lacking the commensal. These reports strongly suggest that *N. lactamica* colonization inhibits carriage of Nme. One of the speculated mechanisms for this inhibition is the presence of cross-reactive antibodies to meningococci (151). However, one study showed that natural antibodies against meningococci occur before the colonization of *N. lactamica* (149). A recent study by our laboratory showed that commensal *Neisseria* DNA kills

Nme, suggesting that nasopharyngeal commensal *Neisseria* may inhibit Nme colonization through this DNA-based killing mechanism (Kim, Higashi, and Goytia et al., 2019, Cell Host and Microbes in press).

Commensals also prevent pathogen colonization indirectly by enhancing the host immune system. In order to colonize, pathogens need to attach to mucosal surfaces. The gastrointestinal mucus layer helps to reduce the attachment of pathogens to intestinal epithelial cells. Acetate, produced by *Bifidobacterium,* helps to reduce gut permeability and bacterial translocation (152). *Bifidobacterium longum* subspecies *Infantis* secretes peptides which can also reduce intestinal permeability (153). The reconstitution of *B. infantis* in a mouse colitis model ameliorated the intestinal pathology (154).

Another example of commensal-induced gut protection is the induction of IL-22, a cytokine that enhances the production of antimicrobial peptides (155). *Lactobacillus reuteri* metabolizes tryptophan and produces a metabolite, indole-3-aldehyde, which, in turn, activates aryl hydrocarbon receptor on innate lymphoid cells (ILCs) (156). This activation leads to the production of IL-22 which upregulates the expression of RegIIIγ in the gut. RegIIIγ inhibits the colonization of pathogenic bacteria/fungi, such as *C. rodentium*, vancomycin-resistant Enterococcus, and *C. albicans* (155–158). RegIIIγ also prevents overstimulation of the immune system by creating the 50μm “demilitarize zone” in the small intestine (117).

As we have mentioned previously, SCFAs produced by commensals can induce Th17 and Treg differentiation. Th17 protect the host from pathogenic colonization through the secretion of IL-22 upon stimulation (125). Both Th17 and Treg maintain a low inflammatory state inside the gut. A balanced microflora provides colonization resistance against pathogens by inhibiting pathogen growth directly and by stimulating the host immune response.

Disturbances in the microbiota lead to outgrowth of pathogens and increase the risk of non-infectious diseases

The composition of the gut microbiota can be affected by the host lifestyle and antibiotic usage. Disturbances in the microbiota lead to the development of chronic inflammation and metabolic dysfunction (Fig 1.1.4).

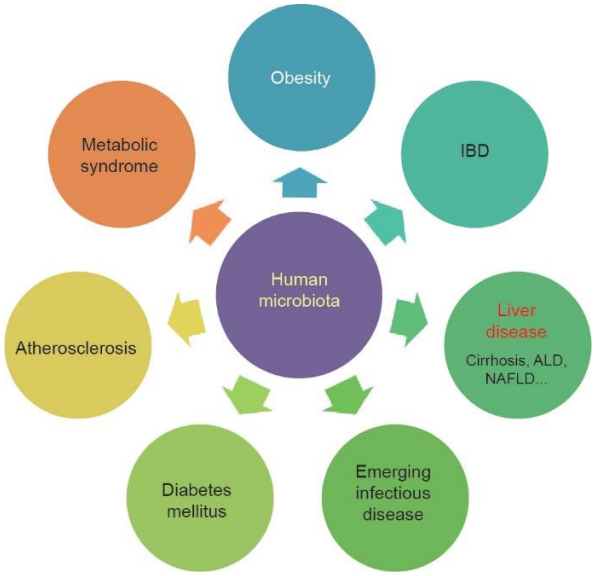

Fig 1.1.4. Dysbiosis of human microbiota might lead to the development of infectious and non-infectious diseases (159). [Reproduced from ref (159)]

Susceptibility to pathogen colonization is significantly increased when the microbiota composition is altered by antibiotics (160, 161). For example, patients taking broad-spectrum antibiotics have a very high incidence of diarrhea and colitis caused by *C. difficile* (162). They usually have an altered microbiota with a reduction in butyrate-producing anaerobic bacteria and an increase in endotoxin-producing opportunistic pathogens (163). Immunocompromised patients also have a higher incidence of sepsis caused by vancomycin-resistant *Enterococcus* (164, 165).

Many studies show that dysbiosis in microbiota is related to the development of many non-infectious diseases, such as obesity (166–168), Type 2 diabetes (T2D) (169–171), and allergic diseases (172). The identical-twins study (173) shows that the fecal composition of the identical twins can influence the adiposity and metabolism of GF mice. Mice that received fecal microbiota from an obese twin had greater body mass and adiposity than those that received fecal microbiota from a "lean" twin. In addition, the lean human microbiota prevented development of obesity in those obese-recipient mice. Pedersen *et al.* (174) showed that the presence of *Prevotella copri* and *B. vulgatus* may affect the serum metabolome and induce insulin resistance. The usage of metformin, a widely used antidiabetic drug, may also alter the composition of the microbiota, which, in turn, affects the development of T2D (171). Finally, infants with a gut microbiota enriched in Clostridia and Firmicutes at the age of 3-6 months are associated with the resolution of cow milk allergy by the age of 8 (175). Infants with lower abundance of *Bifidobacterium*, *Akkermansia*, and *Faecalibacterium*, and a higher relative abundance of particular fungi (*Candida* and *Rhodotorula*) have a higher risk of developing atopy and asthma (176).

The diversity of different subsets of the microbiota is also associated with health outcomes. Increased diversity of gut bacteria has been linked to the absence of inflammatory bowel disease, obesity, and resistance against acute infections by enteropathogens (177, 178). Conversely, low bacterial diversity at the vagina is considered healthy as it is associated with decreased incidences of bacterial vaginosis (23, 179) and premature birth (180). In the respiratory tract, a decrease in bacterial diversity of the upper respiratory tract is also associated with increased incidences of acute upper respiratory tract infections, such as acute otitis media (181, 182), and mucosal inflammation in chronic rhinosinusitis (183). These studies demonstrate the niche-specific effect of biodiversity on human health.

In summary, numerous recent studies have increased our understanding of the relationship between the microbiota and human physiology. Complex interactions between the microflora and the host induce development of the gut and immune system. A healthy microbiota generates metabolites that are important for maintaining physiological processes. Both the microbiota and metabolites enhance host immune responses and prevent the colonization of pathogens. Perturbations in the microbiota are associated with many non-infectious diseases and increase the susceptibility of infections. Missing from this research effort are studies on how commensals colonize and persist in the body in the presence of a robust immune system. The aim of my dissertation research is to address this question. I first addressing this question by developing a natural small animal model that pairs the common laboratory mouse with a wild mouse commensal *Neisseria.* This model allows investigators to study commensal *Neisseria*-host interactions in natural setting. I will introduce the genus *Neisseria* in the next section.

Chapter 1.3.
The genus *Neisseria*

The genus *Neisseria* (Fig. 1.1.5) is a group of Gram-negative, oxidase positive, aerobic β-Proteobacteria within the family *Neisseriaceae*. *Neisseria* species are commonly found in the human oral cavity (184). Keijser et al. showed that *Neisseria* is the most abundant genus within *Proteobacteria*, comprising 8.2% and 3.9% of total sequences in human saliva and plaque, respectively (185). It also constitutes approximately 10% of the bacterial population on the human tongue (75). At least 13 *Neisseria* species are found in humans and many others are isolated from a wide variety of animal species, including mammals, reptiles, , and even from insects species (186–189). Most of the *Neisseria* are commensals that do not cause disease in healthy individuals. Only in rare cases, which is discussed later, will they cause disease (e.g., sepsis in immunocompromised hosts) (190, 191).

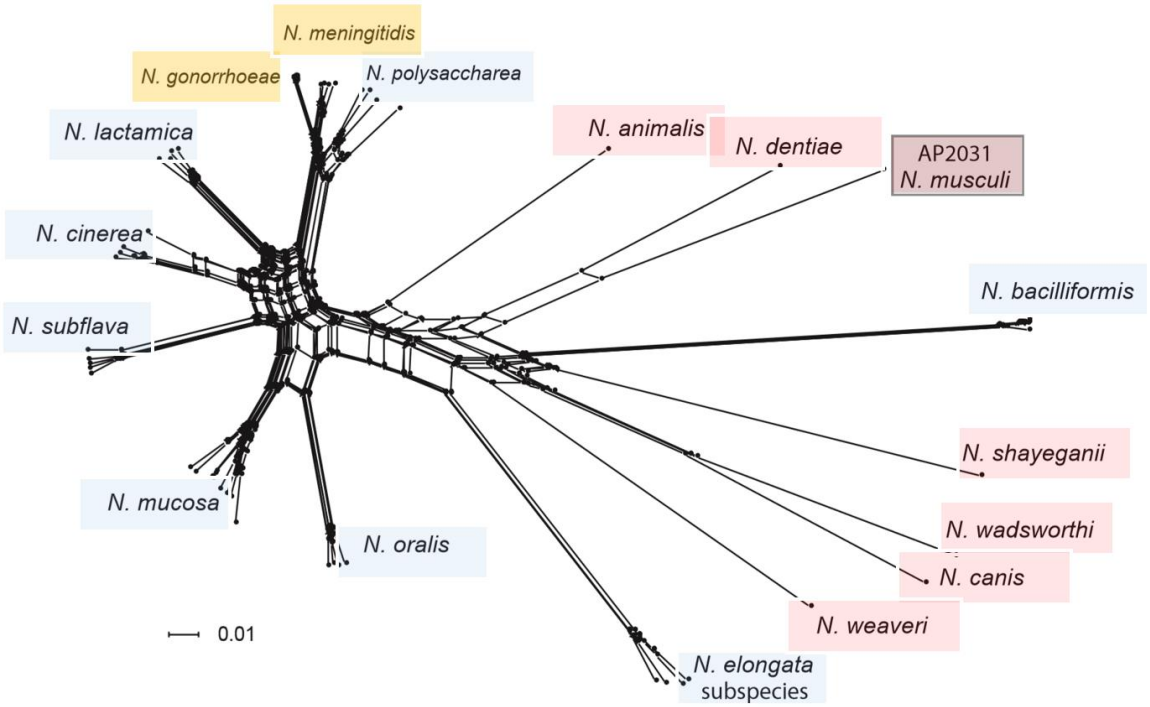

Fig. 1.1.5. Evolutionary relationship of the genus *Neisseria* (188). Neighbor-net tree reconstructed from 51 concatenated bacterial ribosome gene sequences. Animal *Neisseria* are in pink boxes. Commensals of man are in blue boxes. There are only two human-specific pathogens in this genus, *Ngo* and *Nme*. They are in yellow boxes. This neighbor-joining tree indicated that commensals and pathogenic Neisseria are highly related genetically. Bar, 0.01 substitutions per nucleotide position. [Reproduced from ref (188)]

Human Commensal Neisseria

In addition to the well-studied pathogens Ngo and Nme, there are at least 6 different species of human commensal *Neisseria* (*N. lactamica*, *N. polysaccharea*, *N. cinerea*, *N. flavescens*, *N. musoca*, and *N. elongata*). They vary in morphology, from short bacilli to cocci. These commensal *Neisseria* colonize the human nasopharynx and oropharynx asymptomatically. Using metagenomic sequencing, Donati et al. (184) showed that commensal *Neisseria spp*. have different tissue tropisms within the oral cavity and oropharynx. For example, *N. subflava* mainly segregated with the tongue dorsum while *N. mucosa* dominated in the gingival plaque. Recently, researchers identify the presence of *N. flavescens* in the duodenum of adult celiac patients (192). This further suggests that commensal *Neisseria* colonize other mucosal surfaces

besides the human oral cavity. Finally, human commensal *Neisseria* have a wider range of hosts than pathogenic *Neisseria*. For example, *N. mucosa* can be cultured or identified in humans, rhesus macaques, dogs, cats, ducks, woodlouse (186). This data suggests that this species can colonize a wide range of host.

How commensal *Neisseria* affects the host physiology is unknown. However, studies have linked the presence of commensal *Neisseria* with the progression of human disease. For example, the presence of *N. lactamica* is known to reduce the risk of Nme infection by either excluding Nme from the nasopharynx or enhancing the host adaptive immune system through inducing cross-reactive salivary IgA and serum IgG antibodies against the meningococcus (147, 193, 194). Absence of caries is linked to an abundance of *N. flavescens* in the oral cavity (185). *N. cinerea* bronchial colonization reduces the risk of respiratory syncytial virus infection (195). *N. mucosa* is less abundant in inflammatory bowel disease patients, and more abundant in obese patients, compared with healthy controls (196). Although these studies are correlative, they suggest that commensal *Neisseria* can act as biomarkers of certain human diseases.

The vast majority of human dwelling *Neisseria* species are commensals. However, commensal *Neisseria* can also act as opportunistic pathogens (Table 1.4). Occasionally, they cause invasive disease such as septicemia/bacteremia, endocarditis, and pneumonia (190). In addition, *N. lactamica, N. sicca, N. flavescens, and N. mucosa* have been isolated from cases of meningitis/ sepsis in human (190). Most of the diseases caused by commensal *Neisseria* are related to dog/cat bites, oral and heart surgery, and immunosuppressive treatments.

Table 1.4. Examples of commensal *Neisseria* isolated from rare clinical diseases in human (186, 197)**.**

Neisseria species	Native host	Case report(s)	References
N. lactamica	Human	Meningitis or sepsis in adults and children, bacteremic pneumonia	Everts et al. (2010) (198) Zavascki et al. (2006) (199) Wang et al. (2006) (200)
N. subflava	Human	Endocarditis, bacteraemia	Lewin and Hughes (1966) (201) Ramos et al. (1998) (202)
N. sicca	Human	Endocarditis, meningitis, conjunctivitis	
N. flavescens	Human	Endocarditis, septicaemia, once in an outbreak of meningitis, halitosis	Sinave and Ratzan (1987) (203) Wertlake and Williams (1968) (204) Huang et al. (2014) (205)
N. mucosa	Human	Meningitis, endocarditis, septicaemia, visceral botryomycosis	Stotka et al. (1991) (206) Pilmis et al. (2014) (207) Locy (1995) (208)
N. cinerea	Human	Peritonitis, neonatal conjunctivitis, proctitis, nosocomial pneumonia in an immunodeficient patient, tricuspid valve endocarditis, meningitis and septicaemia in patients with facial trauma	Taegtmeyer et al. (2006) (209) Bourbeau et al (1990) (210) Southern and Kutscher (1987) (211) Dossett et al. (1985) (212) Boyce et al. (1985) (213) Benes et al. (2003) (214) Kirchgesener et al. (1995) (215)
N. elongata	Human	Osteomyelitis, endocarditis, septicaemia after oral surgery	Haddow et al. (2003) (216) Hofstad et al. (1998) (217)
N. bacilliformis	Human	Endocarditis	Masliah-Planchon et al. (2009) (218) Abandeh et al. (2012) (219)

N. weaveri	Dog	Septicaemia after a dog bite, cheek abscess	Carlson et al. (1997) (220)
N. canis	Cat	Purulent wound and cellulitis, respiratory tract infection, fever after a cat bite	Guibourdenche et al. (1989) (221) Safton et al. (1999) (222)
N. animaloris	Dog/Cats	Infection following animal bite, Chronic otitis media after ears licked by dog	Holmes et al. (1990) (223) Roebuck and Morris (1999) (224)
N. zoodegmatis	Dog/Cat	Infection following animal bite, Skin ulceration in an immunodeficient patient	Holmes et al. (1990) (223) Grob et al. (1989) (225)

Animal commensal Neisseria

Commensal *Neisseria* species are also found in a variety of animals (Table 1.4) including non-human primates, dogs, cats, ducks, guinea pigs, wild-caught mice, domestic cows, reptiles, and even from dolphins (186). *Neisseria* species will likely be isolated from other animals.

Neisseria is a dominant taxon in the core microbiome in the oropharynx and the nasopharynx of cats and dogs. Next-Generation Sequencing detected *Neisseria* in all oral samples from six and eleven healthy dogs and cats, respectively (226, 227). In one study, 92% of 49 dogs carried *N. zoodegmatis* (228). In 1973, *N. mucosa* var. *heidelbergenesis* was isolated from the blowholes, throat, and mouth of 2 out of 35 dolphins (229).

Neisseria species colonize the avian oral cavity and alimentary tract. For example, *Neisseria* species have been isolated from fecal samples of duck and penguins (230, 231). A new species of *Neisseria, N. tadorna*, was isolated from the liver of the Gaoyou Sheldrake (232). Three strains of *Neisseria* were identified from skin samples of sheep and goats (233).

Some animal commensal *Neisseria* can also be opportunistic pathogens. For example, *N. animaloris* and *N. zoodegmatis* can cause disease within the Felidae family (234–236). *N. iguanae* was first isolated from the liver of a dead rhinocerous iguana with multiple granulomas and later from chronic tail abscesses in common iguanas (237). The same species is also identified in the oral cavities from healthy rhinocerous iguana and common iguana, suggesting this organism is associated with septic lesions but is also a commensal. However, the mechanisms underlying the pathogenesis of these animal *Neisseria* are still unknown.

Human pathogenic Neisseria

Neisseria meningitidis and Neisseria gonorrhoeae

Nme, an obligate opportunistic pathogen, mostly colonizes the human nasopharynx asymptomatically (238). Occasionally, it crosses the epithelial barrier, enters the bloodstream, migrates across the blood-brain barrier, and enters the cerebrospinal fluid meninges to cause meningitis (239). Nme causes endemic and epidemic meningitis and/or sepsis (240). There are 13 known serogroups of Nme, based on different capsular polysaccharide structures, but only 6 serogroups (A,B,C,W-135,X, and Y) cause the majority of invasive meningococcal disease (241). The overall mortality rate in meningococcal disease is between 10 and 15% (238). Even with proper treatment, 10% of survivors suffer neurological damage (238).

Patients with meningococcal disease are promptly treated with benzylpenicillin or a third-generation cephalosporin upon hospitalization (242, 243). If the causative agent is not known at admission, ceftriaxone or cefotaxime is used for the first 24-48 hours (244). Definitive treatment with penicillin G, ampicillin, or an extended-spectrum cephalosporin is recommended once the diagnosis is confirmed (245).

Since the development of the first vaccine for meningococcal serogroup C in 1960s (246), there have been tremendous efforts in developing vaccines against other serogroups. Polysaccharide-protein conjugated vaccines against serogroup A, C, W135, and Y are available and used successfully worldwide (247). These polysaccharide vaccines are safe and immunogenic as they induce an adaptive immune response. More importantly, they are also effective in young children which help to reduce the rate of meningococcal infection within this high-risk group (248). The incidence of serogroup C disease in the UK also significantly reduced with the introduction of MenC vaccine (249). However, polysaccharide vaccine is ineffective towards serogroup B meningococci since the capsule of serogroup B is antigenically similar to a human neuronal antigen (250). Using “reverse vaccinology” approach (251, 252), the 4CMenB vaccine was developed in 2014 (253). This vaccine contains 5 meningococcal protein antigens (factor H binding protein, *Neisseria* heparin binding antigen, Neisserial adhesin A, and Outer membrane vesicle). This 4CMenB vaccine has been licensed in Europe while a vaccine composed of two lipidated factor H binding proteins has been licensed in the USA (254, 255). Nevertheless, there is no universal vaccine that can protect against all serogroups and vaccine development continues.

Ngo is also an obligate pathogen which causes > 800,000 new cases of gonorrhea in the USA each year (Centers for Disease Control and Prevention 2015) and more than 106 million new cases worldwide annually (256). Viable gonococci can be isolated from the urogenital tract, oropharynx, conjunctiva, and rectum of both infected and asymptomatic males and females (257). Patients with gonococcal genital tract infection have dysuria and purulent exudate as acute symptoms (14, 257). Around 40% of untreated cervical infections will result in pelvic inflammatory disease (PID), orchitis, epididymitis, or salpingitis. Infected women with PID are at risk for developing infertility or ectopic pregnancy (14). Ngo is now one of the urgent threats on

the CDC list because it has developed resistance to all antibiotics used for treatment (https://www.cdc.gov/std/gonorrhea/arg/default.htm). Currently, there is no vaccine for Ngo.

Both Ngo and Nme can colonize the mucosal surfaces asymptomatically, a behavior that is shared by their commensal *Neisseria* ancestors. Among Ngo-infected women, 30-50% are asymptomatic while 5-10% of infected men are asymptomatic (14, 258). Nme also colonizes the nasopharynx of 10-40% of the general population asymptomatically (13). The meningococcal carriage prevalence is age-related. The carriage rate of meningococci is less than 1% among children under the age of 4 and peaks at 20-25% in late teenage/early adult life (13, 259, 260). This increasing carriage rate in young adults might be due to the increased exposure to social risk factors, including visiting bars/night clubs, kissing, smoking, and living in closed communities such as military or university dormitory (261–263). Even though commensal and pathogenic *Neisseria* share many common host interaction factors and colonize similar niches, commensal *Neisseria* seldom cause disease. The mechanism(s) by which commensal *Neisseria* establish asymptomatic colonization is still not well understood.

Pathogenesis of Ngo and Nme on mucosal epithelial surfaces

The current understanding of *Neisseria*-host interactions is mostly derived from studies using primary or immortalized cell lines derived from the relevant tissues or human organ culture (264). Mucosal epithelial cells are the primary target of colonization for Ngo and Nme. The infection process can be separated into 7 steps (as shown in Fig.1.1.6) (265).

The first step in Ngo pathogenesis is attachment to the epithelial cell (266). This process is mediated through specific bacterial surface structures such as Type IV pili (Tfp), opacity (Opa) proteins, lipooligosaccharide (LOS), and outer membrane protein Porin B (PorB). Once attached, bacteria crawl together to form microcolonies and microcolonies fuse to form biofilms

(267). Pilus retraction brings the gonococci close to the cell surface and stimulates mechanosensitive host cell signaling pathways (268). Pilus retraction initiates both morphological and transcriptional changes on host cell (269, 270). A variety of cytoskeletal components and signaling molecules are recruited to the site of bacterial adhesion, forming the cortical plaque within 30 minutes post infection (271).

After mediating initial attachment, Opa proteins interact with the mammalian cell surface immunoglobulin-related vertebrate glycoproteins- carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) and heparin sulfate proteoglycan (HSPG) receptors (272, 273). This interaction facilitates the internalization of gonococci by the host cell. During these initial stages of infection, Ngo releases fragments of peptidoglycan, LOS, and OMVs to the extracellular space (274, 275). These antigens activate Toll-like receptor (TLR) and nucleotide-binding oligomerization domain-containing protein (NOD) signaling in epithelial cells, macrophages, and dendritic cells (276–278). NOD and TLR activation lead to the secretion of cytokines and chemokines by these cells. The release of pro-inflammatory cytokine and chemokine signal the recruitment of polymorphonuclear leukocytes, or neutrophils, to the site of infection, where they phagocytose Ngo. Around 24 hours post-infection, Ngo transcytoses through the basolateral/ apical membrane and initiates a new round of infection (279, 280).

To survive and replicate inside host cells, pathogenic *Neisseria* activates Epidermal Growth Factor Receptor, phosphoinositide-3 kinase, and mitogen activated protein kinase signaling pathways. Activation of these signaling cascades protects epithelial cells from apoptosis and stress upon neisserial infection (281–285). Cleavage of lysosomal protein LAMP1 by neisserial IgA protease inhibits lysosomal remodeling which, in turn, facilitates long term intracellular survival and replication (286). In addition, our laboratory shows that cleavage of CD46-cyt1 by metalloproteinases and Presenilin/γSecretase complex during infection reduces the intracellular

pool of CD46-cyt1 (287), which, in turn, diminishes the ability of infected cells to initiate autophagy at a later stage of infection (288). These gonococcal-epithelial interactions allow the pathogen to survive intracellularly.

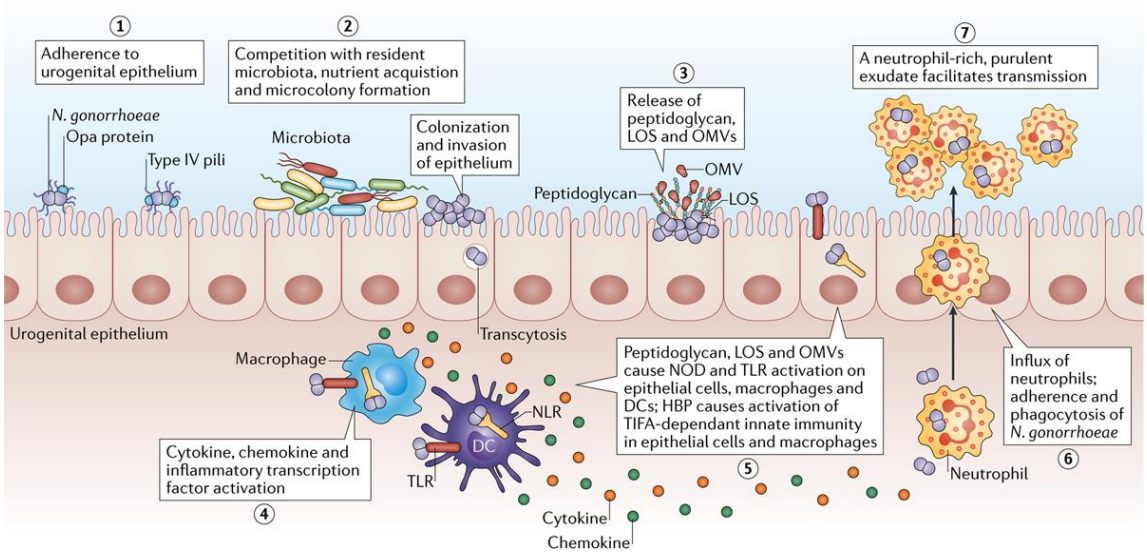

Figure 1.1.6. Overview of Ngo infection (265)**.** Tfp mediate loose attachment (1) to the mucosal epithelial cell, and 2) Ngo replicates and forms microcolonies, likely competes with the microbiota. Opa and PorB facilitate the gonococcal invasion and immune evasion. 3) Ngo releases fragments of peptidoglycan, LOS, and OMVs. 4-5) Secreted antigens activate innate immune responses and stimulate the production of cytokines and chemokines by macrophages, dendritic cells, and epithelial cells. 6) The release of proinflammatory cytokines and chemokines recruits leukocytes or neutrophils to the site of infection where they phagocytose Ngo. 7) The influx of neutrophils makes up a purulent exudate that facilitates transmission. Opa, opacity protein; PorB, outer membrane porin B; LOS, lipooligosaccharide; OMVs, outer membrane vesicles. [Reproduced from ref (265)]

The pathogenesis of Nme is similar to Ngo in that the initial attachment to the nasopharynx is mediated by Tfp (289). The opacity- associated proteins (Opa and Opc) facilitate the invasion process through binding to the CEACAM receptors and extracellular matrix components (290).One major difference between these two pathogens is the ability of certain Nme strains to migrate across the endothelial cells and disseminate in the bloodstream, eventually reaching the cerebrospinal fluid and cause meningitis (291).

How meningococci traverse the blood-brain barrier (BBB) and enter the cerebrospinal fluid is still unclear. However, there are at least 3 possible strategies for Nme to cross the blood-brain barrier (292): 1) transcytosis across endothelial cells, 2) passing between tight junctions, and 3) endothelial barrier disruption due to a cytotoxic effect.

A small subset of piliated meningococci is internalized by endothelial cells following attachment. These internalized meningococci may undergo transcytosis and further enter the meninges. Recent *in vitro* studies also suggest that Nme crosses this BBB through paracellular passage (293). Following the initial attachment and activation of host endothelial CD147- B2-adrenergic receptor pathway by Tfp retraction (294, 295), ezrin is recruited and actin is polymerized. This signaling induces the relocation of cell-cell junction proteins (vascular endothelial cadherin, zona occludens 1, and claudin 5) to the site of bacterial invasion (295, 296). Adhesion of Nme also induces the production of matrix metalloproteinase 8 which proteolytically cleaves the tight junction protein occludin (297). Nitric oxide and reactive oxygen species produced by the host cell also participate in the degradation of cell-cell junctions (298). Disruptions of cell-cell junctions allow Nme to cross the endothelial barrier between cells and gain access to the cerebrospinal fluid. Since all these observations were made *in vitro* on human primary brain endothelial cells or cell lines, whether these events also hold truth *in vivo* at the BBB remains to be determined. Lastly, a breakdown of the BBB due to bacterial cytotoxicity is unlikely, since the increase in permeability of the BBB is minimal and tissue lesions such as hemorrhages in the subarachnoidal space are rare during meningococcal meningitis (292). Nonetheless, Nme remains mostly as commensal within the host (299). Occasionally, they can cause devastating invasive diseases such as septicemia and meningitis in susceptible individuals. However, what precisely determines whether it will remain as commensal or pathogen still need to be elucidated.

Pathogenic *Neisseria* evade immune response through multiple mechanisms. The meningococcal capsule protects the bacterium from phagocytosis and complement-mediated killing (240). Sialylation of terminal residues of LOS also inhibits complement-mediated and antimicrobial peptide killing (300). Phase and antigenic variation of surface antigens also facilitate immune evasion (301). There are around 26-65 gonococcal and meningococcal host interaction factor genes that contain short sequence repeats in their promoter or coding region (302). Some repeat-containing genes of Nme are: *porA*, iron acquisition (*hmb*R, *hpu*AB, *fet*A), adhesin and invasin (*opc*, *opa*, *nad*A, Tfp biogenesis genes), and autotransporters (*mspA*/*asuI, nalP*/*aspA*). Slipped-strand mispairing of the sequence repeat tracts (poly (C) or poly (G)) can cause on/off switches in gene expression or modulation of expression depending on where these tracts are located. By altering the expression of host interaction factors, pathogenic *Neisseria* escape the adaptive immune response and persist inside the host.

Host interaction factors common to human/animal commensal and pathogenic Neisseria

Many host interactions factors of pathogenic *Neisseria* are also found in animal commensal *Neisseria*. Both human and animal commensal *Neisseria* have a comprehensive range of host interaction factors from pathogenic *Neisseria*. Marri *et al.* reported that out of 177 *Neisseria* "virulence" genes, 133 of them are in at least one or more sequenced human commensal *Neisseria*, and 70 genes are in all of them (303). Over 70% of the genes known to contain repeat sequences for phase variation in pathogenic *Neisseria* are present in commensals (303). However many of these genes do not have tandem repeats, suggesting recombination-based phase switching of host interaction factors is less frequent in commensals (303).

Weyand *et al.* reported that *Neisseria macacae*, an animal *Neisseria* isolated from rhesus macaques in 1983, encodes 61 out of 70 pathogenic host interaction neisserial genes (304). In addition, Nmus, a new species of commensal mouse *Neisseria,* also encodes many host

interaction genes of pathogenic *Neisseria* (305, 306). These include proteins important for pathogenic *Neisseria* attachment, invasion, and intracellular survival. For example, all commensal *Neisseria* encode and express Tfp, which is important for attachment, DNA transformation, and host cell signaling. Using biophysical analysis, Tfp of *N. elongata* retract at half the speed of Ngo (Biais, personal communication). In addition, commensal and pathogenic *Neisseria* use different Sigma factors to initiate pilin transcription (307, 308). These studies suggest that differences in Tfp retraction dynamics and transcriptional regulation between commensal and pathogenic *Neisseria* may affect how these bacteria interact with their hosts and vice versa.

N. polysaccharea, *N. flavenscens*, and *N. lactamica* have *opa* genes that promote cell attachment, invasion, immune cell signaling and inflammation (303). All these *opa* variants contain variable numbers of the CTCTT pentameric repeat which leads to Opa phase and antigenic variation in pathogenic *Neisseria* (309). Commensal Opas also have high affinity for human CEACAM1 receptor, which, in turn, might inhibit the pathogenic Opa-CEACAM1 interaction (310). In addition to genes for acquiring iron from human transferrin and lactorferrin, human commensal *Neisseria* also have genes for the transport of hemin, Fe^{3+} and Fe^{2+}, and for siderophore receptor and transport. Many homologs of vaccine antigens, including genome derived *Neisseria* antigens, are found in both human and animal commensal *Neisseria* (*N. macacae* and Nmus) (197). Previously, the capsule biosynthesis locus was thought to be unique to Nme. Recently, Clemence el al. (311) and Ma et al. (306) have identified the presence of capsule biosynthesis genes among human and animal commensal *Neisseria*. However, *in vivo* functions of these commensal host interaction factors are still to be studied.

The damage response curve of Neisseria spp.

Using damage either from host immune responses or intrinsic microbes properties, or both, Arturo Casadevall and Liise-anne Pirofski proposed the damage-response framework of microbial pathogenesis to describe host-microbe interactions (4). They used different parabolic damage-response curves to represent “classes” of microbes. Each curve can be viewed as a possible outcome of host-microbe interactions in a given host.

Based on damage as a function of immune response, Casadevall and Pirofski classified microbes into six classes (4). Class 1 microbes are microorganisms that cause damage only in hosts with weak immune responses. Class 2 are those that cause damage either in hosts with weak or normal immune responses. Class 3 are pathogens that cause damage in normal host responses and disease at extremes of immune responses (weak and strong). Class 4 are pathogens that cause symptomatic infections only at the extremes of both weak and strong immune responses. Class 5 are pathogens that cause damage across the spectrum of host responses and result in chronic infections eventually. Finally, Class 6 microbes can cause damage only in strong immune responses. Despite differences at the level of phylogeny, growth conditions or presence of specific virulence factors, microbes with similar types of damage profile or disease can be grouped together.

Most of the *Neisseria* spp. are commensals and there are only two pathogens, Ngo and Nme. In both weak and normal host immune responses, Ngo and Nme cause gonorrhea and meningitis, respectively. Besides causing infectious disease, they also have the ability to colonize the host asymptomatically (13, 14, 258). Occasionally, commensal *Neisseria* cause invasive disease such as septicemia/bacteremia, endocarditis, and pneumonia in hosts with weak immunity (190). In rare cases, commensal *Neisseria* have been isolated from cases of meningitis/ sepsis

in human with normal immunity (190). According to the damage-response framework, both pathogenic and commensal *Neisseria* can be classified as a Class 2 microbe (Fig. 1.1.7). The framework proposed by Casadevall and Pirofsky allows us to define a range of host interactions and clinical symptoms in reference to *Neisseria* pathogenesis and commensalism, as well as generate relevant information on the range of infection phenotypes of pathogenic *Neisseria*.

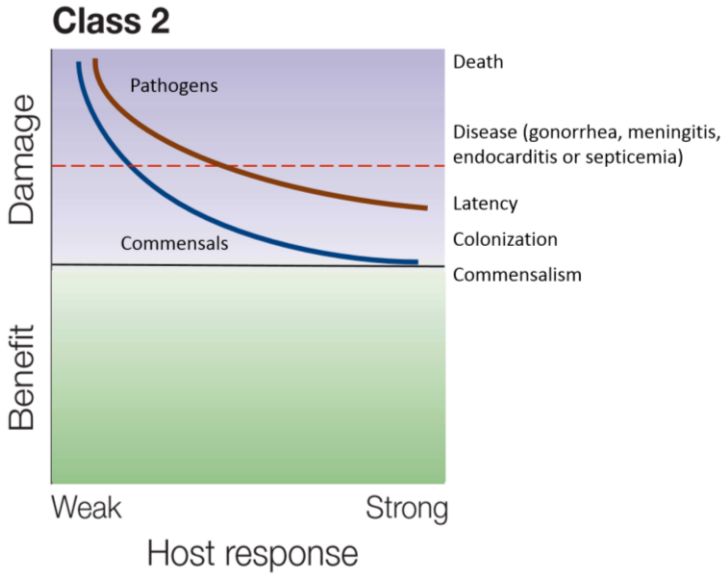

Fig. 1.1.7. Damage-response curve of *Neisseria* species (313). The *y*-axis denotes host damage as a function of the host response. In this scheme, host damage can occur throughout the host response, but is magnified at the weak extreme. The host response is represented by a continuum from 'weak' to 'strong'. Weak responses are those that are insufficient, poor or inappropriate — that is, they are not strong enough to benefit the host. Strong responses are those that are excessive, overly robust or inappropriate — that is, they are too strong and can damage the host. When a threshold amount of damage is reached (red dash line), the host can become symptomatic and if damage is severe, death can ensue. Class 2 microorganisms have the ability to cause damage in normal hosts but are frequently associated with severe infections in hosts with weak response. Brown and blue curve represents the pathogenic *Neisseria* and commensal *Neisseria* damage-response curves, respectively. Host response prevents significant damage and asymptomatic colonization of pathogenic and commensal *Neisseria* occurs. Green and purple represent health and progression of host damage, respectively. [Modified from ref (313)]

Chapter 1.4
Neisserial Tfp and capsule and their cognate host components

To establish colonization and persistence within the host, *Neisseria* use many host interaction factors which help them during attachment/invasion and immune evasion. Within the content of this dissertation, I will focus on two major host interaction factors: Tfp and capsule.

The Neisseria Type IV pilus

Tfp is a macromolecular structures that spans the bacterial cell envelope (Fig. 1.1.8). Tfp is produced by a range of Gram-negative and Gram-positive species (reviewed in Giltner et al.) (312). It is a highly dynamic structure that extends and retracts in cycles. Tfp promotes adhesion (313–315), horizontal gene transfer (316–320), bacterial twitching motility (321–324), bacterial aggregation/biofilm formation (325–328), and immune evasion (329, 330). These diverse functions are caused by the extension and retraction of the Tfp fiber.

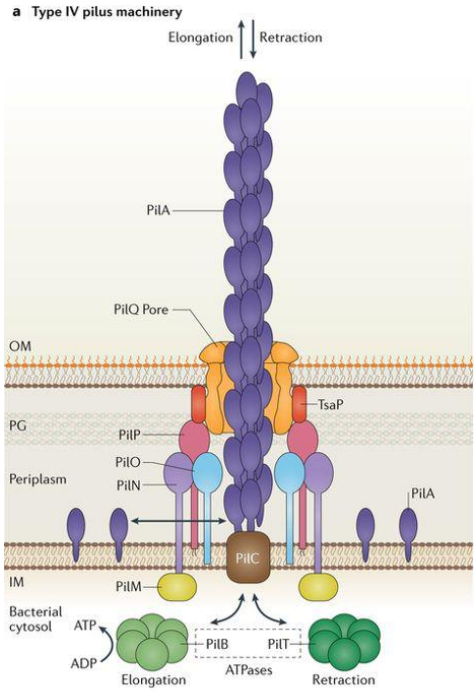

Fig. 1.1.8. Structure of the *Myxococcus xanthus* Type IV pilus (331)**.** Proteins forming the Tfp are highly conserved among the Tfp-expressing bacteria, but the nomenclature varies a lot between species. Pilins (PilA) inserted into the inner membrane are then incorporated into the base of the growing pilus. The Tfp is composed of the outer membrane pore complex (PilQ,

TsaP; the pilotin PilF is not included in this figure), the alignment complex (PilM–PilN–PilO–PilP), and the inner membrane motor complex (PilC–PilB–PilT). ATP hydrolysis by PilB and PilT provides energy for pilus elongation and retraction. [Reproduced from ref (331)]

The gonococcal Tfp can be separated into four components: the pilus fiber, the outer membrane pore, the inner membrane assembly complex, and the cytosolic motor proteins. The fiber polymer consists of repeating subunits of the 18-22kDa pilin, or PilE, monomers (332). Neisserial pilin is encoded by *pilE* and synthesized as a precursor protein. Prepilin subunits are produced in the cytoplasm and translocated into the periplasmic space. PilD, a peptidase/transmethylase, cleaves and methylates prepilin into a mature and functional pilin monomer (333). Mature pilin monomers are then incorporated into the base of the growing pilus fiber with help from the ATPase PilF (334). The pilus fiber is anchored by the outer membrane PilQ/PilP pore proteins. The PilQ pore opens and allows the growing fiber to pass through the outer membrane and into the extracellular space (335). Once assembled, a conserved set of minor-pilin proteins, including PilC and PilH/I/J/K, help to stabilize the fiber (336). PilT, a member of the ATPase Associated with various Activities (AAA) protein family, mediates retraction of the pilus, likely by disassembling pilins at the fiber base (339, 340). Mature pilin has an alpha helical N-terminus and a globular C-terminus (332). The fiber is made up of three subunits and each subunit is arranged in a stacking-spiraled position. The N-terminal alpha-helices and polar interactions between the globular domains help to maintain the structure of the fiber. Tfp fibers form bundles of up to 8 fibers and these bundles are very strong, stable, and flexible (339). When the pilus fiber is subjected to force, it can be stretched 3 times longer and its diameter can be 40% narrower than the original structure, this can cause conformational changes in the fiber (340). The PilE globular domain can be modified post-translationally by glycosylation, acetylation, and phosphorylation (341, 342). These modifications affect the bundling and function of Tfp. Upon deletion of *pilE*, *Neisseria* become non-piliated and the cells are defective in microcolony formation, DNA transformation, and attachment/invasion *in vitro*. However, Ngo with *pilE* deletion is still able to colonize the male urethra but does not induce

urethritis (Cannon JG, unpublished data). Therefore *in vitro* data may not fully recapitulate how *Neisseria* colonize and persist *in vivo*.

One major difference between pathogenic and commensal Tfp is the ability of pathogenic Tfp to undergo antigenic variation (343). In pathogenic *Neisseria*, antigenic variation of *pilE* involves the recombination of a one of many silent copies of variant pilin pseudogenes (*pilS*) with *pilE*, the pilin gene in the expression locus (346). Antigenic variation of PilE allows Ngo to evade the memory response against the pilin type expressed by the infecting Ngo strain, which, in turn, allows the infecting strain to continue residing in the body and/or reinfect the same individual repeatedly. Pathogenic *Neisseria* have approximately 19 copies of *pilS*, while commensals only have 2-5 copies (303). In addition, the commensal *pilE* locus does not have the guanine-repeat element that is required for *pilE*/*pilS* recombination (345). Pilin antigenic variation via the guanine-repeat element is unlikely to occur in commensal *Neisseria.*

The PilT motor

Proteins belonging to the AAA family of ATPases form a hexameric ring that facilitates protein degradation and refolding. PilT, a bacterial AAA ATPase, forms a ring that is 115Å in diameter and 70Å in height, and a central opening of 15-35Å diameter. The PilT hexamer is located at the cytoplasmic side of the inner membrane (346). ATP hydrolysis by the subunits causes conformational changes in the ring that provide mechanical energy for pilin fiber disassembly (347, 348). The energy generated by ATP hydrolysis breaks the protein-protein contact between pilin monomers (337, 346, 349). These monomers are then recycled for the next fiber assembly process (337, 346, 349). Using laser tweezers microscopy, Merz et al. showed that a gonococcal pilus retracts at a speed of 1 μm/s and exerts a 100pN force (337). Retraction of the Tfp fiber can be regulated by environmental signaling. Anaerobic conditions reduce the force of

Tfp retraction (350) while Tfp fiber bundling increases the force to 1 nN (339). The availability of ATP also affects Tfp retraction dynamics (351, 352).

Many *Neisseria* interactions with host cells involve Tfp retraction. For example, twitching motility, microcolony formation, horizontal gene transfer, attachment/invasion, and host cell signaling require pilus retraction (353). To move over a surface, *Neisseria* cells extend their Tfp through the pilus assembly, bind to a receptor, and retract Tfp through the PilT-dependent pilin disassembly pathway. These steps pull the bacterium forward. Tfp extension and retraction also allows bacterial cells to interact with each other and with its neighboring cells, ultimately forming spherical microcolonies of 50 to over 100 cells. Higashi et al. showed that these microcolonies are motile and move together to fuse with each other (354). Tfp retraction also functions in horizontal gene transfer. ComP, a minor Tfp protein, binds a *Neisseria*-specific 10 bp sequence that is abundant in neisserial genomes (316, 355, 356). Tfp retraction causes ComP-bound DNA to cross the bacterial envelope and enter the cytosol (357, 358). Finally, Tfp retraction induces cytoprotective host cell signaling pathways. The magnitude and duration of Ca^{2+} fluxes on host cell is enhanced by Tfp retraction (359, 360). Activation of host cell pro-survival signaling pathways (e.g., PI3K/MAPK) is also induced by Tfp retraction (268). Cumulatively, this perturbation of host cell signaling facilitates Ngo host cell entry and/or intracellular survival.

Neisserial *pilT*-null mutants are hyperpiliated, non-motile, defective in invasion, microcolony and biofilm formation, and non-transformable (337, 361–363). Fewer *pilT*-null mutants are recovered from inside the host cells. Since activation of Epithelial Growth Factor (EGF) signaling pathway mediates *Neisseria* invasion (364, 365), such defect in cell invasion with *pilT*-null mutant might be due to the failure in activating (phosphorylating) the EGF receptor. Hockenberry et al. showed that *pilT*-null mutant infected cells have less phosphorylated EGFR, produce and shed less HB-EGF compared to WT infected cells infection (269). These results indicate that Tfp

retraction is required for EGFR activation and cell invasion. Proteolytic cleavage of CD46, a type I integral membrane protein, is stimulated by pilus retraction. CD46 cleavage is greatly reduced in cells infected with *pilT*-null mutants compared to those infected with WT Ngo (287, 366). This leads to reduced Ca^{2+} fluxes and PI3k/MAPK signaling pathways activation. Tfp retraction also downregulates innate immune responses by controlling the production of proinflammatory ctyokines (268, 270). Without proper pilus retraction, epithelial cells produce more proinflammatory cytokines (IL-6, MCP-1, IL-1B, and IFN-γ) which can lead to both innate and adaptive immune responses. Therefore, proper Tfp retraction maintains functional *Neisseria*-*Neisseria* and *Neisseria*-host cell interactions.

The Neisseria Polysaccharide Capsule

Many Gram-negative and Gram-positive bacteria produce a polysaccharide capsule (369–372). The negatively-charged capsular polysaccharides form a highly hydrated capsule layer that envelops bacterial cells. Due to their surface exposure, capsules are usually the first bacterial structure to interact with the host immune system. One of the roles of capsule is to protect bacteria from desiccation during aerosol transmission. Capsule also protects invasive bacteria in the bloodstream by evading phagocytosis by macrophages and preventing complement-mediated killing and cationic peptide killing, as well as escaping antibody recognition through antigenic variation and immune mimicry (371–380).

Nme, but not Ngo, has the ability to produce a capsule. Nme isolated from the nasopharynx of meningococcal carriers may be capsulated or non-capsulated. However, all isolates from blood and cerebrospinal fluid express capsule (381). Capsule helps Nme to survive in the host by resisting complement-mediated and antimicrobial peptides killing as well as opsonic and non-opsonic phagocytosis (379, 382, 383). In Nme, capsular genes are clustered in a single

chromosomal locus (384). This capsule biosynthesis locus is divided into three regions: Region A, B, and C (Fig1.1.9A). Region A encodes enzymes for biosynthesis and polymerization of polysaccharides in the cytoplasm. Region B and C contain genes for capsule translocation/transportation from the cytoplasm to the cell surface. Based on structural differences in capsule polysaccharides, Nme can be divided into at least 13 serogroups (A,B,C,D,29E,H,I,K,L,W135, X,Y, and Z) (386). Capsules of serogroups B, C, W, and Y contain sialic acid while the capsule of serogroup A is non-sialylated. Approximately, 90% of meningococci that cause invasive disease produce capsules belonging to serogroup A, B, C, W, X, or Y (378).

Capsular genes organization and regulation

The Nme capsule biosynthesis operons are transcribed divergently from promoters located between the capsule synthesis locus (Region A) and capsule transport locus (Region C) (Fig.1.1.9A). In Nme FAM18, the capsule biosynthesis operon contains three genes (*cssA, B* and *C*) that are highly conserved among the Nme serogroups B, C, Y, W, and X. The three enzymes encoded by these genes produce the capsular substrates. The fourth gene, *csC*, which is highly divergent among Nme serogroups, encodes the capsule polymerase that catalyzes the elongation of capsular polymers (387). Capsule polymerases also determine serogroup-specific polymer linkages. The capsule transport operon (Region C) is composed of four polycistronic genes, *ctr*A-D. They encode proteins that transport capsules across the inner and outer membrane (Fig. 1.1.9B) (386). CtrA and CtrB form an export channel that spans the inner and outer membrane. CtrC-D are homologous to the ATP-dependent transporters. Finally, CtrE and CtrF in Region B are responsible for lipid modification at the reducing end of the polymers and capsule translocation to the cell surface (387).

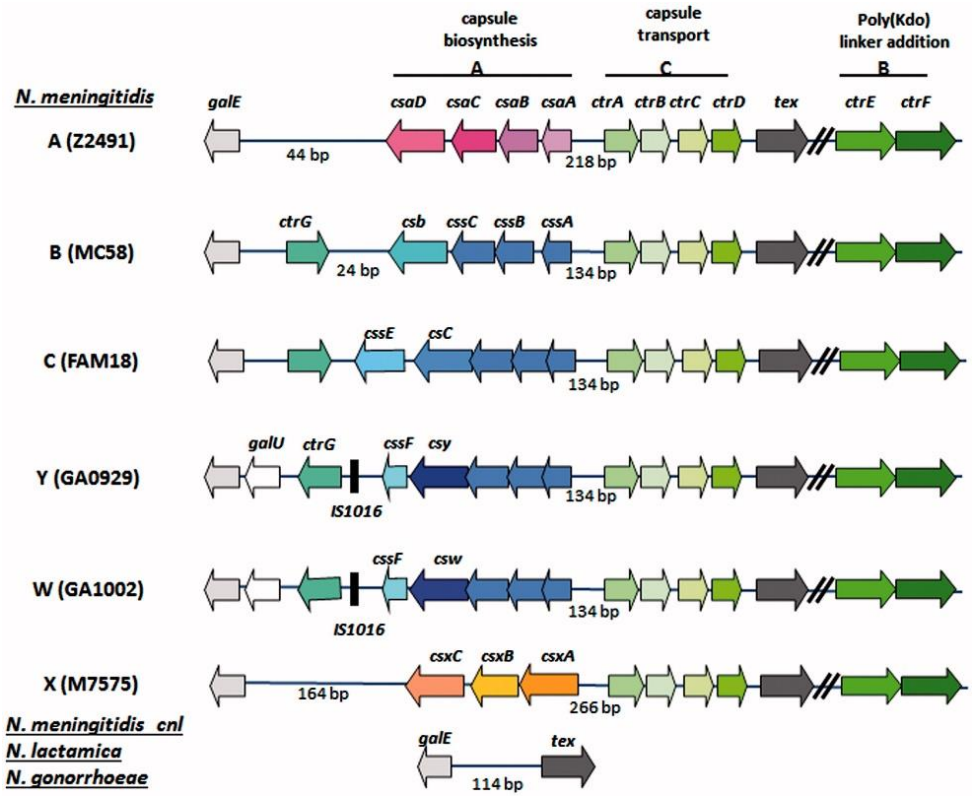

Fig. 1.1.9A Genetic organization of the capsule loci of Nme serogroups A, B, C, Y, W, and X (388)**.** Within the *cps* locus, there are 3 gene clusters. They are capsule synthesis (Region A), capsule transport (Region C), and translocation (Region B). They are all required for the production of capsule in Nme. Enzymes encoded by *cssA-C* synthesize CMP (cytidine-5'-monophosphate)-sialic acid substrates used for elongation of capsular polymers. The capsule polymerase is encoded by the fourth gene of the *css* operon, and this gene varies in nucleotide sequence from serogroup to serogroup. The capsule polymerases determine serogroup-specific polymer linkages. *ctrA-D*, encode proteins that form the channel spanning between the inner and outer member and facilitated the transport of CPS across the membrane. Meningococcal CtrE and CtrF encoded in region B were shown to be the β-Kdo transferases. They also enable the proper translocation of completely assembled capsular polysaccharides. Instead of the capsule biosynthesis locus, the capsule null Nme, *N. lactamica*, and Ngo contain the capsule null locus which comprises of the 114bp intergenic region, *galE* and *tex* genes. [Reproduced from ref (388)]

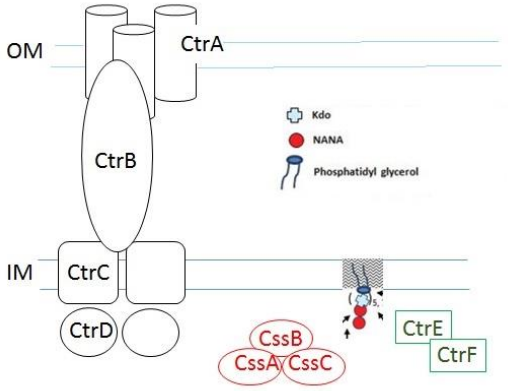

Fig. 1.1.9B Hypothetical model of capsule assembly and translocation using Nme serogroup B capsule as an example (388)**.** NANA, sialic acid; Kdo, 3-deoxy-D-manno-octulosonic acid; IM, inner membrane; OM, outer membrane. [Reproduced from ref (388)]

As mentioned, capsule expression is regulated in Nme. Nme has developed multiple strategies to regulate the expression of capsule in order to adapt to different environmental stimulates/ colonization niches. Meningococcal capsule expression is known to be affected by three events (Fig.1.2.0): 1) On-OFF phase variation of capsule biosynthesis genes, 2) alterations in capsular polysaccharides structure, and 3) regulation of the amount of capsule expressed at the bacterial surface. Capsule expression is regulated differently among various meningococcal serogroups. For example, the phase variation of capsule caused by slipped strand mispairing is observed only in serogroup B. Insertion (IS) element affecting the expression of *css*A is found in both serogroups B and C. However, some regulatory mechanisms, including, transcriptional regulation of the divergent capsule promoter, a two-component regulatory system, an IS element insertion into the untranslated region of *css*A, and post-transcriptional RNA thermoregulation of *css*A translation, can all occur in multiple serogroups.

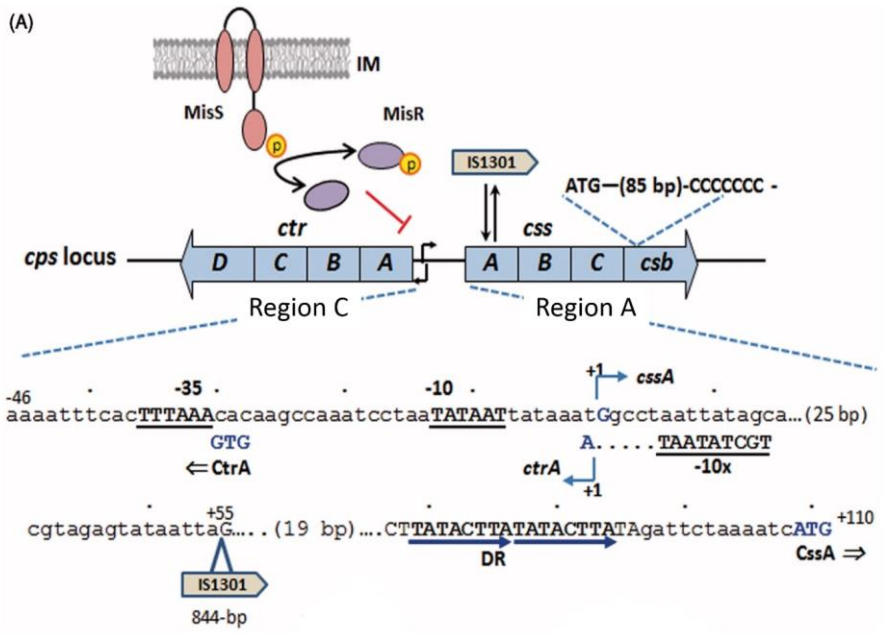

Fig. 1.2.0. Regulatory mechanisms controlling capsule expression (388)**.** Schematics of promoter region between Region A and C of the serogroup B *cps* locus are shown with the partial intergenic sequence displayed below. The transcriptional start site is marked as +1 with the nucleotides G (*css*), A (*ctr*) and the start codons of CssA and CtrA showed in blue and in capital letters. The sigma recognition sites are underlined and in bold. The IS1301 insertion within UTR occurred at the +55 position. The 8-bp direct repeat moiety served as RNA thermosensor is marked by blue solid arrows. The MisR/MisS two-component system mediated regulation, the reversible IS1301 insertion/excision event within *cssA* and the slipped strand mispairing poly(C) track are also indicated. [Modified from ref (388)]

The promoter region between Region A and C controls the transcription of capsule. This 134-bp intergenic region is highly conserved among serogroups B, C, W, and Y (388). Both Region A and C are transcribed using the housekeeping Sigma factor, sigma 70 (389). IS element, IS1301, can insert or excise within the *cps* and affect the expression of capsule (390). Around 10-50% of non-capsulated Nme recovered from epithelial cells have an IS1301 insertion within the *css*A ORF (388). IS1301 was observed in the untranslated region of *css*A from invasive isolates of serogroup C ST-11 (382). IS1301 insertion increases production of capsule by up-regulating expression of genes in Region A and C (382). Slip-strand mispairing is another common mechanism of phase variation in *Neisseria* species. It can affect capsule expression in Nme. Hammerschmidt et al. identified a stretch of seven cytidine residues within the polysialyltransferase coding sequence of non-capsulated serogroup B (391). Frameshifts within

this region can be caused by insertion or deletion of a single cytidine, resulting in premature translational termination. Ultimately, capsule expression is turned off. Bacteria also use two-component systems (TCS) to sense changes in the microenvironment and regulate gene expression accordingly. The TCS MisR/MisS system regulates capsule expression in Nme (392). The response regulator MisR binds the capsule promoter region and blocks the transcription of *cps* genes (393). Binding of this negative regulator leads to reduction in capsule production. The environmental signal(s) detected by the MisR/MisS system is unknown. Lastly, capsule expression can be post-translationally regulated. Loh et al. found an 8-bp direct repeat upstream of the *cssA* start codon (394). This direct repeat forms a hairpin structure in the RNA that acts as a thermosensor to regulate capsule biosynthesis post- translationally. At normal body temperature, the repeat forms a hairpin that blocks translation of capsule genes. At higher body temperature, such as in a fever, the hairpin is destabilized, allowing translation of the capsule biosynthesis locus. Together, controlling capsule expression could provide an adaptive advantage to meningococci during colonization.

The meningococcal capsule functions in immune evasion. The capsule protects Nme from being recognized/killed by the immune system, mostly through three different strategies: molecular mimicry, preventing phagocytosis, and inhibiting killing by antimicrobial peptides and complement pathways. The serogroup B capsular polysaccharides are structurally identical to the human neural cell-adhesion molecule that is essential for the central and peripheral nervous systems (397). Serogroup B is therefore poorly immunogenic as the immune system recognizes the B capsule as self. Capsular polysaccharides are essential for allowing cells to escape engulfment by professional phagocytes (macrophages, neutrophils, and dendritic cells) and nonphagocytic cells (383). However, the mechanism(s) allowing a capsulated Nme cell to evade phagocytosis remain to be defined. Capsules also inhibit antimicrobial peptide killing by preventing the deposition of cationic peptides onto the bacterial cell surface, probably through

electrostatic hindrance. The minimal inhibitory concentration of LL-37, a cationic antimicrobial peptide, is almost ten-times lower in the capsule-minus mutant than WT (379), suggesting that the capsule protects the bacterial cell from being killed.

Meningococci induce a strong inflammatory response and activate the complement pathways within the bloodstream. There are three main complement pathways: the classical pathway (CP), the lectin pathway, and the alternative pathway (AP). Patients with complement deficiencies are highly susceptible to Nme infection (380, 396, 397). On the other hand, capsule mutants are highly sensitive to serum killing, suggesting that capsules are essential for meningococcal survival in the blood. For example, the sialic acid capsules of serogroups B and C inhibit AP activation by limiting C3 deposition on the bacterial membrane. Capsules of serogroups B, C, W, and Y inhibit CP by inducing less C4b deposition (398). Together, capsules prevent the formation/insertion of the membrane attack complex on the bacterial surfaces. As a result, capsules inhibit complement-mediated killing and increase Nme survival inside the bloodstream.

Chapter 1.5.
Models for studying *Neisseria*-host interactions

Human and animal models are important for understanding *in vivo* bacteria-host interactions and evaluating vaccine efficacy. Due to the strict human tropism of pathogenic *Neisseria*, there is no animal model that can fully recapitulate the pathogenesis of Ngo and Nme *in vivo*.

Models of gonococcal infection

Human volunteer studies have provided a few insights into early gonococcal infection in the male urethra (401–403). Volunteers in these studies are limited to males. Subjects are inoculated with 10^6 colony forming units of Ngo into their urethra. Urine samples are collected

from the subjects periodically. The study is terminated with antibiotics on day 7 or on the day polymorphonuclear leukocytes are detected in the urine or in genital secretions. These studies show that viable Ngo can be recovered from the urine and genital secretions of some but not all infected subjects. The presence of viable Ngo from the urine sample is a strong predictor of urethral infection. Subjects with active infection develop urethritis with purulent exudates and/or dysuria between 1 and 5 days post-inoculation. The number of Ngo recovered from an infected subject does not correlate with severity of symptoms or the timing of symptoms development (401, 402). These studies have many limitations. They used a laboratory adapted Ngo strain, FA1090; they studied only male volunteers, and the infection is allowed to proceed for a limited time. Nevertheless, they identified several host interaction factors (IgA, pilin, and transferrin receptor) that are important for early colonization in the urethra of men. Among them, only the gonococcal transferrin binding protein, Tbp, is required for Ngo FA1090 infection (403). In addition, an increase in pro-inflammatory cytokines IL-8, IL-6, TNFa, and IL-1β is observed after 2 hours of inoculation (404).

The usefulness of human studies is limited to the early stages of gonococcal infection because infected subjects are treated with antibiotics once they show signs of infection. In addition, experimental infections can be performed only in men due to the potential complications in women (14). Therefore, there is no human study for endocervical infection. Even though there are monkey species that Ngo can colonize and infect, the mucosal infection observed in the urethra is not identical to human. In addition, not all human isolates of Ngo are virulent for chimpanzees (405, 406). Due to the ethical concerns, high cost, and difficulties in handling, the use of primates for *in vivo* studies is impossible.

Many efforts have been put into developing a small animal model which can mimic the early stages of gonococcal infection. Rabbits, rats, guinea pigs, chicken embryos, and laboratory

mice have been used for studying *Neisseria*-host interaction (407). Of these species, the laboratory mouse is one of the more adaptable animals in terms of methods for inducing infection. The variety and availability of mutant mice as well as their sequenced genomic data further support using laboratory mouse as a surrogate host for Ngo.

Female BALB/c mice support Ngo colonization for 10-12 days, provided they are pretreated with 17β-estradiol and antibiotics (408). Hormonal treatment extends estrus during the reproductive cycle. Antibiotics help to suppress the overgrowth of microflora due to the presence of additional estradiol. Infected mice recapitulate many early aspects of human vaginal and cervical infection, including Ngo attachment to epithelial cells and neutrophils, production of pro-inflammatory cytokines (IL-6, TNF-α, KC, and MIP-2), and influx of neutrophils to the site of infection (408). In contrast to human cervical gonococcal infection, mice naturally clear the infection within 7-10 days, and asymptomatic persistent colonization has not observed in this animal (409). The effectiveness of the mouse model is limited by host restriction. Gonococcal host interaction factors bind specifically to human variants of receptors and acquire iron only from human transferrin and lactoferrin. Mice do not express CD46/CEACAMs/human transferrin which are essential for Ngo infection. Thus the interactions between Ngo and mouse cervical cells do not fully represent natural gonococcal infection. These restrictions are eased by transgenic mice that express human CD46/CEACAMs/human transferrin/ human factor H/ C4-binding protein (410–414). The use of transgenic mice will improve the robustness of the mouse model of *Neisseria* infection.

Models of meningococcal infection

Infant Swiss CD1 mice have been used for modeling meningococcal disease (415–417). In these models, a high inoculum and supplementation of mice with human iron sources are required to establish meningococcal colonization (418). In addition, mice over 10 days old no

longer respond to Nme infection (416, 419). Yi *et al.* developed a mouse model of meningococcal colonization using outbred female Swiss Webster adult mice and daily iron injections (420). Intranasal local priming with iron dextran on day 0 is necessary to establish colonization on day 1 after inoculation. Around 42% of the mice inoculated with 10^7 CFU remain colonized with meningococci after 13 days and no viable meningococci is detected in the lung. Antibiotic treatment in the nasopharyngeal cavity before inoculation does not affect colonization frequency. Elevated level of mouse IgA is detected from colonized mice, suggests that there is active mucosal immune responses. However, they did not describe any clinical signs from mice colonized with meningococci. Nonetheless, this mouse model will allow investigators to study the early pathogenesis of meningococcal infection.

Using human CEACAM1 transgenic mice, Johswich et al. showed that the expression of CEACAM1 is necessary and sufficient to establish intranasal meningococcal infection (421). This mouse model allows colonization of Opa expressing Nme up to 10 days without causing invasive disease. Depleting polymorphonuclear cells and complement cascades prolonged meningococcal colonization up to 14 days. The CEACAM1 model also shows that nasal colonization of Nme induced sterilizing immunity against closely related strains. This model is useful for studying immune responses caused by a natural route of infection. The CEACAM1 mouse model is an asymptomatic colonization model.

Conversely, damage of epithelial tissues was observed in the human CD46 transgenic mouse model (412). Thinning of olfactory epithelium was noticed at 3 days post-intranasal inoculation. Nme disseminated to the basal membrane, lamina propria, cribriform plate, and the olfactory bulb of CD46 transgenic mice. Nme also crosses the BBB after intraperitoneal and intranasal infection without addition of iron resources (412, 422). Viable meningococci from the cerebrospinal fluid can be detected after they cross the BBB. However, this CD46 transgenic

mouse model still requires large infectious inocula (10^8 to 10^9 CFU) in order to detect bacteria in the cerebrospinal fluid.

Lastly, human transferrin transgenic mice are also used to study meningococcal disease (410). Six week old female C57Bl6/SJLJ human transferrin transgenic mice were intraperitoneal injected with 10^6 CFU of Nme. Bacteremia and bacterial growth in the blood were observed after 2 hours of injection. Higher level of IL-6 in the blood was observed after 6 hours of infection. In addition, inflammatory lesions and infiltration of polymorphonuclear cells were found in the brain samples of infected mice after 24 hours of infection. These data suggest that there are active immune responses toward meningococcal infection in this mouse model. However, this mouse model does not support long term colonization since they did not detect viable meningococci after 48 hours of injection. Therefore, this mouse model is used to study the acute meningococcal pathogenesis and immunogenicity factors.

Developing a natural animal model for studying Neisseria asymptomatic colonization

So far all animal models developed for studying Neisseria-host interactions are heterologous systems that involve surgically and hormonally manipulating the animals, and administering antibiotics. Moreover, many of these models are focused on disease, and not persistent asymptomatic colonization, which is a major attribute of *Neisseria* spp.

One approach to overcome these restrictions is to pair animal *Neisseria* with their natural hosts. Commensal *Neisseria* are abundant in the upper respiratory tract of rhesus macaques (RMs). Weyand et al. isolated numerous *Neisseria* AP206, AP678, and AP312 from RMs (423). Through spontaneous mutation, they generated rifampicin or streptomycin resistant strains from AP206, AP678, and AP312. These RM *Neisseria* derivatives, as well as the well-defined RM Neisseria, *N. macacae*, encode many host interaction factors found in pathogenic *Neisseria*

(described above). Weyand et al. paired AP206 and AP312 with RM to study *Neisseria*-host interactions (423). Two RMs were pre-treated with enrofloxacin to remove any indigenous *Neisseria* microbiota. Derivatives of *N. macacae* AP206 and AP312 were orally and nasally inoculated into two RMs (Fig 1.2.1). They showed that *N. macacae* can recolonize RM after laboratory passage and horizontal transmission of *N. macacae* between cage mates was observed after 14 days post-pairing. The presence of dual antibiotic resistance strains among cage mates demonstrates that horizontal gene transfer happens *in vivo.* Importantly, strains transmitted between cage mates were isolated from the uninoculated distal nasal cavity. This migration pattern is similar to the ascending gonococcal infections in the upper female reproductive tract in humans and to the meningococcal migration along olfactory nerves. The *N. macacae* macaque colonization model circumvents host restrictions. It allows the study of neisserial asymptomatic colonization and persistence, transmission, horizontal gene transfer, and molecular requirements for colonization. Since animal *Neisseria* share many host interaction factors with pathogenic *Neisseria*, observations made using this pairing strategy might translate to mechanisms of colonization and infection in humans.

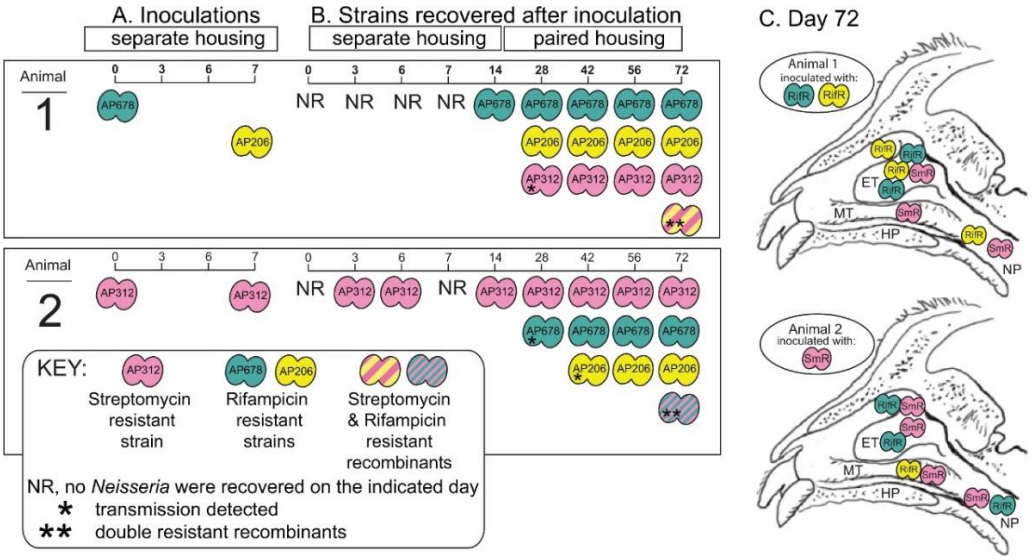

Fig. 1.2.1. Rhesus macaque *Neisseria* studies reveal colonization, transmission, persistence and *in vivo* horizontal gene transfer (197, 423)**.** (A) Inoculations on days 0 and

7. (B) Recovered bacteria days 0 to 72. (C) Nasal cavity sites where transmission was detected. Abbreviations in panel C: ET, ethmoturbinate; HP, hard palate; MT, maxilloturbinate; NP, nasopharynx; RifR, Rifampicin resistant; SmR, streptomycin resistant. The illustration in part C is based on a figure in Harkema, Carey and Wagner (2006). [Reproduced from ref (197, 423)]

However, the use of non-human primate in biomedical research has become increasingly controversial. Besides the ethical and logistics issues, the cost of conducting non-human primate studies, in terms of labor and animal care, has been drastically increasing. One of the factors that contributed to the cost induction is the increasing resistance among airlines to transport live non-humane primates (424). Therefore, there is an urgent need to develop small animal models to study *Neisseria*-host interactions in a natural setting.

Chapter 2 - Manuscript 1

A mouse model for *Neisseria* colonization

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Abstract

Commensals are important for the proper functioning of multicellular organisms. How a commensal establishes persistent colonization of its host is little understood. Studies of this aspect of microbe-host interactions are impeded by the absence of an animal model. We have developed a natural small animal model for identifying host and commensal determinants of colonization - and of the elusive process of persistence. Our system couples a commensal bacterium of wild mice, *Neisseria musculi*, with the laboratory mouse. The pairing of a mouse commensal with its natural host circumvents issues of host restriction. Studies are performed in the absence of antibiotics, hormones, invasive procedures or genetic manipulation of the host. A single dose of *N. musculi*, administered orally, leads to long-term colonization of the oral cavity and gut. All mice are healthy. Susceptibility to colonization is determined by host genetics and innate immunity. On the part of *N. musculi*, colonization requires the Type IV pilus. Reagents and powerful tools are readily available for manipulating the laboratory mouse, allowing easy dissection of host determinants controlling colonization resistance. *N. musculi* is genetically related to human-dwelling commensal and pathogenic *Neisseria* and encodes host interaction factors and vaccine antigens of pathogenic *Neisseria*. Our system provides a natural approach for studying *Neisseria*-host interactions, and is potentially useful for vaccine efficacy studies.

Introduction

Commensals (a.k.a. microbiota) play a critical role in the physiology of multicellular organisms. They are required for the homeostasis of many bodily processes, and they participate in gut and immune system development and prevent pathogen colonization. Perturbations in these microbial communities are strongly linked to obesity, inflammatory bowel disease, diabetes and autoimmunity (142, 173, 425–429).

The mechanisms underlying host and commensal determinants of persistent colonization are little understood. The majority of commensals cannot be cultured or manipulated genetically (430–432). Because of host restriction barriers, few animal models provide a natural setting for probing commensal-host interactions. The *Neisseria*, a genus of Gram-negative *β*-Proteobacteria, provides an opportunity to develop a natural small animal model for this purpose.

The *Neisseria* genus contains a large number of genetically related species (241). The vast majority of these are commensals of hosts ranging from rodents, canids and bovines to nonhuman primates and man (186, 299, 304, 305, 433, 434). *Neisseria gonorrhoeae* (Ngo) and *Neisseria meningitidis* (Nme) are the only two species that cause disease. These pathogens, which only infect man, also behave like a commensal in that they have a tendency to colonize asymptomatically (435–437). Commensal *Neisseria* are little studied, and there are no small animal models for colonization. Several mouse models have been developed for pathogenic *Neisseria* infection; but due to the strict tropism of Ngo and Nme for humans, they are necessarily heterologous systems that require invasive procedures, antibiotics, hormones, direct administration of human homologs proteins such as transferrin, and/or the use of transgenes expressing human proteins (409, 412, 421, 438).

We recently isolated a new species of commensal *Neisseria*, *Neisseria musculi* (Nmus), from the oral cavity of healthy wild mice (305). Nmus is easily cultured and manipulated *in vitro*, and is genetically related to other *Neisseria*. Towards developing a small animal model for *Neisseria* colonization, we determined whether Nmus could be paired with inbred laboratory mice. We report that Nmus colonizes the oral cavity and gut of laboratory mice for at least 1 year without causing disease. Long-term colonization is achieved with a single oral dose. Using this model, we discovered that permissiveness to Nmus colonization is strongly influenced by host genetics and by innate, but not adaptive, immunity. On the part of Nmus, colonization requires its Type IV pilus (Tfp). Finally, we present evidence that Nmus encodes homologs of host interaction factors and vaccine antigens of pathogenic *Neisseria*, and that it expresses one of the vaccine targets, the capsular polysaccharide. We discuss the power of our natural small animal model for broadening our knowledge of commensal and pathogenic *Neisseria* biology, and of host components that restrict/permit colonization.

Results

N. musculi colonizes the oral cavity and gut of mice in a mouse strain specific manner.

We isolated Nmus from the oral cavity of a wild mouse, *Mus musculus domesticus* (305). Our repeated attempts to culture *Neisseria* from the oral cavity of inbred mice from Jackson Labs and Taconic were unsuccessful. Since inbred laboratory mice do not harbor *Neisseria*, this provided an opportunity to test the susceptibility of these animals to Nmus colonization.

The Collaborative Cross (CC) is a new powerful tool in mouse genetics that allows the linkage of alleles with phenotypic traits (439). We tested Nmus on selected CC founder strains. These strains include 5 conventional, widely used inbred strains, and 3 wild-derived inbred strains from distinct *Mus musculus* subspecies (Table 2.1). The wild derived strains are CAST, from wild mice trapped in Thailand belonging to a distinct subspecies, *Mus musculus castaneous*; PWK, which was trapped in the Czech Republic and belonging to subspecies *Mus musculus musculus*; and WSB/EiJ (WSB), which was trapped in Maryland, USA, and belonging to *Mus musculus domesticus*. The conventional inbred strains are chimeras with varying degrees of genetic relatedness to CAST, PWK and WSB, although their genetic origin is overwhelmingly *Mus musculus domesticus*25.

The mouse inoculation protocol is shown in Fig. S1. Prior to inoculation, the presence of *Neisseria* in these animals was determined by plating oral cavity (OC) and fecal pellet (FP) samples on selective agar. Mice have always been culture-negative. The next day, AP2365, a naturally occurring Rifampicin resistant (Rif^R) rough variant of Nmus, was gently pipetted into the OC of the animals, and Nmus counts in OC and FP were determined weekly for 3 months by plating the samples on selective agar. CAST/EiJ (CAST) and A/J mice were very susceptible to colonization (Table 2.1): the OC and FP of 35/40 (87%) of CAST mice and 26/28 (92%) of A/J mice were continuously culture-positive. C57BL/6J (B6) mice were partially resistant to

colonization (12/23; 52%). In contrast, NOD, NZO, PWK, WSB and 129S1 mice were highly resistant at the same infectious dose and route of inoculation.

Table 2.1 Susceptibility of Collaborative Cross Founder Strains to colonization by *N. musculi*.

Strain	# Colonized/Inoculated (%)[a]	*p* value[b]
CAST/EiJ	35/40 (87)	-
A/J	26/28 (92)	ns[c]
C57BL/6J	12/23 (52)	<0.006[c]
NOD/LtJ	0/4 (0)	<0.0004[c]
NZO/HILtJ	0/4 (0)	<0.0004[c]
PWK/PhJ	0/9 (0)	$<10^{5}$ [c]
WSB/EiJ	0/9 (0)	$<10^{5}$ [c]
129S1/SvImJ	0/4 (0)	<0.0004 [c]
MyD88$^{-/-}$	21/21 (100)	<0.001[4]
RAG-1$^{-/-}$	3/14 (21)	ns[d]

[a]Mice were scored for the presence of *N. musculi* in the oral cavity and fecal pellet each week for 3 months. [b]x^2 with Yates correction for small numbers and Bonferroni for multiple pairwise comparisons. [c]Compared to CAST. [d]Compared to WT BL/6. ns: not significant.

A representative colonization experiment, involving inoculation of 10 CAST mice, is shown in Fig. 2.1. Nmus colony forming units (CFUs) in the OC and FP quickly reached a plateau and remained steady thereafter, indicating the commensal had adapted to these niches and replication and turnover reached equilibrium. Generally, when Nmus was cultured from the OC, it was also recovered from the FP. Samples reisolated from colonized mice were Nmus, as judged by multilocus sequence typing of 51 ribosomal genes (rMLST) of 10 OC and 10 FP colonies recovered from CAST mice at 5 weeks post-inoculation (data not shown). All Nmus reisolated from the OC and FP had the rough colony phenotype like the inoculation strain.

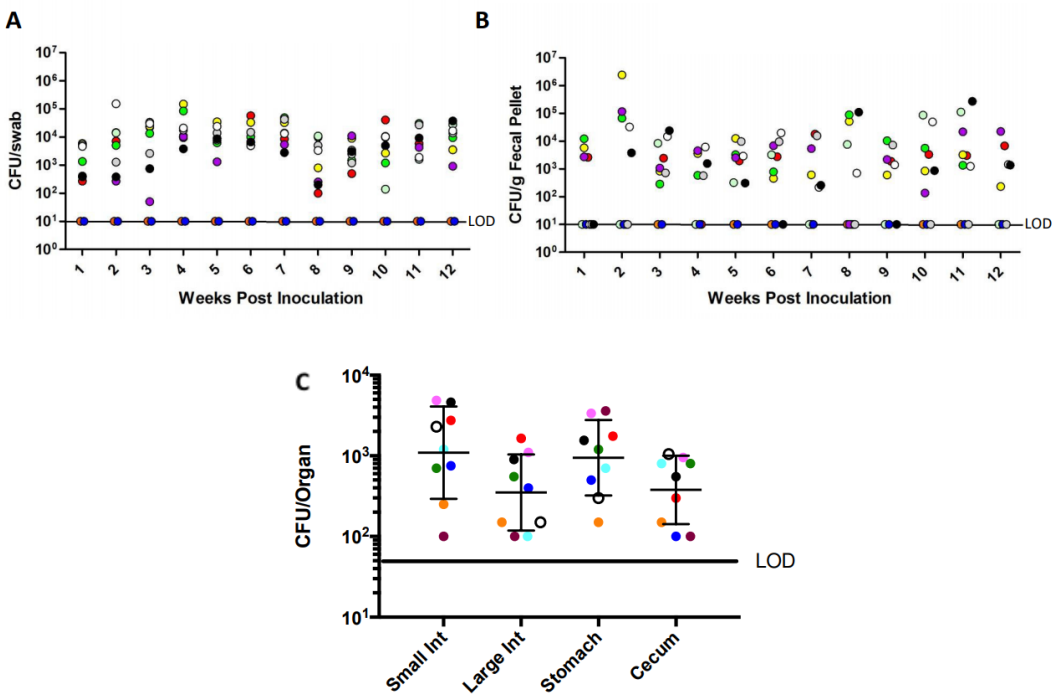


FIGURE 2.1. *N . musculi* colonizes the oral cavity (A) and gut (B) of CAST mice, and different sections of the gastrointestinal tract (C). Samples in (A) and (B) are from the same experiment; each mouse is assigned a unique color. Samples in (C) are taken from 3-month colonized CAST mice from a different experiment. Plots indicate geometric mean with geometric standard deviation. CFU: colony forming units; LOD: limit of detection.

Two colonized CAST mice were followed long-term. Nmus was continuously recovered from their OC and FP for 52 weeks (Fig. S2). Colonized B6 mice yielded similarly high Nmus counts weekly for 52 weeks (data not shown). Throughout our studies, all inoculated and uninoculated mice remained healthy: none lost weight and all maintained healthy coats and normal activity. At necropsy (performed by D. Beselson, Director, UA Animal Care Facility) the organs of the 52-week colonized mice reflected those of healthy mice.

Nmus was not cultured from the peripheral blood of 4 CAST and 4 A/J mice 4 hours or 28 days post-inoculation. Although this experiment does not address whether Nmus enters the bloodstream, the result suggests the commensal does not survive at this site.

Taken together, these results demonstrate that the susceptibility of a mouse to Nmus colonization is strongly influenced by its genetic background. In susceptible mouse strains, Nmus easily colonizes their OC and gut, and persists in these niches for lengthy periods without causing disease.

***N. musculi* colonizes the entire gastrointestinal tract of mice.**

We examined the location of Nmus in the gastrointestinal tract of 3-month colonized CAST mice. The stomach, small intestine, large intestine and cecum of necropsied animals were flushed with sterile saline to remove the luminal content, and the tissues were homogenized and plated on selective agar. Nmus was recovered from all sampled sections of the gut (Fig. 2.1C). It is impossible to sample organs from the same animal on successive days, or to determine whether Nmus populations in these organs were self-sustaining. However, the large numbers of Nmus recovered from tissue-associated gut samples long after inoculation strongly suggests the commensal was not simply in transit from the OC.

To determine whether Nmus could be horizontally transmitted, we cohoused 2 colonized CAST mice with 3 naïve CAST or B6 mice for 12 weeks. None of the uninoculated mice became colonized. To determine whether the endogenous flora influenced colonization, we cohoused 4 B6 and 4 CAST mice for 12 weeks before inoculation. This did not alter colonization susceptibility of either mouse. Moreover, CAST and B6 mice bred in-house or purchased from The Jackson Labs were always colonized at the same frequency. Although these experiments involved small numbers of mice, the evidence suggests that the preexisting flora did not play a significant role in determining colonization susceptibility; final evidence awaits fecal transplant

studies. To determine whether *in vivo* passage of Nmus would increase its colonization efficiency, we inoculated 4 naïve B6 mice with Nmus isolated from the OC of a persistently colonized CAST mouse. This *in vivo* passage did not alter Nmus colonization efficiency. Taken together, these results suggest that neither housing conditions nor the endogenous microbiota are significant roadblocks to Nmus colonization.

Innate immunity determines susceptibility to *N. musculi* colonization.
The partial resistance of B6 mice to Nmus colonization (Table 2.1) provided an opportunity to investigate the role of the immune system in determining colonization susceptibility. Nmus was assayed in two strains of immunodeficient B6 mice: B6-MyD88$^{-/-}$ mice, which lack the MyD88 adaptor that mediates signaling through many Toll Like Receptors; and B6-Rag-1$^{-/-}$ mice, which lack T and B cells and cannot mount an adaptive immune response (Table 2.1). Strikingly, MyD88$^{-/-}$ mice were exquisitely susceptible to Nmus colonization, unlike the B6 parental strain (MyD88$^{-/-}$ 21/21 mice colonized vs B6 mice 12/23, p <0.001). MyD88$^{-/-}$ mice also had higher Nmus burdens than the parental WT strain (p=0.0026; Fig. 2.2). In contrast, Rag-1$^{-/-}$ mice were no more susceptible than WT B6 mice. These results indicate that the innate, but not adaptive, immune system is a major determinant of Nmus colonization. The increased numbers of Nmus recovered from MyD88$^{-/-}$ mice, compared to WT B6, suggests that the innate response plays an ongoing role in controlling Nmus numbers.

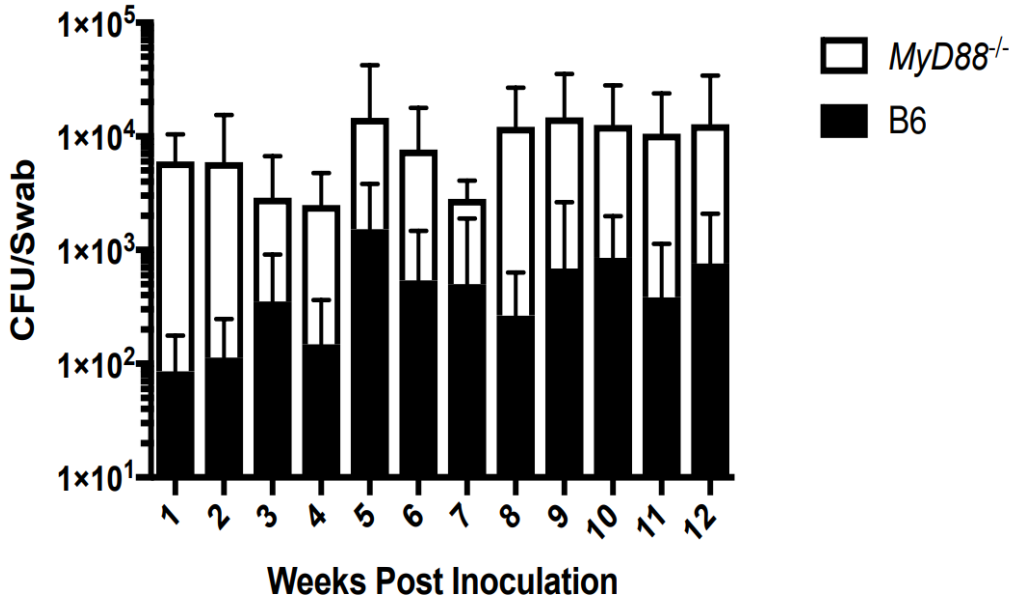


FIGURE 2..2 MyD88-/- mice have higher *N. musculi* burdens than parental B6 mice. *N. musculi* colony forming units (CFU) in oral swabs taken from B6 (black bars) and MyD88-/- (white bars) mice. n= 9-10 mice/group. Plots indicate the mean with SD. Significance was determined using Student's t-test on the average burden per strain over the lifetime of the experiment. Representative of 2 independent experiments.

***N. musculi* colonization requires the Type IV pilus.**

To test the usefulness of our model for studying commensal determinants of colonization, we focused our attention on the Type IV pilus (Tfp). All *Neisseria* species have a complete set of Tfp biogenesis genes (303, 305, 425). In the case of pathogenic *Neisseria*, Tfp is implicated to promote colonization, based on experiments using cultured human cells and a limited number of human challenge studies (317, 324, 401, 442, 443). The function of Tfp has never been tested in a natural animal model.

For this experiment, a nonpiliated mutant of Nmus, Δ*pilE*, was constructed by deleting the gene encoding the Tfp fiber subunit; a complemented strain, Δ*pilE*::*pilE*$_{WT}$-C10, was also constructed. The piliation status of Δ*pilE* and complement was validated by several methods. Unlike WT and complement, Δ*pilE* did not produce *pilE* mRNA, as judged by RT-PCR (Fig. S3). Nmus Δ*pilE*

exhibited phenotypes characteristic of nonpiliated mutants: it was defective in DNA transformation (Table S1), and attached less well to surfaces (Fig. 2.3). These results indicate Nmus Δ*pilE* does not produce the Tfp fiber. Finally, the growth of Δ*pilE* was examined. WT, Δ*pilE* and complement grew equally well (Fig. S4). The slightly lower OD_{600} of Δ*pilE* cultures was not statistically different at any time point; it likely reflects the slight tendency of Δ*pilE* cells to aggregate in liquid culture.

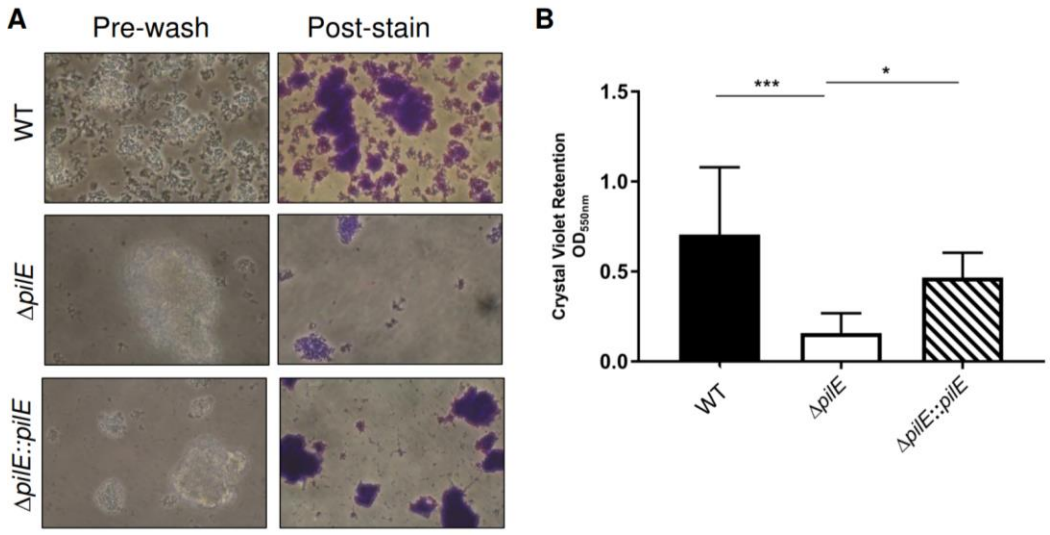


FIGURE 2.3. *N. musculi* Δ*pilE* is defective in attachment (A) and biofilm formation (B). D*pilE*::Δ*pilE*: complemented strain. Statistical analysis of (B) was performed on GraphPad Prism 7 by One-way ANOVA with Tukey's multiple comparison test. *** $p < 0.001$. * $p < 0.05$. No significant difference was detected between the WT and complemented strain.

Nmus Δ*pilE* was defective in colonizing the OC and gut of CAST and B6 mice, compared to WT and complement (Fig. 2.4, Table S3; $p<0.0001$, WT vs Δ*pilE* for both CAST and B6). The few OC and FP reisolates were Nmus, as judged by *pilE* sequencing primer (NP246F and NP246R2) and their mutated *pilE* locus was unaltered (data not shown). The complemented strain Δ*pilE*::$pilE_{WT}$-C10 colonized the OC and FP of CAST mice like WT Nmus (OC, p=0.3316; FP, p=0.9916). In B6 mice, the colonization behavior of the complemented strain did not fully revert to WT Nmus, even after taking into account the partial colonization resistance of B6 mice

(OC, p=0.0013; FP, p=0.0008). We cannot explain this behavior. During the transformation/recombination process that inserted the wt *pilE* sequence into the Δ*pilE* strain, a mutation may have occurred elsewhere in the genome that affected the colonization behavior of the complemented strain. The transformation/recombination process may have had a polar effect on a gene immediately downstream of the complemented *pilE* site. In the annotated Nmus genome, *pilE* is at the end of the contig; the identity of the downstream gene is unknown. Other explanations are also possible, but we note that this colonization behavior of the complemented strain is observed only in the B6 genetic background, and not in the CAST background. Finally, we note that in these experiments, the rough variant of Nmus (WT, ∆*pilE* and complemented strains) was used, and all reisolated Nmus exhibited the rough colony phenotype.

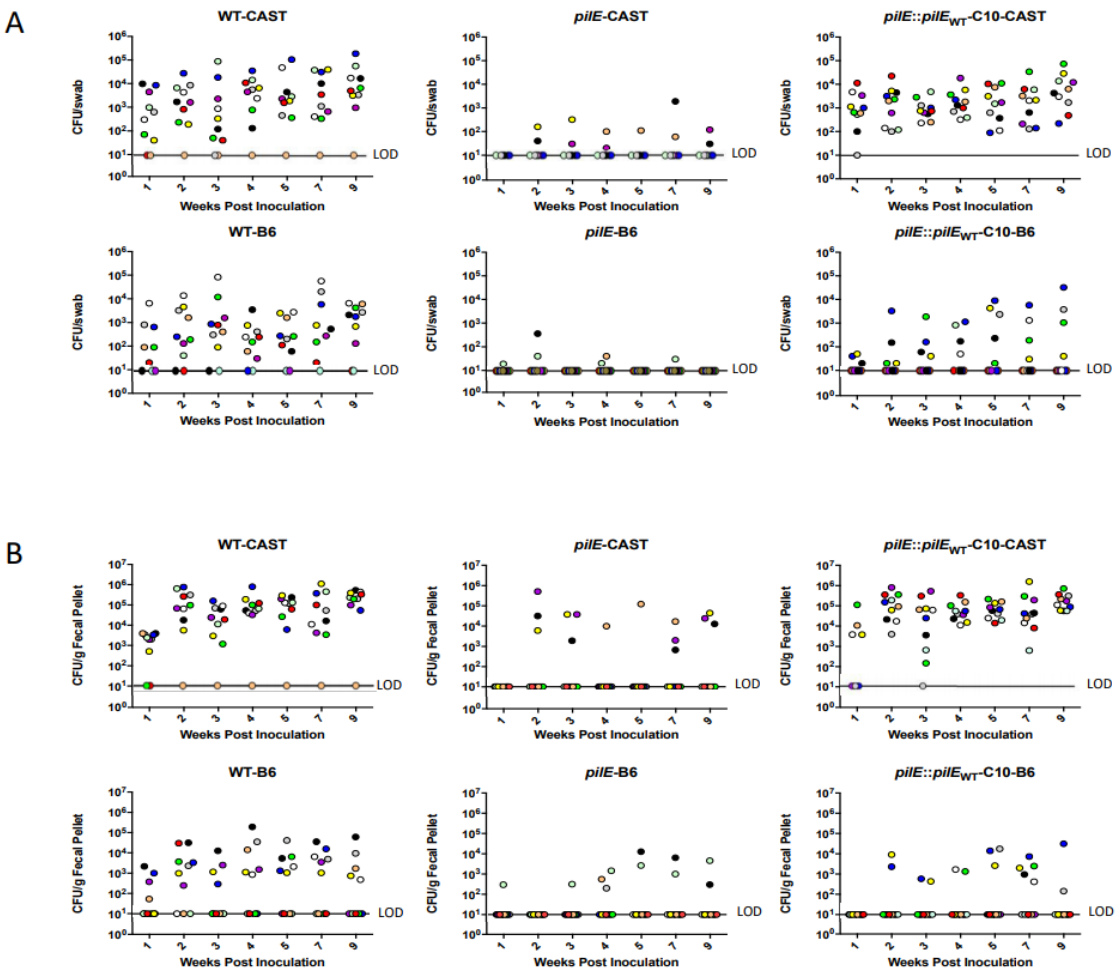


FIGURE 2.4. *N. musculi* Δ*pilE* is defective in colonizing the oral cavity (A) and gut (B) of CAST and B6 mice. p<0.0001 for both the oral swab (OC) and fecal samples (FS) of CAST and B6 (WT vs Δ*pilE*); p= ns, not significant, for OC and FS of CAST (WT vs Δ*pilE*::*pilE*$_{WT}$-C10); p=0.0013 for OC and p= 0.0008 for FS of B6 (WT vs Δ*pilE*::*pilE*$_{WT}$-C10). CFU: colony forming units; WT: wild type N. musculi; AP2365Δ*pilE*::*pilE*$_{WT}$-C10: *pilE* complemented strain. Each *N. musculi* strain was assayed in 10 mice. OC and FS from the same mouse are assigned the same color. LOD: limit of detection. Statistical differences were determined by using Mantel-Cox Order Test.

***N. musculi* encodes host interaction factors and vaccine candidates of human-dwelling *Neisseria*.**

Finally, we determined whether Nmus could be used to model human-dwelling species of *Neisseria*. To date, *Neisseria* colonization studies have focused almost exclusively on the two pathogens, Ngo and Nme. Great efforts have been made to identify host interaction factors, with the goal of identifying vaccine antigens capable of stimulating protective immune responses. Chief among these vaccine development efforts was the use of reverse vaccinology to identify

genome-derived *Neisseria* antigens (GNA) in Nme (251). Currently, similar work is conducted to identify vaccine antigens in Ngo (442, 443).

Many homologs of pathogenic *Neisseria* host interaction factors and candidate vaccine antigens, including GNAs, were found in Nmus and human-dwelling commensal *Neisseria* (Table 2.2, 2.3). Two GNAs with high identity and query coverage were GNA1220, a membrane protein of unknown function containing a stomatin-like domain; and GNA33, membrane-associated lytic transglycosylase required for cell separation (444, 445). Nme GNA1946 and Ngo ortholog NGO2139, which are methionine-binding subunits of ABC transporters, both retrieved the same Nmus ortholog with greater than 75% identity and 95% query coverage. GNA1946 and NGO2139 (MetQ) induce the production of serum bactericidal antibodies (251, 446). Nmus also has a homolog for Nme LpdA, a high molecular weight protein, P64k, which is very immunogenic and is used frequently as a carrier protein for weaker immunogens (447, 448).

Table 2.2 Putative orthologs of protective antiens encoded in the AP2031 genome.

Protein query	Query accession	Query species	Nmus		Npo		Nla		Nci		Nsu		Nor		Nmu		Nel		Nba	
			% id.[a]	% qc.[b]	% id.[a]	% qc.[b]	% id.[a]	% qc.[b]	% id.[a]	% qc.[b]	% id.[a]	% qc.[b]	% id.[a]	% qc.[b]	% id.[a]	% qc.[b]	% id.[a]	% qc.[b]	% id.[a]	% qc.[b]
LctP	CBA04244	Nme	25	95	25	95	98	100	92	100	92	100	92	100	94	100	82	99	40	42
LpdA	CAA57206	Nme	74	100	74	100	85	100	84	100	85	100	84	100	84	100	71	100	72	100
GNA1030[c]	NP_274064	Nme	48	23	29	79	95	88	90	100	74	100	86	100	91	86	65	88	62	100
GNA1220	NP_274245	Nme	81	99	81	99	93	100	93	100	34	31	82	98	84	100	75	99	78	96
GNA1946	NP_274940	Nme	77	95	77	95	88	100	85	100	79	97	82	97	82	98	80	96	67	96
GNA2091[c]	NP_275079	Nme	63	79	63	79	88	100	80	100	86	79	67	99	85	80	63	79	65	79
GNA33	NP_273099	Nme	77	85	77	85	91	100	87	100	78	100	73	99	77	100	74	85	67	94
NadA[c,d]	NP_274986	Nme	43	15	43	15	27	30	67	28	ND[e]	ND[e]	38	15	69	46	48	7	35	27
PorA P1[c,d]	NP_273150	Nme	53	97	53	97	86	100	85	100	71	98	59	98	71	100	61	99	56	99
ExbB	NP_274732	Nme	68	99	68	99	96	100	93	99	73	99	73	99	76	99	58	99	57	98
GNA992	NP_274028	Nme	48	23	48	23	91	94	39	79	62	23	64	14	65	29	63	67	64	18
GNA2001	NP_274993	Nme	68	65	68	65	65	100	60	100	60	97	56	100	56	97	79	98	76	57
GNA1870 (fHbp)[c,d]	NP_274866	Nme	30	38	30	38	34	61	91	100	39	83	31	62	38	82	28	89	29	64
NspA	NP_273705	Nme	42	86	42	86	84	87	48	86	45	86	29	36	40	43	47	86	45	86
TBP2	CAA55541	Nme	25	13	25	13	74	100	35	98	26	8	28	17	31	12	26	10	28	47
TbpA	AAF81744	Nme	30	54	30	54	94	100	75	100	26	58	28	61	30	71	31	67	32	54
GNA2132 (NHBA)[c,d]	NP_275117	Nme	ND[e]	ND[e]	ND[e]	ND[e]	75	100	40	40	32	25	34	9	35	51	32	29	28	34
GNA1162	NP_274189	Nme	33	40	33	40	95	100	88	100	48	13	35	14	54	99	48	35	52	10
PilC1	YP_207232	Ngo	36	68	36	68	46	100	40	89	40	65	36	67	38	66	24	65	26	31
PilQ	YP_207267	Ngo	56	100	56	100	81	100	77	100	61	100	57	99	58	100	49	97	49	97
AniA	YP_208345	Ngo	79	79	79	79	93	79	92	79	87	79	80	79	89	79	80	79	78	79
OpaD	YP_208563	Ngo	32	69	32	69	66	100	29	86	32	85	30	19	23	16	32	89	29	85
OpcA	CAB45007	Ngo	28	16	28	16	40	64	41	83	25	76	20	76	31	15	26	82	23	38
LptD	YP_208748	Ngo	60	98	60	98	91	98	81	100	63	98	61	98	61	98	55	100	55	92
BamA	YP_208831	Ngo	75	100	75	100	90	100	94	100	82	100	80	100	82	100	71	100	69	100
TamA	YP_208979	Ngo	68	90	68	90	95	100	83	98	77	89	74	88	75	88	63	89	62	89
NGO2054	YP_209073	Ngo	64	76	64	76	94	100	78	100	62	100	57	81	60	57	54	100	62	74
NGO2139 (MetQ)	YP_209148	Ngo	78	95	78	95	90	100	78	100	82	97	86	97	85	98	82	96	77	82

a. %id., % Identity. Yellow cells indicate >50% identity.

b. %qc., % Query coverage. Green cells indicate >75% query coverage.

c. components of rMenB-OMV vaccine, Novartis

d. components of the Bexsero and Trumemba vaccines by GlaxoSmithKline/Novartis and Pfizer.

e. ND, significant similarity not detected.

We also conducted BLAST searches using three β-barrel-containing outer membrane proteins as queries: Nme NspA, a factor H ligand, and Ngo adhesins OpaD and OpcA. (The Nme OpcA ortholog is a lectin capable of interacting with vitronectin (449, 450). NspA and OpaD retrieved the same Nmus homolog (NspA: 42% identity, 86% query coverage; OpaD: 32% identity, 69% query coverage). The bulk of shared identity in these proteins localized to the β-barrel strands. OpcA did not have a significant hit.

The capsular polysaccharide is a target of several meningococcal vaccines (451). BLAST searches using Nme capsule proteins showed that Nmus have genes for capsule biosynthesis, transport and translocation proteins (Table 2.3). With the exception of the putative capsule

polymerase (CssC), all the capsule-related proteins have high sequence homology with their Nme orthologs (≥ 56% identity and ≥ 75% query coverage).

Table 2.3. Orthologs of *N. meningitidis* capsule synthesis, transport, and translocation proteins, and the presence of selected capsule transcripts, in *N. musculi*.

Protein query	Query accession	Query species	MAXIMUM IDENTITY (%)	QUERY COVERAGE (%)	Genome Annotation	mRNA
CssA	WP_002233375.1	Nme	72	96	UDP-N-acetylglucosamine-2-epimerase	+
CssB	WP_002233374.1	Nme	87	99	UDP-N-acetyl-D-mannosamine dehydrogenase	ND[a]
CssC	CCP19843.1	Nme	28	13	Capsule polymerase	ND[a]
CtrA	NP_273135	Nme	56	93	Capsule transport complex	+
CtrB	NP_273136	Nme	67	91	Capsule transport complex	ND[a]
CtrC	NP_273137	Nme	73	100	Capsule transport complex	ND[a]
CtrD	NP_273138	Nme	84	98	Capsule transport complex	ND[a]
CtrE	NP_273145	Nme	60	93	Capsule translocation	+
CtrF	NP_273146	Nme	61	99	Capsule translocation	+

Query coverage >75% is boxed in green. Sequence identify >50% is boxed in orange. Nme, *Neisseria meningitidis*. a. ND, Not determined.

N. musculi expresses a polysaccharide capsule.

We determined whether Nmus produces a capsule, using two biochemical tests, india ink and alcian blue staining, that are widely used to detect capsulated organisms (452). After india ink treatment, Nmus cells (smooth and rough variants) were surrounded by a clear halo against a dark background, which is indicative of capsulated organisms (Fig. 2.5A). India Ink stained cells of capsulated Nme 8013 similarly, but not theunencapsulated Nme FAM2 (Fig. 2.5A). To further confirm that the refractile zone of these cells corresponds to the capsular polysaccharide, we stained extracts from these cells with alcian blue (453). Results indicate that a high molecular weight alcian blue-reactive smear is present in the capsulated Nme 8013 and Nmus AP2365 smooth and rough variants, but not in the unencapsulated Nme FAM2 (Fig. 2.5B).

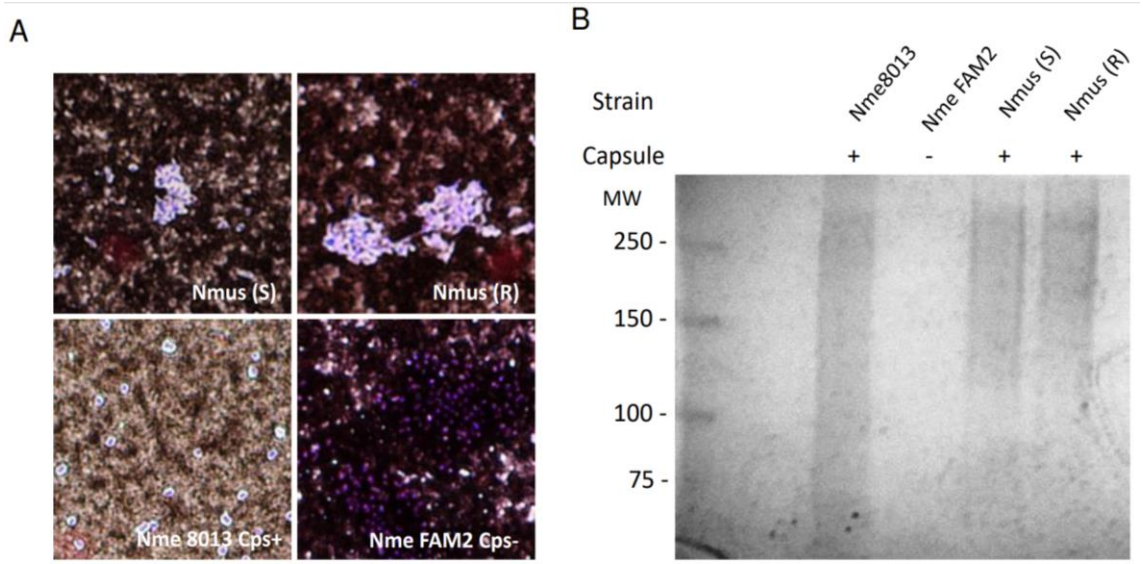


FIGURE 2.5. *N. musculi* produces a capsule. (A) India ink staining of *N. musculi* smooth (S) and rough (R) strains, capsulated (Cps+) N. meningitidis strain 8013 and unencapsulated (Cps-) strain FAM2. Cells were counterstained with crystal violet. (B) Alcian blue staining of lysates of these strains separated by 6% SDS-PAGE.

Finally, we determined whether capsule genes are transcribed in Nmus, by means of RT-PCR of selected capsule biosynthesis, transport and translocation genes Fig. S5, Table S2) Transcripts for *ccsA*, *ctrA*, *ctrE* and *ctrF* are detected using this method (Tab le 3). Taken together, these results indicate that the capsule genes in Nmus are expressed.

Discussion

We have developed a genetically tractable small animal model for identifying host and microbial determinants of colonization and persistence. The system pairs the laboratory mouse with a commensal of wild mice, *Neisseria musculi,* which is closely related to human-dwelling species of *Neisseria* (305) (Table 2.2, 2.3; see also below). The protocol does not require antibiotics, hormones, invasive procedures or genetic manipulation of the animal. A single oral dose of *N. musculi* results in long-lasting colonization of the oral cavity and gut of the mouse (Fig. S2). All animals are healthy throughout the study.

Using this model, we showed that host genetics and innate immunity strongly control susceptibility to colonization by *N. musculi* (Table 2.1). Reagents and tools are readily available for the laboratory mouse, allowing easy dissection of host components that restrict/permit *N. musculi* colonization. The mice in this study are founder strains of the Collaborative Cross, a new powerful tool that can be used to link genetic traits with biological phenotypes. As susceptibility of these strains to *N. musculi* colonization ranges from sensitive to highly resistant, the Collaborative Cross will allow us to identify host alleles that determine colonization resistance.

We also used the model to examine the role of the *N. musculi* Type IV pilus in colonization. All *Neisseria* and many bacteria belonging to other genera express Tfp. In the case of pathogens *N. meningitidis* and *N. gonorrhoeae*, cell culture experiments and a small number of human challenge studies strongly implicate a role for the Tfp in colonization (315, 322, 399, 440, 441). Here, we corroborate these findings, providing *in vivo* proof that the *N. musculi* Tfp is an important colonization determinant (Fig. 2.4). *In vitro* studies have identified other, more subtle activities of the *N. gonorrhoeae* Tfp, including the reprogramming of the host transcriptional profile and activation of immune signaling pathways (267, 268). Our model provides the first

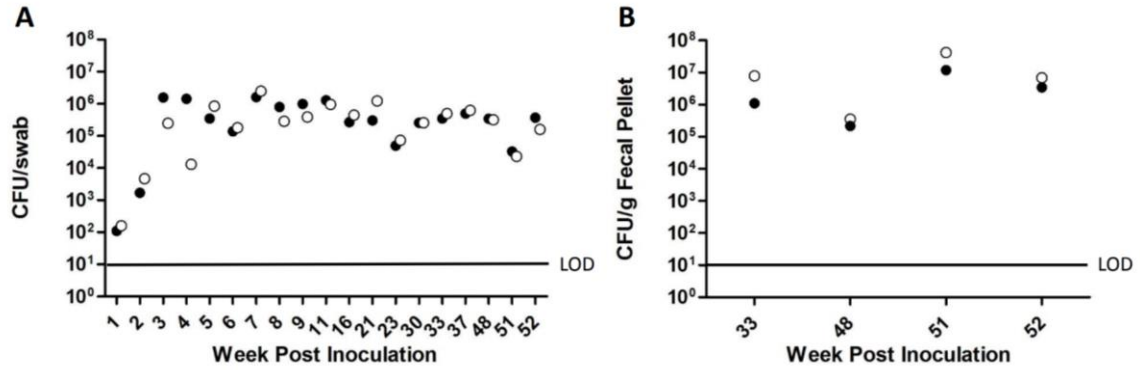
opportunity to identify the *in vivo* endpoints of these activities, as well as the function of host interaction factors held in common between *N. musculi* and human commensal *Neisseria* (Table 2.2, 2.3).

The animal models currently in use to study *N. gonorrhoeae* and *N. meningitidis* are heterologous systems that pair a mouse with a human-specific pathogen (409, 421, 438). These approaches limit the ability to utilize the full breadth of mouse genetics techniques available for studying host determinants of persistent colonization. Although *N. musculi* does not cause disease, it does encode many pathogenic *Neisseria* host interaction factors and candidate vaccine antigens (TABLE 2.2, 2.3). Indeed, *N. musculi* expresses one of these candidate vaccine antigens, the capsular polysaccharide (Fig. 2.5). Our model will be a useful tool for characterizing the *in vivo* functions of these host interaction factors, and is potentially useful for evaluating vaccine candidates for the pathogens.

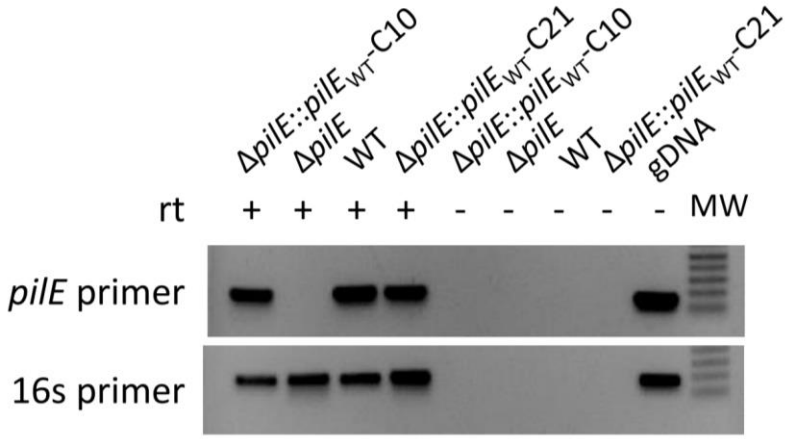
That *N. musculi* colonizes the gastrointestinal tract of laboratory mice should not be a surprise. *N. musculi* colonizes the oral cavity of wild mice and is detected in their gut (305), and *Neisseria* species have been detected in animal feces (186). Human niches for *Neisseria* are generally assumed to be the nasopharynx (*N. meningitidis*, *N. gonorrhoeae* and commensal species), genital tract (*N. gonorrhoeae* and occasionally *N. meningitidis*) and rectum (*N. gonorrhoeae*), but to our knowledge studies have not been done to determine the presence of *Neisseria* in the human gut, either by direct culture or molecular speciation. The large numbers of *N. musculi* recovered from the oral cavity and gut of mice over a long period indicates this organism is able to adapt to a variety of environments within the animal. Taken together, these observations suggest that *Neisseria* is a more successful and adaptable organism than had previously been suspected.

There is currently a great interest in the microbiome. In spite of the large number of papers on the subject, little is known about how changes in the microbiome are brought about. Conspicuous by its absence are data concerning the acquisition of a new commensal in the presence of an existing microbiota or after antibiotics treatment. Our model opens the door to these investigations.

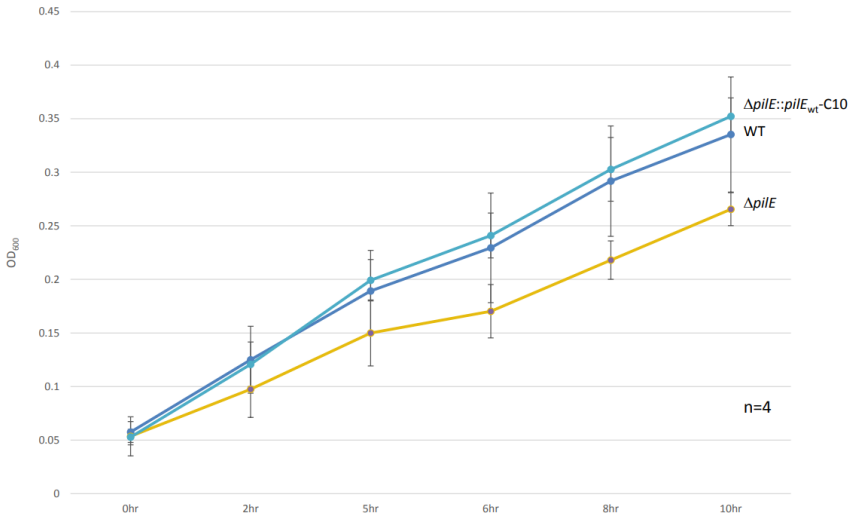
Supplemental Figures and Tables



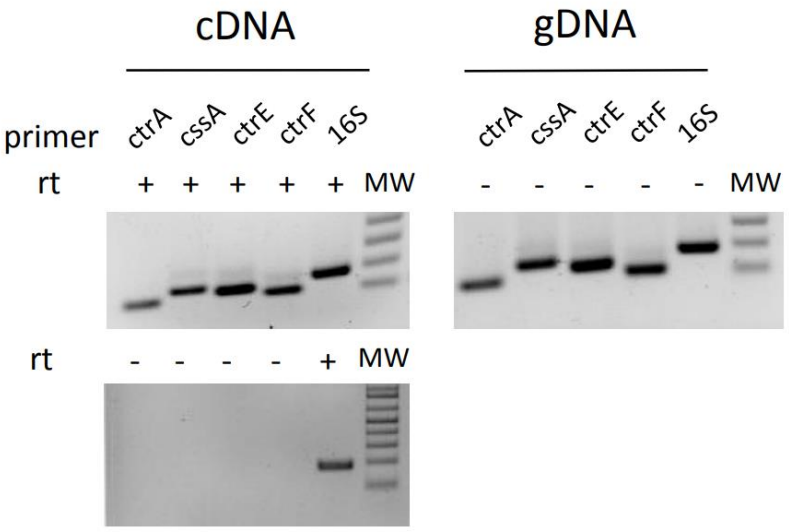
SUPPLEMENTAL FIGURE 2. *N. musculi* persistently colonizes the oral cavity (A) and gut (B) of CAST mice. LOD: limit of detection.



SUPPLEMENTAL FIGURE 3. Nmus Δ*pilE* does not produce *pilE* mRNA. WT: parental wild type N. musculi; AP2365Δ*pilE*::*pilE*$_{WT}$-C10 and AP2365Δ*pilE*::*pilE*$_{WT}$-C21: *pilE* complemented strains; rt: reverse transcriptase; gDNA: genomic DNA control.



SUPPLEMENTAL FIGURE 4. OD600 of cultures of *N. musculi* WT, Δ*pilE* and complemented strain Δ*pilE*::*pilE*$_{WT}$-C10. Values are the average of 4 independent experiments.



SUPPLEMENTAL FIGURE 5. Transcripts of *ctrA, cssA ctrE* and *ctrF* are detected in *N. musculi*. rt: reverse transcriptase; cDNA: complementary DNA; gDNA: genomic DNA control.

SUPPLEMENTAL TABLE 1. Transformation frequency of Nmus WT, Δ*pilE* and complemented strain AP2365Δ*pilE*::$pilE_{WT}$-C10.

Strain	DNA	Transformation frequency [a]
AP2365 (WT)	AP2098-Sm^R gDNA [c]	$6.16 \times 10^{-4} \pm 3.19 \times 10^{-4}$
AP2365Δ*pilE*	AP2098-Sm^R gDNA	$<6.98 \times 10^{-7} \pm 3.06 \times 10^{-8}$ [b]
AP2365Δ*pilE*::$pilE_{WT}$-C10	AP2098-Sm^R gDNA	$8.94 \times 10^{-5} \pm 1.93 \times 10^{-5}$
AP2365 (WT)	No DNA	$<6.42 \times 10^{-8} \pm 1.79 \times 10^{-8}$ [b]
AP2365Δ*pilE*	No DNA	$<7.03 \times 10^{-7} \pm 6.71 \times 10^{-8}$ [b]
AP2365Δ*pilE*::$pilE_{WT}$-C10	No DNA	$<2.21 \times 10^{-7} \pm 2.79 \times 10^{-9}$ [b]

[a]Transformation frequency is expressed as the number of Sm^R CFU/total CFU. Values are the mean of 3-5 independent experiments ± SEM. [b]Limit of detection. [c]Genomic DNA from AP2098, a naturally-occurring Streptomycin resistant *N. musculi* isolate.

SUPPLEMENTAL TABLE 3. *p*-values for the frequency of colonization of CAST and C57BL/6J by *N. musculi* WT, Δ*pilE* ,and complemented strain AP2365Δ*pilE*::$pilE_{WT}$-C10 using Mantel-Cox Rank Order Test.

	WT vs Δ*pilE*	WT vs Δ*pilE*::$pilE_{WT}$-C10	Δ*pilE* vs Δ*pilE*::$pilE_{WT}$-C10
CAST-OC [a]	<0.0001	0.3316	<0.0001
CAST-FP [b]	<0.0001	0.9116	<0.0001
C57BL/6J-OC [a]	<0.0001	0.0013	0.0030
C57BL/6J-FP [b]	<0.0001	0.0008	0.3316

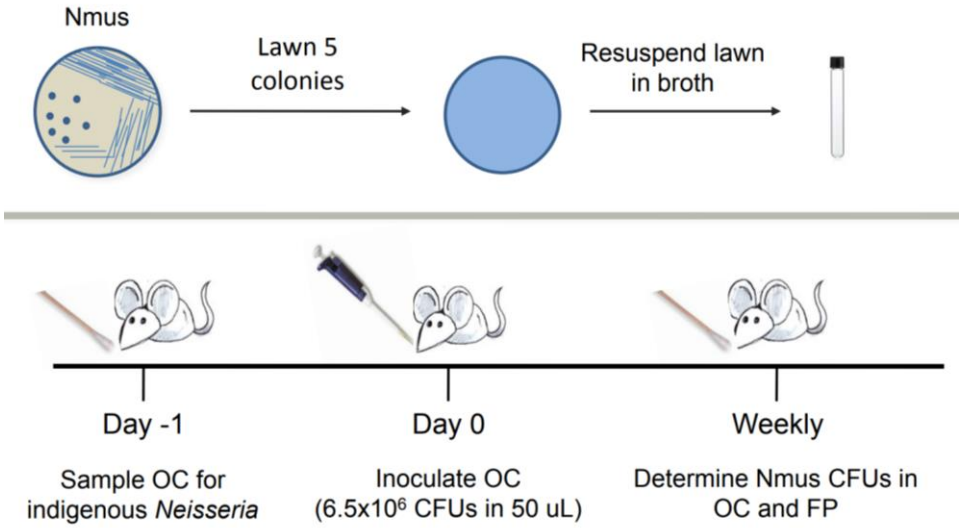
(a) OC, Oral cavity. (b) FP, Fecal pellet.

Materials and Methods

Generation of the Rifampicin-resistant *N. musculi* strain. AP2365, a naturally occurring Rifampicin resistant (Rif^R) rough variant of *Neisseria musculi* type strain (305), was isolated by plating AP2031 (AP2031T)on GCB (Becton Dickinson) agar containing Rifampicin (50 mg/L).

Mouse strains. All inbred mouse strains and Collaborative Cross parental strains were obtained from The Jackson Laboratory (Bar Harbor, ME). All animal protocols were approved by The University of Arizona IACUC.

Mouse inoculation protocol. Mice were rested in the University of Arizona mouse facility for two weeks before inoculation. The inoculation protocol is shown in Fig. S1.



SUPPLEMENTAL FIGURE 1. Protocol for inoculation and sampling of *N. musculi* in mice. CFU: colony forming units; OC: oral cavity; FP: fecal pellet.

To determine the presence of *Neisseria* species in the indigenous flora of the animals, the oral cavities of mice were swabbed using the BD BBL™ CultureSwab Plus Transport System (Fisher

Scientific); the swabs were suspended in GCB medium base (Becton Dickinson) plus Kellogg's Supplement I and II, and dilutions of the suspensions were plated on GCB agar containing Vancomycin (2 mg/L) and Trimethoprim (3 mg/L). The plates were counted after incubation for 48 h at 37°C, 5% CO_2. *Neisseria* has never been recovered from mice before inoculation. Fecal pellets of mice were suspended and processed similarly. On the day of inoculation, AP2365 was swabbed from agar plate and resuspended in PBS at an OD_{600} of 2.0. Inbred mice were manually restrained and 50 µl of the bacterial suspension was pipetted into the oral cavity. The oral cavities of the inoculated mice were swabbed weekly or biweekly. Swab suspensions in GCB medium base (Becton Dickinson) were plated on GCB agar containing Rifampicin (40 mg/L), and the plates were incubated for 48 h at 37°C, 5% CO_2.

Verification of *Neisseria musculi* in oral swab suspensions. Samples from each colony growing on GCB Rifampicin agar were used for verification of *Neisseria musculi* as described. Briefly, ITS primers specific to sequences that are highly conserved among *Neisseria* species were used for colony PCR (305). The ITS sequences of sample isolates were compared to the type strain AP2031 for species validation, and found to be identical.

Construction of *N. musculi* Δ*pilE* and its complemented strain. Table S2 lists the primers used for these constructions.

SUPPLEMENTAL TABLE 2. Primers used in this study.

Primer pairs	Use	Sequence
IM011F*	To delete *pilE*	**GAGGCCAAGCCCAAGCCCAAGCCCAGG CAGAAGCAGCAGCCAAAAGCGGCAAGC TGTAAATTCCTTCTTCTTTTGATTGCAGT ACAACGTGCCAAGCACATTACGGTTTTA CAACTATATAAATTTTATTCACTTTTAAA CTGGAGTTTTAAC**CTCGAGGGCTTGACA CTTTATG
IM0012R*		**CAGCGTTGTTTTATTTTGTGTATTTAGGT GATACCTTCCAATAAGGCATCAGTCCAA ACCCTGTGCGGTAGTTGCCTGTATCGGA TAAAAGCGCACCATATTGCTATAGTGCG CTTTTCGGTTAAACCTTAAAAGTTATTAA GGTTGTTGA**ATCGATGTTTAAACTTCAGA CGGC
NP246F	Detect *pilE* mutant	TGAAACACAAGGCCGTCTGA
NP246R2		TGACTTAAACGACTTTTTCTCATAGGG
IM013	Amplify *CmR* gene	atcatc**ttaattaa**CTGCCACTCATCGCAGTA
IM014		atcatc**gatatc**GGGATGCATAAACTGCATCC CGAGATTTTCAGGAGCTAAGGAAG
MR485	To complement *pilE*	atcatc**agcgct**GCAAGGCCGTCTGAAACAC
MR486		atcatc**ggtacc**CTGTGCGGTAGTTGCCTGTA
IM015	Amplify *pilE*-CmR	GCTGCAAGGCCGTCTGAAACAC

IM016		TACAGCCCCTAAAGTTAAGCCTGCCGTG TTTCAAATGCGG CATACGCTTTATCGGCGTTTCTGCCAGAA TTTCCCGAATGTTTCACTCTTGCCGCTGC ATCGGCAGCGTTGTTTTATTTTGTGTATT TAGGTGATACCTTCCAATAAGGCATCAGT CCAAACCCTGTGCGGTAGTTGCCTGTCT GCCACTCATCGCAGTA
MR493 MR494	Detect *16S* transcript	ATCCTGGCTCAGATTGAACG CCGCTTTCCTTCTCAAAGTG
MR489 MR490	Detect *pilE* transcript	GGCTTTACCCTGATCGAGTTG CCGTCTAAAACGCAGGTTTC
IM017 IM018	Detect *ctr*A transcript	AGTATCCGTATGCCGCTCAC AGCTGCACCGAAATATCCTG
IM019 IM020	Detect *cssA* transcript	CAGATGGACGTTGTGTTTGC AGGGAAAATTTCGGAGAAGG
IM021 IM022	Detect *ctrE* transcript	CATAAAAAGGCAGGCCGTAG GACCAAACCGTAACCGAATG
IM023 IM024	Detect *ctrF* transcript	AGCTGCCTTGAAAGGTGATG GTTACGCTCAACAGCACCAG

*The regions of primers IM011F and IM0012R that anneal to the *pilE* locus of AP2031 are inbold.

In AP2365 Δ*pilE*, the *pilE* open reading frame was replaced with a Kanamycin (Kan) resistance cassette. Primers IM011F and IM012R containing flanking sequences for the *pilE* gene in *Neisseria musculi* AP2031T were used to amplify the Kan cassette from plasmid pNBNeiKan (305) (synthesized by Genescript). The amplified DNA was purified and transformed into WT *Neisseria musculi* AP2031T by spot or liquid transformation as described, and transformants were selected on GCB agar containing Kellogg's Supplements I and II and Kan (50 mg/L). The Δ*pilE*::*kan* locus in AP2031T was transferred to the Rifampicin resistant *Neisseria musculi* strain AP2365 as follows. Primers NP246F and NP246R2 were used to amplify Δ*pilE*::*kan* from AP2031T, and the amplified DNA was cloned into pGEMT (Promega). The recombinant plasmid

DNA was introduced into AP2365 by spot transformation. Transformants were selected on GCB containing GCB agar containing Supplements I and II and Kan (50 mg/L). The Δ*pilE*::kan locus in AP2365 was confirmed by Sanger sequencing of PCR products generated with primers NP246F and NP246R2.

The complemented strains AP2365Δ*pilE*::*pilE*$_{WT}$-C10 and AP2365Δ*pilE*::*pilE*$_{WT}$-C21, independent clones, were constructed as follows. Primers IM013 and IM014 were used to amplify the *Chloramphenicol* (Cm) cassette from plasmid pLES94 (454) . Primers MR485 and MR486 were used to amplify the WT *pilE* locus in AP2031T. The Cm PCR product was digested with PacI and EcoRV (New England Biolabs) and the *pilE* PCR product was digested with AfeI and l KpnI (New England Biolabs). The two digested DNAs were ligated into similarly digested pUC19 (New England Biolabs) using T4 ligase (New England Biolabs). Primers IM0015 and IM0016 were used to amplify the *pilE*::*cm* region in the recombinant plasmid, and the amplified DNA was cloned into pGEMT (Promega). DNA from the resulting plasmid was electroporated into the AP2365 Δ*pilE*::*kan* to replace the mutated *pilE* locus. Transformants were selected and maintained on GCB agar containing Supplements I and II and Chloramphenicol (2.5 mg/L). The *pilE* locus in the complemented strains was confirmed by Sanger sequencing of PCR products generated with primers NP246F and NP246R2.

Transformation assays. DNA transformations were performed as described (305). Briefly, recipient strains AP2365, AP2365Δ*pilE*, and AP2365Δ*pilE*::*pilE*$_{WT}$-C10 were grown for 16 h at 37°C on GCB agar containing Supplements I and II and the appropriate selective antibiotic(s). Bacterial cells were suspended in GCB broth containing $MgSO_4$ (5 mM). 30 µL of each suspension, previously diluted to an OD_{600} of 1.5, was added to 0.2 mL of GCB liquid containing $MgSO_4$ (5 mM) and 1 µg of chromosomal DNA from *Neisseria musculi* strain AP2093, a

naturally occurring isolate whose *rpsL* contains a point mutation conferring resistance to Streptomycin (305). Following incubation at 37°C for 20 min, bacteria were added to 2 mL of GCB liquid containing Supplements I and II, and incubated at 37°C, 5% CO_2 for 4 h. Transformants were enumerated by plating cells onto GCB agar containing Supplements I and II and Streptomycin (100 µg/mL), and total input bacteria were enumerated by plating an equal volume on supplemented GCB agar without antibiotics.

RNA extraction, cDNA synthesis, and RT-PCR. Bacterial cells were grown to mid-log phase in GCB broth containing Supplements I and II, and total RNA was extracted using Trizol (Invitrogen) according to manusfacturer's instructions. Contaminating DNA was removed using DNA-free (Ambion). The quality and amount of RNA was determined by spectrophotometry (NanoDrop, Therm Scientific). For RT-PCR, 1000 µg of RNA were used to generate the first strand using M-MLV reverse transcriptase (Promega), according to manufacturer's instructions. This was followed by a PCR reaction using GoTaq green master mix (Promega). Nmus *pilE* was amplified using primers MR489 and MR490. Nmus 16S was amplified using primers MR493 and MR494. Nmus *ctrA* was amplified using primers IM017 and MR018. Nmus *cssA* was amplified using primers IM019 and IM020. Nmus *ctrE* was amplified using primers IM021 and IM022. Nmus *ctrF* was amplified using primers IM023 and IM024. Nmus 16S was amplified using primers MR493 and MR494. The primer sequence is listed in Table S2.

Growth curves. Bacterial cells were grown for 16 h at 37°C, 5% CO_2 on GCB agar containing Supplements I and II and the appropriate selective antibiotics. Cells were scraped from the plates, suspended in supplemented GCB, and diluted to an OD_{600} of 0.05. 2 ml of each bacterial sample was added to 60mm dishes and incubated at 37°C, 5% CO_2. Bacterial density was measured every two h for 10 h using a Beckman Coulter DU730 spectrophotometer (Brea, CA).

The cell density at each time point was expressed by subtracting the OD_{600} value at t=0 from the OD_{600} value at that time of collection.

Adherence assay. A static biofilm assay adapted from Merritt (455) was used to measure adherence. Briefly, 2 ml supplemented GCB liquid was added to each well of a 6 well dish (Corning) and $1x10^7$ CFU of *N. musculi* WT, Δ*pilE* or the complement strain was introduced into the wells. The plates were incubated at 37°C 5% CO_2 for 16 h. Each well was gently washed 3 times with 1 ml sterile PBS. Any residual wash buffer was forcibly shaken from the plate to remove all planktonic bacteria. One ml of 0.1% crystal violet was added to each well and the plate was incubated for 30 min at RT. The excess dye was removed, and all wells were washed with 10 ml of PBS. Retained crystal violet was solubilized by the addition of 1 ml of 30% glacial acetic acid, and the OD_{550nm} was measured on a Beckman Coulter DU730 spectrophotometer (Brea, CA). Three fields were imaged per before the initial washes and after crystal violet staining. Results are representative of three independent experiments performed in technical triplicate.

Blast searches. TBlastn searches were conducted using TBLASTN 2.7.1+ (456, 457). Protein query sequences from *N. meningitidis* and *N. gonorrhoeae* were used to search Nmus strain AP2031's genome sequence (PubMLST ID 29520(460)). Many Nme queries used for the analysis were retrieved from the Protegen protective antigen database (459). The accession numbers for the commensal human-dwelling *Neisseria* genome data used for BLAST searches are: Npo (*Neisseria polysaccharea* ATCC 43768, NZ_ADBE00000000), Nla (*Neisseria lactamica* 02-06, NC_014752), Nci, (*Neisseria cinerea* ATCC 14685, NZ_ACDY00000000), Nsu, (*Neisseria subflava* NJ9703, NZ_ACEO00000000), Nor (*Neisseria oralis* CCUG 26878, PubMLST ID 19091), Nmu (*Neisseria mucosa* ATCC 25996, NZ_ACDX00000000), Nel

(*Neisseria elongata* ATCC 29315, NZ_CP007726), Nba (*Neisseria bacilliformis* ATCC BAA-1200, NZ_AFAY00000000).

India ink stain and light microscopy. *Neisseria meningitidis* capsulated strain 8013 (Nme 8013), unencapsulated strain FAM2 (Nme FAM2), and Nmus AP2365^T were suspended in India ink (BD Diagnostic) and spread as thin films on a microscope slide (452). After the films were allowed to air dry, the bacteria were counter-stained with crystal violet (GIBSON) for 1 minute. The slides were gently rinse with water and examined under a light microscope at 100X magnification.

Capsule extraction. Capsule was extracted as described (453). *Neisseria meningitidis* capsulated strain 8013, unencapsulated strain FAM2, and Nmus AP2365 rough and smooth variants were grown on GCB agar for 17 to 18 h at 37°C, 5% CO_2. Cells were suspended in phosphate-buffered saline (PBS) to an OD_{600} of 0.8. 1 ml of the suspension was pelleted by centrifugation (10,000 x g, 5° C) for 2 min. The pellet cells was resuspended in 0.5 ml PBS and incubated at 55°C for 30 min to allow release of capsular material. Bacteria were again pelleted, and the supernatants were concentrated 10-fold in an Amicon Ultra centrifuge filter with a 10,000 molecular weight cutoff. Capsular material was separated by 6% SDS-PAGE and stained with the cationic dye Alcian Blue (0.125% alcian blue in 40% ethanol/5% acetic acid; Sigma) for 2 h, and destained overnight in 40% ethanol/5% acetic acid.

Acknowledgments

This work was supported by grants from the BIO5 Institute and the College of Medicine at the University of Arizona, and by NIH R56AI124665.

Chapter 3

Type IV Pilus retraction is required for colonization and persistence of commensal *Neisseria in vivo*

(Data presented in this chapter will support future manuscript that contains additional analysis on Type IV pilus retraction (PilT) function)

Abstract

The Type IV pilus (Tfp) is produced by many Gram-negative and Gram-positive bacteria, both free living and commensal and pathogenic bacteria. This surface structure allows bacteria to sense and interact with other bacterial cells and with their immediate environment. It allows bacteria to crawl on surfaces and aggregate into microcolonies and biofilms, to take up exogenous DNA (horizontal gene transfer), and to attach to and invade host cells. Although the role of Tfp retraction in the biology of pathogenic *Neisseria* (*N. meningitidis* and *N. gonorrhoeae*) has been studied extensively *in vitro*, its role in commensal *Neisseria* biology is poorly understood. Using our recently developed natural small animal model for studying commensal *Neisseria* colonization and persistence, I showed that Tfp is required for *N. musculi* (Nmus), a mouse commensal, to colonize mice. The Nmus *pilE*-null mutant (Δ*pilE*), which does not produce Tfp (nonpiliated), fails to colonize mice (306). Here, I show that Nmus *pilT*-null mutant (Δ*pilT*) which cannot retract Tfp, also fails to colonize mice. Nmus Δ*pilT* cells are hyperpiliated and form aberrant microcolonies and biofilms. From these results I conclude that PilT, and, by implication, pilus retraction, plays an important role in *Neisseria* colonization and persistence, most likely through its role in biofilm formation.

Introduction

Type IV pili (Tfp) are fimbriate organelles that are produced by many Gram-negative and Gram-positive free living and host-adapted bacteria (312, 331, 460). Tfp are essential for bacterial motility, microcolony and biofilm formation, and DNA uptake (horizontal gene transfer) (313, 323, 361, 363, 461–466). They activate host cell signaling pathways that mediate bacterial adhesion, invasion, and intracellular survival (267, 268, 285). These interactions require both the presence of the Tfp fiber and the ability of the fiber to retract.

In *Neisseria*, Tfp biogenesis and function requires over 20 proteins. With the exception of PilE (pilin) the structural subunit of the fiber, the sequence of these proteins are highly conserved (188, 303, 423). Tfp biogenesis has been studied in detail in *Neisseria gonorrhoeae*. Signal peptidase (PilD) cleaves the N-terminal leader peptide from pre-pilin and ATPase (PilF) incorporates the mature pilin subunits (PilE) into the Tfp fiber (334). The pilus fiber is anchored in the outermembrane through the PilQ outermembrane pore complex and the fiber extends into the extracellular space (335, 467, 468). The Tfp fiber undergoes cycles of extension, substrate tethering, and retraction. Tfp retraction is mediated by the retraction motor, which is composed of six AAA ATPase subunits of the PilT protein (337, 469). Rounds of ATP binding, hydrolysis, and release causes the Tfp to retract , by a poorly understood mechanism (470–472).

Due to the strict tropism of pathogenic *Neisseria* for their human host, the biological consequences of Tfp retraction are mostly studied *in vitro* using cultured human cells and a limited number of human challenge studies (315, 322, 399, 440, 473). PilT retraction upregulates classes of host cell transcripts and downregulates inflammatory pathways, some of which have been shown to promote invasion and intracellular survival (267–270). A *pilT*-null

mutant of *N. gonorrhoeae* (Δ*pilT*$_{Ngo}$) is hyperpiliated and adheres to host cells but is nonmotile, nontransformable, forms aberrant microcolonies, reduced in host cell invasion and intracellular survival, and is defective in host cell signaling (267–270, 285, 337, 361). Using the meningococcal transposon mutant library (474) and the CB17 Severe Combined Immunodeficiency humanized mouse model (475), Capel et al. showed that the disruption of *pilT* does not affect the initial attachment to human endothelial cells (476). Meningococcal PilT is also important for full virulence *in vivo* (477). However, whether Tfp retraction is essential for *in vivo* colonization and persistence in a natural setting is unknown.

N. musucli (Nmus), a new speices of commensal *Neisseria* isolated from the oral cavity of wild mice, is genetically related to other *Neisseria* (188). Nmus genome is annotated and it has a complete set of Tfp biogenesis genes (188). Nmus PilT shares high homology with *Neisseria meningitidis* PilT (87% amino acid identity, 100% coverage) and is required for Nmus genetic competence (188). To study commensal *Neisseria*-host interactions, we developed a natural mouse model by pairing Nmus with laboratory mice (306). Using this mouse model of commensal Neisseria colonization and persistence, I sought to determine the role of Tfp, in particular the role of PilT in commensal host cell interactions. I demonstrated the loss of competence of Δ*pilT*$_{Nmus}$ is not caused by second site mutations generated during construction of the deletion mutation. I showed that the complemented strain Δ*pilT*$_{Nmus}$::*pilT*$_{WT}$ restores genetic competence. I also showed that Δ*pilT*$_{Nmus}$ fails to colonize mice in our mouse model of *Neisseria* colonization and persistence, and this defect is reversed in the complemented strain.

Results

N. musculi DNA uptake requires Tfp retraction.

All species of *Neisseria* are naturally competent for DNA uptake/transformation. Competence requires Tfp. It involves the binding of the DNA Update Sequence (DUS- a 10 nt sequence 5'-GCCGTCTGAA -3') to the Tfp associated protein ComP, and Tfp retraction mediated by the motor protein (PilT) (318, 363, 480, 481). To determine the role of *pilT* in *Neisseria musculi* (Nmus) competence and to confirm the phenotype observed is not due to second site mutations generated during mutant construction, we compared the transformation frequency of WT Nmus, the *pilT*-null mutant (Δ$pilT_{Nmus}$), and the *pilT* complemented strain (Δ$pilT_{Nmus}$::$pilT_{WT}$). Following the transformation protocol (304), genomic DNA (gDNA) from a streptomycin-resistant Nmus variant (Sm^R) (188) was used in the liquid transformation assay. Transformation frequency was calculated by dividing the colony forming unit (CFU) of SmR over the total CFU per µg of DNA. Cells incubated with medium without DNA was the negative control. The frequency of Nmus AP2365 (WT) was 3.33×10^{-4} (SEM±1.11×10^{-4}) in the presence of Sm^R gDNA. In contrast, Δ$pilT_{Nmus}$ transformation frequency was approximately 3 logs lower than the WT transformation frequency and the frequency of the negative controls were below the limit of detection (WT: $<5.29 \times 10^{-8} \pm 2.11 \times 10^{-8}$; Δ$pilT_{Nmus}$: $<3.77 \times 10^{-7} \pm 1.52 \times 10^{-7}$; and Δ$pilT_{Nmus}$::$pilT_{WT}$: $<7.74 \times 10^{8} \pm 8.47 \times 10^{9}$) (Table 3.1). Δ$pilT_{Nmus}$::$pilT_{WT}$ had a similar transformation frequency as the WT (Table 3.1). These results indicate PilT, and by implication Tfp retraction, is critical for DNA uptake/transformation in Nmus.

TABLE 3.1. Transformation frequency of *N. musculi* WT, Δ*pilT*$_{Nmus}$ and complemented strain Δ*pilT*$_{Nmus}$::*pilT*$_{WT}$.

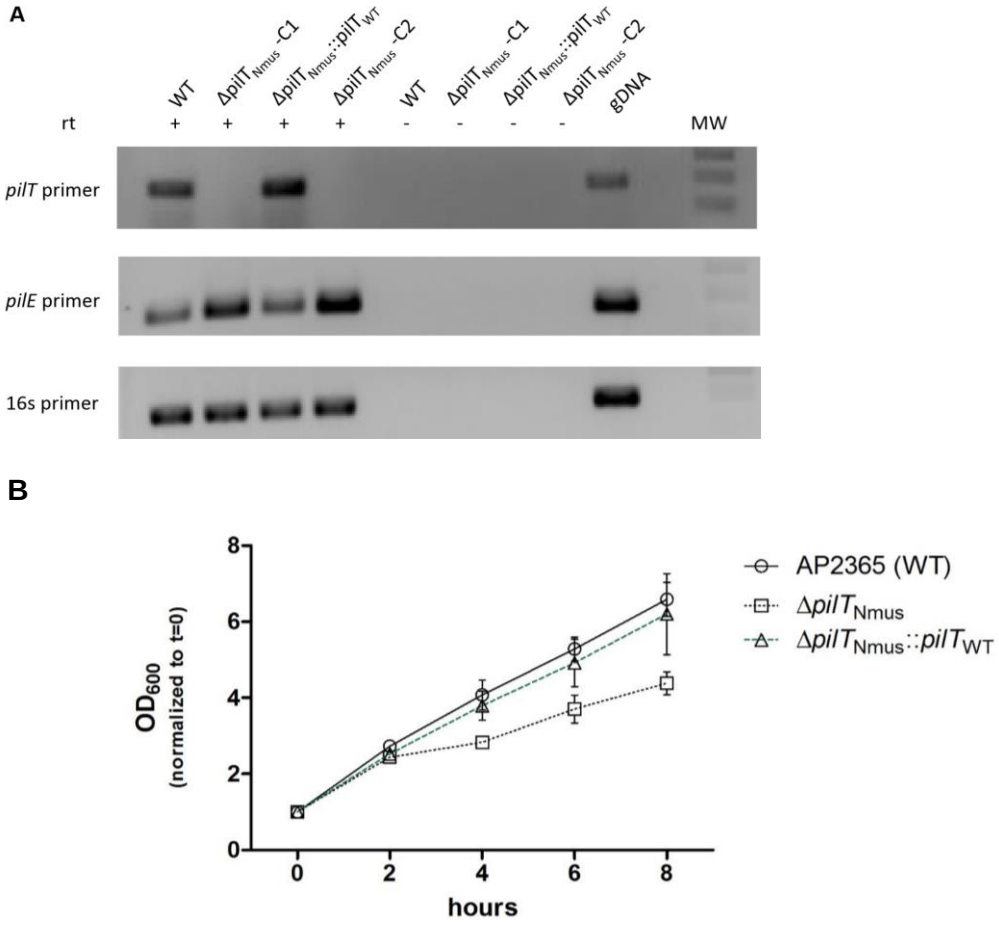
Strain	DNA	Transformation frequency [1]
WT	AP2098-Sm^R gDNA [3]	$3.33 \times 10^{-4} \pm 1.11 \times 10^{-4}$
Δ*pilT*$_{Nmus}$	AP2098-Sm^R gDNA	$< 3.45 \times 10^{-7} \pm 1.98 \times 10^{-7}$ [2]
Δ*pilT*$_{Nmus}$::*pilT*$_{WT}$	AP2098-Sm^R gDNA	$7.44 \times 10^{-4} \pm 6.01 \times 10^{-4}$
WT	No DNA	$< 5.29 \times 10^{-8} \pm 2.11 \times 10^{-8}$ [2]
Δ*pilT*$_{Nmus}$	No DNA	$< 3.77 \times 10^{-7} \pm 1.52 \times 10^{-7}$ [2]
Δ*pilT*$_{Nmus}$::*pilT*$_{WT}$	No DNA	$< 7.74 \times 10^{8} \pm 8.47 \times 10^{9}$ [2]

[1]Transformation frequency is expressed as the number of Sm^R CFU/total CFU. Values are the mean of 3 independent experiments ± SEM. [2]Limit of detection. [3]Genomic DNA from AP2098, a naturally-occurring Streptomycin resistant *N. musculi* isolate.

N. musculi colonization requires Tfp retraction.

All *Neisseria* species encode a complete set of Tfp biogenesis genes. Tfp initiates host cell attachment *in vitro* (289, 341, 353, 480) and *in vivo* (420, 476). Using our natural mouse model of commensal *Neisseria* colonization and persistence, we showed that the presence of the Tfp fiber is necessary for *in vivo* colonization and persistence (306). Tfp undergoes cycles of extension and retraction through ATPase proteins, PilF and PilT, respectively (336, 472, 483). Tfp retraction, controlled by PilT, activates immune and cytoprotective signaling pathways *in vitro* (267, 268, 270, 285, 354, 482). In the absence of PilT, a *N. gonorrhoeae pilT*-null mutant (Δ*pilT*$_{Ngo}$) is defective in Tfp retraction (337), forms aberrant microcolonies (363), and is hyperpiliated (362). A meningococcal *pilT*-null mutant adheres to host cells better than WT during early infection. However, it fails to disperse as monolayers at later time point and does not form lesions on T84 human epithelial cells (483). Based on these *in vitro* studies, we hypothesize that the presence of the Tfp fiber and the ability of the fiber to retract both influence *Neisseria* colonization and/or persistence *in vivo*. Due to the strict host tropism for human-adapted *Neisseria*, the function of Tfp retraction has never been tested in a natural animal model.

To test the hypothesis stated above, we analyzed the colonization phenotype of WT Nmus, Δ*pilT*$_{Nmus}$, and Δ*pilT*$_{Nmus}$::*pilT*$_{WT}$ in our mouse model. First, we characterized the mutant and complemented strains. Unlike the WT and complemented strains, Δ*pilT*$_{Nmus}$ did not produce *pilT* mRNA (Fig. 3.1A). Consistent with the Δ*pilT*$_{Ngo}$ hyperpiliation phenotype, transcription of *pilE* in Δ*pilT*$_{Nmus}$ was also upregulated (Fig. 3.1A). Whether this increase in *pilE* transcription reflects the increase in production of Tfp pilin remains to be tested. Δ*pilT*$_{Nmus}$ is also defective in DNA transformation (Table 3.1). Together, these results showed that Nmus Δ*pilT* produces Tfp fiber but is defective in pilus retraction. Finally, there was no difference in the growth rates of WT, Δ*pilT*$_{Nmus}$, and the complemented strain (Fig. 3.1B). The optical density (OD) of the Δ*pilT*$_{Nmus}$ culture is lower than the other strains, but this difference is not statistically significant. The lower OD likely reflects the hyperaggregative phenotype of the *pilT* mutant in liquid culture (Fig. 3.1C).



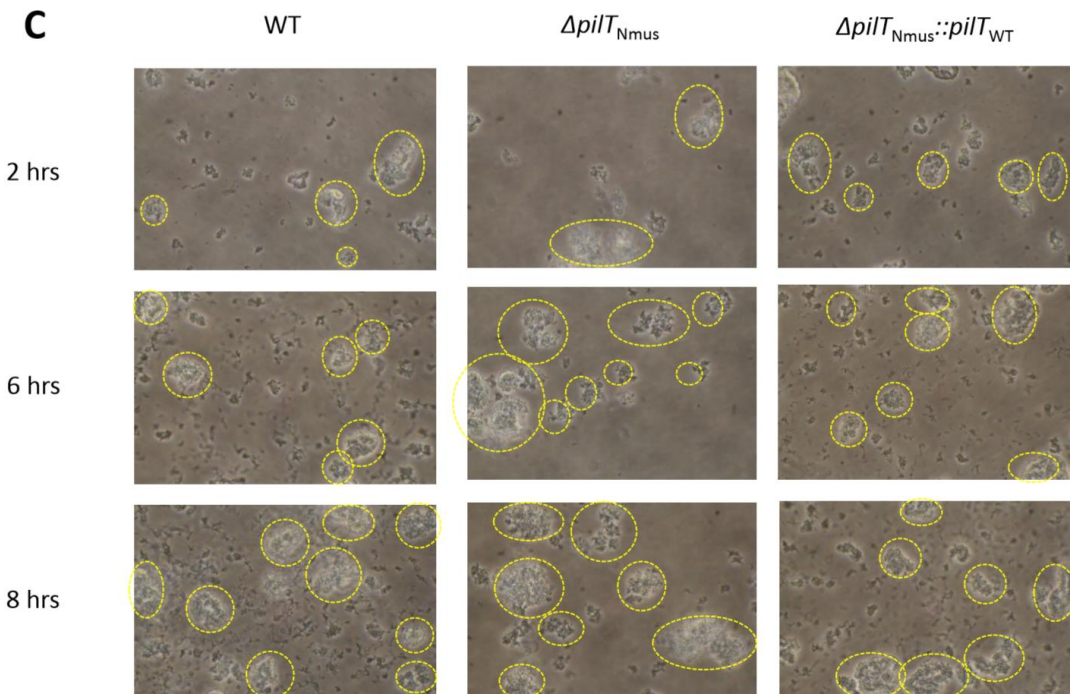


Fig. 3.1. **Characterization of *pilT* transcript and growth in *N. musculi pilT*-null mutant.** (A) Δ*pilT*$_{Nmus}$ does not produce *pilT* mRNA. Δ*pilT*$_{Nmus}$ -C1: *N. musculi pilT* mutant Clone 1; Δ*pilT*$_{Nmus}$ -C2: *N. musculi pilT* mutant Clone 2; WT: parental wild type *N. musculi*; Δ*pilT*$_{Nmus}$::*pilT*$_{WT}$ complemented strains; rt: reverse transcriptase; gDNA: genomic DNA control. MW: 1kb plus DNA ladder. Representative data from 5 independent experiments. (B) Optical density (OD_{600}) of cultures of *N. musculi* WT, Δ*pilT*$_{Nmus}$ and complemented strain Δ*pilT*$_{Nmus}$::*pilT*$_{WT}$. Values are the average of 3 independent experiments. Significance was determined using Student's *t*-test on the average OD_{600} per strain at each time point. (C) Δ*pilT*$_{Nmus}$ form larger aggregates of microcolony when compared to WT and the complemented strain. Representative images were taken on bacteria growing in GCB medium with supplement I+II. Microcolonies are highlighted with yellow dashed line.

To test whether Tfp retraction is essential for colonization and persistence, WT Nmus, Δ*pilT*$_{Nmus}$, and the complemented strain Δ*pilT*$_{Nmus}$::*pilT*$_{WT}$ were each inoculated into the oral cavity of 30 CAST mice (10 mice per strain). Their viable counts (CFUs) in oral cavity swabs (OC) and fecal pellets (FP) were determined daily for 12 weeks. Δ*pilT*$_{Nmus}$ was defective in colonizing the OC and gut of CAST compared to the WT and the complemented strain (Fig. 3.2, Table 3.2) (Mantel-Cox Rank Order Test, $P < 0.0001$ for both WT versus Δ*pilT* and Δ*pilT* versus Δ*pilT*$_{Nmus}$::*pilT*$_{WT}$). The Δ*pilT*$_{Nmus}$ cells recovered from OC and FP swabs were confirmed to still have the *pilT* deletion by PCR analysis using *pilT* primers (NP242F and NP242R) (data not

shown). The complemented strain colonized the OC and FP of CAST mice like WT Nmus (Mantel-Cox Order Test, OC, P = 0.1351; FP, P = 0.8416; Table 3.2). These results show that Nmus colonization requires PilT.

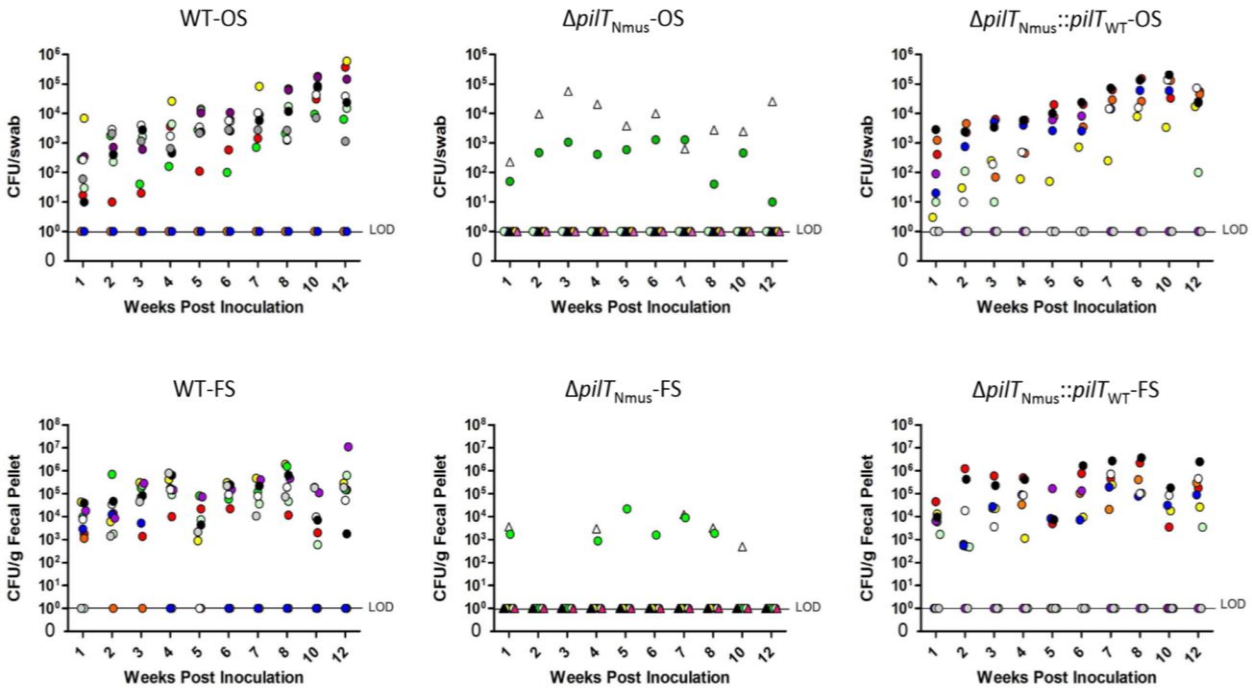


Fig. 3.2. ***N. musculi* Δ*pilT* is defective in colonizing the oral cavity (top row) and gut (bottom row) of CAST mice.** CFU: colony forming units; WT: wild type *N. musculi*; Δ$pilT_{Nmus}$::$pilT_{WT}$: *pilT* complemented strain. Each *N. musculi* strain was assayed in 10 mice. Oral swab and fecal samples from the same mouse are assigned the same color. LOD: limit of detection. OS: Oral swab; FS: Fecal Sample. Circle is female and triangle is male CAST.

TABLE 3.2. *p*-values for the frequency of colonization of CAST by *N. musculi* WT, Δ*pilT*, and complemented strain Δ$pilT_{Nmus}$::$pilT_{WT}$ using Mantel-Cox Rank Order Test.

	WT vs Δ*pilT*	WT vs Δ$pilT_{Nmus}$::$pilT_{WT}$	Δ*pilT* vs Δ$pilT_{Nmus}$::$pilT_{WT}$
CAST-OC[a]	<0.0001	0.1351	<0.0001
CAST-FP[b]	<0.0001	0.8416	<0.0001

(a) OC, Oral cavity. (b) FP, Fecal pellet.

Discussion

In this study, we used the natural mouse model (306) to investigate the role of Nmus PilT in commensal *Neisseria* colonization and persistence. *In vitro* studies have shown that inactivation of *pilT* in pathogenic *Neisseria* (*N. gonorrhoeae* and *N. meningitidis)* and human-adapted commensal *Neisseria* (*N. elongata*) results in loss of motility and DNA uptake, and the mutants form aberrant microcolonies. As microcolonies are precursors of biofilms, the defect in microcolony formation is likely to lead to the formation of less robust biofilms, which in turn could explain the colonization defect of the mutant. PilT-deficient mutants of *Neisseria* are hyperpiliated, hyperaggregative, and attach to epithelial cell similarly or even better than WT (267–270, 285, 337, 361, 484, 483). I showed that Nmus PilT also mediates DNA uptake, Tfp pilin expression, and microcolonies formation (Table 3.1; Fig. 3.1A, 1C). Unlike human-adapted *Neisseria*, Nmus Δ*pilT* mutant displays decreased adherence to CMT93 mouse rectal epithelial cells and forms less robust biofilms compared to WT Nmus (Kate Rhodes, unpublished data).

The importance of PilT *in vivo* has been examined in the Tfp-expressing species *Pseudomonas aeruginosa*, *Dicheloacter nodosus*, *enteropathogenic Escherichia coli* (EPEC), and *N. meningitidis* (314, 477, 486, 487). In *P. aeruginosa*, PilT is necessary for corneal colonization and dissemination from the lung to the liver in an acute pneumonia mouse model. However, the loss of *pilT* in *P. aeruginosa* does not significantly affect the survival rate of infected mice. Meningococcal Δ*pilT* mutant is less virulent than the WT in a human CD46 transgenic mouse model. In this study, we investigated the role of commensal *Neisseria* PilT in colonization and persistence. Using a natural mouse model of commensal Neisseria colonization and persistence, we showed that the colonization frequency of Δ$pilT_{Nmus}$ in both the oral cavity and fecal sample is significantly lower than the WT and the complemented strain (Fig. 3.2; Table 3.2). Combined with our previous published results (306) (Chapter 2, current dissertation),

colonization of commensal *Neisseria* requires not only the presence of the Tfp but also the ability of the Tfp to retract. The reduced colonization frequency of the *pilT*-null mutant might be due to the defect in biofilm formation which, in turn, affect the capability of Nmus to resist shear stress along the gastrointestinal tract. Our laboratory is currently testing this hypothesis (Kate Rhodes, ongoing experiment).

All *Neisseria* express Tfp. How Tfp of commensal *Neisseria* interact with the host is unknown. Unpublished data (Hockenberry PhD dissertation; Biais, personal communication) showed that commensal *Neisseria elongata* Tfp retracts at half the speed, with the same force and ATP hydrolysis rate as *N. gonorrhoeae* Tfp. *N. elongata* stimulates cytokine production (IL-6, IL-8, MCP-1, and TNFα) through Tfp retraction while *N. gonorrhoeae* inhibits cytokine responses. These results indicate that commensal and pathogenic Tfp retraction induce a different host cell response. They further suggest that how neisserial Tfp retraction regulates bacterial-bacterial interaction might also be species specific. In addition, the absence of PilT will have different effects among different bacterial species. Nevertheless, studying Nmus in its natural host will provide information on how Tfp influences commensal *Neisseria* interactions with the host.

In summary, the Nmus Δ*pilT* mutant produces more pilin, grows like WT but forms aberrant microcolonies. It is non-motile and does not take up DNA. We were able to show that Tfp retraction is essential for commensal *Neisseria* colonization *in vivo*. However, the underlying mechanisms still remain to be identified. Based on our results, we will be able to further investigate whether altering the rate of ATP hydrolysis of Tfp will affect the colonization and persistence *in vivo*. Since Nmus PilT has the same ATP-binding Walker A and B motifs as human-adapted *Neisseria* (data not shown), we can generate the same amino acid mutation (L201C) (269) in the Walker B motif of Nmus PilT. Using our mouse model of commensal

Neisseria for colonization and persistence, we can test whether Nmus *pilT*$_{L201C}$ is defective in establishing colonization and/or persistence *in vivo* (Kate Rhodes, ongoing experiments).

Materials and Methods

Bacterial strains and growth conditions. *Neisseria musculi* AP2365 (188) (WT) and Δ*pilT*$_{Nmus}$ (188) were used throughout this study. All strains were grown on Gonococcal Broth (GCB) agar plates supplemented with containing Kellogg's supplements I and II at 37°C with 5% CO_2.

Mouse strains. CAST mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All animal protocols were approved by The University of Arizona IACUC.

Mouse inoculation protocol. Mouse experiments were performed as described (306). Briefly, mice were rested in the University of Arizona mouse facility for two weeks before inoculation. The oral cavities of mice were pre-swabbed using BD BBLTM CultureSwab Plus Transport System (Fisher Scientific) and suspensions were plated GCB agar containing Vancomycin (2 mg/L) and Trimethoprim (3 mg/L). *Neisseria* has never been recovered from mice before inoculation. On the day of inoculation, AP2365, Δ*pilT*$_{Nmus}$, Δ*pilT*$_{Nmus}$::*pilT*$_{WT}$ were swabbed from GCB agar plate and resuspended in PBS at an OD600 of 2.0. CAST and A/J mice were manually restrained and 50 µl of the bacterial suspension was pipetted into the oral cavity. The oral cavities of the inoculated mice were swabbed weekly or biweekly. Swab suspensions in GCB medium base (Becton Dickinson) were plated on GCB agar containing Rifampicin (40 mg/L), and the plates were incubated for 48 h at 37 °C, 5% CO_2.

Growth curves. WT, Δ*pilT*$_{Nmus}$, Δ*pilT*$_{Nmus}$::*pilT*$_{WT}$ were grown for 16 h at 37 °C, 5% CO_2 on GCB agar containing Supplements I and II and the appropriate selective antibiotics. Cells were scraped from the plates, suspended in supplemented GCB, and diluted to an OD600 of 0.05. 2 ml of each bacterial sample was added to 60mm dishes and incubated at 37 °C, 5% CO_2. Bacterial density was measured every 2h for 10 h using a Beckman Coulter DU730

spectrophotometer (Brea, CA). The cell density at each time point was expressed by normalizing the OD_{600} value at that time of collection over the OD_{600} value at t=0. Pictures of bacterial liquid cultures were taken every 2 h for 8 hr.

Transformation assays. DNA transformations were performed as described (188). Briefly, recipient strains WT, Δ*pilT*$_{Nmus}$, and Δ*pilT*$_{Nmus}$::*pilT*$_{WT}$ were grown for 16 h at 37 °C on GCB agar containing Supplements I and II and the appropriate selective antibiotic(s). Bacterial cells were suspended in GCB broth containing $MgSO_4$ (5 mM). 30 µL of each suspension, previously diluted to an OD_{600} of 1.5, was added to 0.2 mL of GCB liquid containing $MgSO_4$ (5 mM) and 1 µg of chromosomal DNA from *N. musculi* strain AP2093, an isolate which is Streptomycin resistant (188). Following incubation at 37 ° C for 20 min, bacteria were added to 2 mL of GCB liquid containing Supplements I and II, and incubated at 37 °C, 5% CO_2 for 4 h. Transformants were enumerated by plating cells onto GCB agar containing Supplements I and II and Streptomycin (100 µg/mL), and total input bacteria were calculated by plating an equal volume on supplemented GCB agar without antibiotics.

RNA extraction, cDNA synthesis, and RT -PCR. Bacterial cells were grown to mid-log phase in GCB broth containing Supplements I and II, and total RNA was extracted using Trizol (Invitrogen) according to the manufacturer's instructions. Contaminating DNA was removed using DNA -free (Ambion). The quality and amount of RNA was determined by spectrophotometry (NanoDrop, Therm Scientific). For RT -PCR, 1000 µg of RNA were used to generate the first strand using M -MLV reverse transcriptase (Promega), according to manufacturer's instructions. This was followed by a PCR reaction using GoTaq green master mix (Promega). *N. musculi pilT* was amplified using primers MR491 and MR492. *N. musculi pilE* was amplified using primers MR489 and MR490. *N. musculi* 16S was amplified using primers MR493 and MR494. The primer sequence is listed in Table 3.3.

TABLE 3.3. Primers used in this study.

Primer pairs	Use	Sequence	Source
MR493	Detect *16S* transcript	ATCCTGGCTCAGATTGAACG	Ma et al., 2018
MR494		CCGCTTTCCTTCTCAAAGTG	
MR489	Detect *pilE* transcript	GGCTTTACCCTGATCGAGTTG	Ma et al., 2018
MR490		CCGTCTAAAACGCAGGTTTC	
NP242 F	To delete *pilT*	**TCTTTCCGTTTTTGGCACGTTAGCAAAAATAAT TAAAAT TTTACTTGGTTTAACATGAGTTATCTATTTAAAA TAATGC ACCGAAACAACCCGCCGCCGAAACCGGCAGC GGCCGT CTGAAAAATTTTAACTGAAAACGCAAGGCTAC ACATT**CTCGAGGGCTTGACACTTTATG	Weyand et al., 2016
NP242 R		**CTGTGAATAAGCCTGCACCATTTCGGAAAGCA GGTTGT GCAGTTGGTTGGATTCGCTCATGGTGTTTTCCC TGAGAT TTTTCGGTATCGGTTGTTTCAGACGGCCTGATG GCCGT GTGGGGGATTCGGCCGTCTGAAAAGAATCAAA TCAA**ATCGATGTTTAAACTTCAGACGGC	
NP245 F	To complement *pilT*	TTTGTTCGACTGCACATCGC	Weyand et al., 2016
NP245 R		TTCCAGATCGCGGTTGAAGG	
MR491	Detect *pilT* transcript	AAGACCCCGATGTGATTCTG	This study
MR492		GAGCGCACCATTTCTTTTTC	

*The regions of primers NP242F and NP242R that anneal to the *pilT* locus of AP2031 are in bold.

Construction of Δ*pilT*$_{Nmus}$ and its complemented strain. Table 3.3 lists the primers used for these constructions. Δ*pilT*$_{Nmus}$ was constructed as described (188). Briefly, the *pilT* open reading frame (ORF) was replaced with a Kanamycin (Kan) resistance cassette. Primers NP242F and NP242R containing flanking sequences for the *pilT* gene in *N. musculi* AP2031T were used to amplify the Kan cassette from plasmid pNBNeiKan. The amplified DNA was purified and transformed into AP2031T by spot or liquid transformation as described, and transformants were selected on GCB agar containing Kellogg's Supplements I and II (399) and Kan (40 mg/L). The Δ*pilT*::Kan locus in AP2031T was transferred to the Rifampicin resistant *N. musculi* strain

AP2365 and 2 independent clones were isolated as Δ*pilT*$_{Nmus}$-C1 and Δ*pilT*$_{Nmus}$ -C2. Besides detecting *pilT* transcripts, Δ*pilT*$_{Nmus}$ -C2 (Δ*pilT*$_{Nmus}$) was used throughout this study.

The complemented strain Δ*pilT*$_{Nmus}$::*pilT*$_{WT}$ was constructed as follows. Primers NP245F and NP245R were used to amplify the *pilT* ORF from AP2365, and the amplified DNA was cloned into pGEMT (Promega). DNA from the resulting plasmid was electroporated into the AP2365 377 Δ*pilT*$_{Nmus}$ to replace the mutated *pilT* locus. Transformants were plated on GCB agar containing Supplements I and II and Kan sensitive isolates were selected on GCB agar containing Supplements I and II and Kan (40 mg/L). The *pilT* locus in the complemented strains was confirmed by Sanger sequencing of PCR products generated with primers NP245F and NP245R.

Acknowledgments

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Chapter 4

Neisseria musculi produces a polysaccharide capsule

Abstract

The Capsular polysaccharide (CPS) is a major host interaction factor of *Neisseria meningitidis* (Nme). This surface structure promotes *N. meningitidis* survival in the bloodstream and cerebrospinal fluid. In the genus *Neisseria*, the capsular polysaccharide was previously considered a unique trait of Nme. Analysis of human and animal commensal *Neisseria* genomes revealed the presence of homologues of capsule genes in some of the species, including *N. musculi* (Nmus), a mouse commensal *Neisseria* (306, 311). To investigate capsule expression in commensal *Neisseria*, I compared Nmus and Nme *cps* genes and gene organization and found that they are similar to each other. However, the two *cps* loci differed in the intergenic region (IGR) that separates the transportation locus from the capsule biosynthesis/translocation locus. The IGR in Nme is known to regulate *cps* expression. It contains binding site for Sigma factors RpoD and a 8bp direct repeat (388). The Nmus IGR contains potential binding sites for Sigma factors RpoD and RpoN and for transcription activator Npa, and a 17bp direct repeat. However, whether these additional regulatory elements are functional is yet to be tested. I deleted the 227 bp intergenic region (ΔIGR) in Nmus and found that this mutant fails to produce capsule. These results suggest that the Nmus IGR regulates *cps* expression, and that capsule expression in Nmus and Nme may be regulated by different mechanisms. Finally, I addressed the relevance of Nmus capsule for biofilm formation. Nmus ΔIGR forms more biofilms on an abiotic surface indicating capsule production can affect overall surface properties which, in turn, might affect how Nmus colonizes and persists inside the host.

Introduction

The genus *Neisseria* is a diverse group of Gram-negative bacteria. Most of the *Neisseria* are commensals which colonize the mucosal surfaces of animals (186, 187, 189, 305) and man (184, 185). Only when passively introduced into the bloodstream of immunocompromised hosts will cause infections (190). *Neisseria gonorrhoeae* and *Neisseria meningitidis* (Nme) are the only two obligate human pathogens within the genus. Besides being the causative agent of epidemic meningitis, Nme is also a human nasopharynx commensal which can be isolated from 3-20% healthy individuals (238). Factors determining the switch between the carriage state and the invasive status involve a combination of specific capsule structure, other bacterial host interaction factors, and host susceptibility, however, the underlying mechanisms are still not clear.

Nme produces 13 number of antigenicallly different capsules. Of the 13 capsule serogroups, 6 (serogroups A, B, C, W, X, and Y) are most often associated with invasive Nme (384). Nme capsular polysaccharide (CPS) facilitates transmission by preventing desiccation. CPS also allows meningococci to survive in the bloodstream by evading phagocytosis by macrophages and preventing complement-mediated killing and cationic peptide killing, as well as escaping antibody recognition through antigenic variation and immune mimicry (371–380).

Controlling CPS expression is essential for *Neisseria*-host interactions, therefore, the biosynthesis pathway and regulation of meningococcal capsules have been studied (384, 389, 488–491). The Nme *cps* locus is divided into three regions. Region A contains four polycistronic capsule biosynthesis genes (*css*A-D), while Regions B and C contain the capsule translocation (*ctr*E-F) and transport genes (*ctr*A-D), respectively. Transcription of the *cps* locus, except

serogroups I and K, is initiated within the intergenic region (IGR) between Region A and Region C (389, 488, 490). The two operons are divergently transcribed from the promoter for RpoD, the housekeeping Sigma factor, located inside the IGR (389).

The capsule is thought to be one of only a few traits that make Nme unique among other *Neisseria* spp. Recently, Clemence et al., 2018 showed that 13 human and animal commensal *Neisseria* have a complete set of *cps* genes (311). I showed that *N. musculi* (Nmus), a mouse commensal *Neisseria* which is closely related to human-dwelling commensal and pathogenic *Neisseria*, produces a capsule (306). How capsule expression is regulated in commensal *Neisseria* is not known.

Based on the transcriptional regulation of meningococcal capsule, I hypothesize that the IGR separating the capsule biosynthesis and transport loci in Nmus controls capsule expression in this commensal. In this study, I replaced the 227-bp IGR with a Kanamycin cassette and showed that the 227-bp IGR controls capsule production. I also demonstrated that capsule expression reduces biofilm formation on abiotic surface.

Results

Analysis of the *Neisseria musculi* capsule loci.

Genomic sequences for a large number of *Neisseria* spp. including *Neisseria meningitidis* (Nme) and *Neisseria musculi* (Nmus) are available to the public in the *Neisseria* pubmlst.org database (www.pubmlast.org/neisseria) (305). Analysis of commensal *Neisseria* capsular polysaccharide (*cps*) homologues was previously performed by Clemence et al., 2018 (311). Based on their analysis, I generated a schematic diagram of Nmus *cps* genes and compared their arrangement with the *cps* loci of Nme FAM18 (Fig. 4.1A). The diagram shows that Nmus capsule transport genes (*ctr*A, *ctr*B, *ctr*C, and *ctr*D) and capsule synthesis genes (*css*A, *css*B, and *cs*C) are arranged in a similar order as in Nme. Capsule translocation genes (*ctr*E and *ctr*F) in Nmus are contiguous and are in the same orientation. They are located downstream of the capsule synthesis genes, which are also contiguous and in the same orientation. The capsule transport and synthesis genes (*ctr*A-D and *css*A-*cs*C, respectively) are arranged in the opposite orientation to these two loci. They are separated by 227 bp noncoding region, the intergenic region, or IGR. The IGR of meningococcal sialic acid producing serogroups is 134-bp (491). Downstream of Nmus capsule synthesis genes are 4 additional genes (A4, A5, A6, and A7) encoding hypothetical proteins, whose deduced amino acid sequences with motifs common to sugar modification proteins (311), are flanked upstream by the capsule synthesis genes and downstream by the capsule translocation genes.

The Nme IGR is 134 bp and contains transcriptional and translational regulatory elements, including binding sites for the RpoD housekeeping Sigma factor (391, 494) and for a two-component regulatory system MisS/R (392, 493), it also contains the insertion element IS1301 (382, 390, 494) and a 8-bp direct repeat that functions as an RNA thermosensor (394) (Fig. 4.1B, top panel). My analysis of the Nmus 227 bp IGR dentified additional potential

transcriptional and translational regulatory elements (Fig. 4.1B, top and bottom panels). There are consensus RpoD (-35, -10) and RpoN recognition sequences (307), a 20-bp Thymidine repeat, and a 17-bp direct repeat. Consensus sequences for the transcription activator (Npa) are also located inside the coding region of *css*A. Together, these data suggest that Nmus and Nme capsule expression may be regulated by different mechanisms. Future experiments will test whether these regulatory elements are functional.

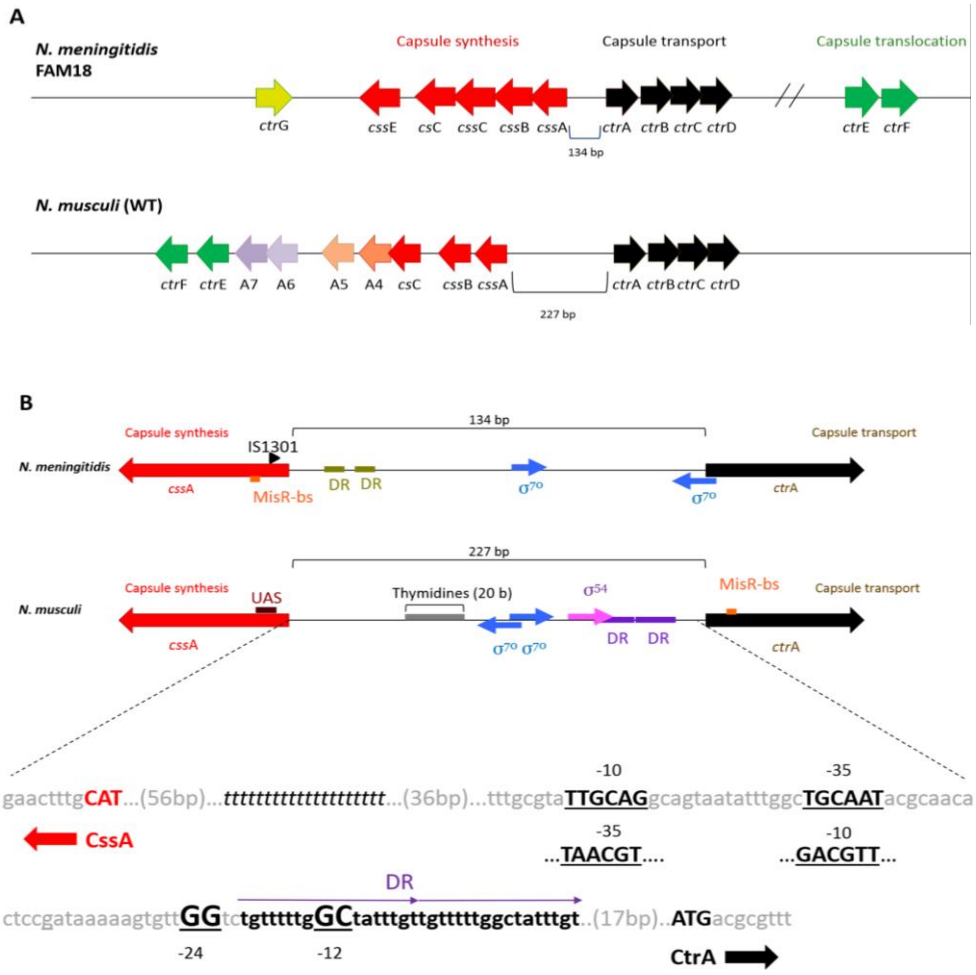

Fig. 4.1. Genetic organization of the capsule loci of *N. meningitidis* FAM18 and *N. musculi*. A) Diagram of meningococcal and Nmus *cps* genes. The genes responsible for capsule polymer synthesis are in red arrows, capsule transport are in black, and capsule translocation are in green. In Nme FAM18, the capsule synthesis and transport genes are divergently transcribed in operons. In Nmus, there are 4 additional genes (A4, A5, A6, and A7) which encode glycosyl transferase GT4, riboflavin synthase subunit *β, N*-acetyltransferase, and spore coat protein GT4 respectively (311). Capsule transport and translocation proteins are highly conserved between these two *Neisseria* species (> 50% amino acid identity and query coverage). Two diagonal lines represent >4kb between genes. Arrows depict gene orientation. Arrows overlapped means the start and stop codons for those genes are also overlapped. B)

Summary of potential regulatory elements within the meningococcal and Nmus IGR. Schematics of Nmus and meningococcal IGR are shown with partial Nmus intergenic sequence displayed below. The start codons of CtrA and CssA are shown in black and red capital letters, respectively. The putative promoter elements are underlined and in bold. The IS1301 insertion happened within the coding region of meningococcal *css*A. The 8-bp direct repeat (DR) moiety served as RNA thermosensor is marked by green bars. In Nmus, the poly(T) track which might serve as the transcriptional terminator is italicized. The 17-bp direct repeat moiety is marked by purple bars. The potential MisR-binding sites are marked in orange.

N. musculi capsule synthesis, transport, and translocation genes to form potential operons.

The similarity in genetic organization of Nmus and meningococcal *cps* genes suggests that the four *ctr*A-D open reading frames (ORFs) comprise an operon while the ORFs in the capsule synthesis region comprise another operon. To test this hypothesis, I performed RT-PCR reactions from log phase bacteria to determine whether these genes are cotranscribed. Using primer sets that span two or more contiguous ORFs (Table 4.1), I confirmed that the capsule transport genes (*ctr*A, *ctr*B, *ctr*C, and *ctr*D) are co-transcribed (Fig 4.2). The capsule synthesis genes (*css*A-*cs*C), the 4 additional ORFs (A4-A7), and the capsule translocation genes (*ctr*E and *ctr*F) are also co-transcribed (Fig 4.2). Even though the longest transcript I could amplify was 1340bp (A5-A7), I was able to show that all three regions (capsule synthesis, hypothetical ORFs, and capsule translocation) are transcribed together, by using primers that amplify two neighboring genes at a time.

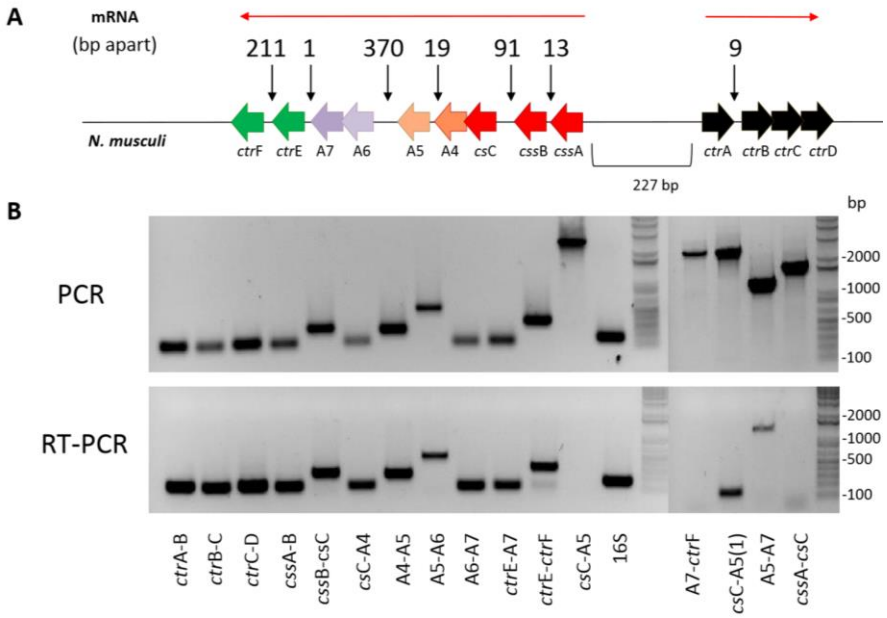

Fig. 4.2. The capsule transport, capsule synthesis, and capsule translocation operons in *N. musculi*. A) Diagram of Nmus *cps* genes. The two thin red arrows above the genes indicate the mRNA transcripts mapped to this locus. The number above each black arrow indicates the number of nucleotides between each ORF. B) Top panel: Products from the *cps* primer sets, which served as positive controls. Amplicons were produced by PCR using primers listed in Table 4.1. Bottom panel: Each transcript was produced by RT-PCR using primers sets that covered the *cps* genes indicated at the bottom of the panel. The capsule transport genes (*ctr*A to *ctr*D) are cotranscribed as one operon. The capsule synthesis genes, the A4-A7 ORFs, and the translocation genes (*css*A to *ctr*F) are transcribed as a separate single long transcript.

The 227-bp intergenic region is required for production and expression of *N. musculi* capsule.

To test whether the 227-bp *ctr*A-*css*A IGR is required for the production of Nmus capsule, I generated the capsule mutant (Δ*cps* 227::Kan) which has the Kanamycin resistance cassette inserted in place of the IGR, and this insert is flanked by transcriptional stops. I also constructed the corresponding complemented strains (Δ*cps*::WT_{cps}) (see Materials and Methods). They are shown schematically in Fig. 4.3A. These mutants are examined for capsule production using the Alcian blue dye (453) to stain extracts from WT, Δ*cps* 227::Kan, and Δ*cps*::WT_{cps} . A high molecular weight alcian blue-reactive smear is detected in the WT and complemented strains, but not in the capsule mutant (Fig. 3B). To further confirm the transcription of *cps* genes are initiated from the IGR, I examined the expression of individual genes within the *cps* loci by RT-PCR. Transcripts for the *cps* genes are detected from WT and complement strain but not in the

Δ*cps* 227::Kan (Fig. 4.4). This result also supports that the capsule synthesis genes, A4-A7 ORFs, and the capsule translocation genes are transcribed as one operon (Fig. 2, Fig. 4). Taken together, these results show that the 227 bp IGR controls the expression and production of the Nmus capsule.

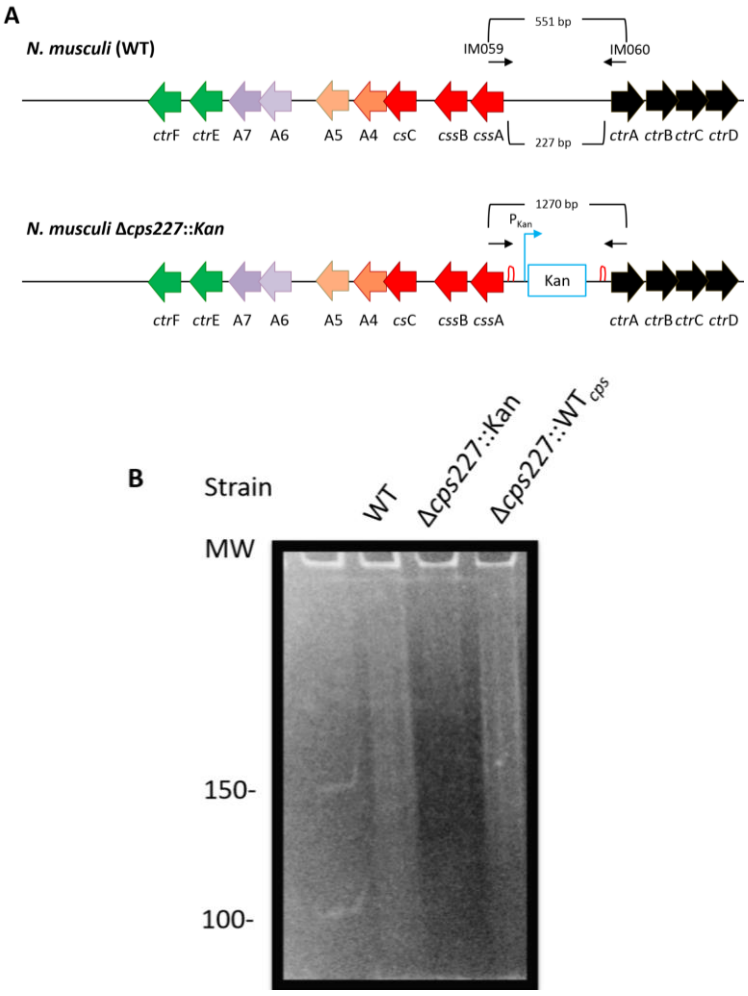

Fig. 4.3. The 227-bp intergenic region is required for the production of *N. musculi* capsule. A) Diagram of the *cps* genes in WT and capsule mutant. The 227-bp IGR was replaced by a kanamycin cassette and flanked by two transcriptional terminators. The deletion was verified by PCR, using primers IM059 and IM060. The expected size for this region is

551bp and 1270bp for the kanamycin construct. B) Alcian blue staining of a 6% SDS-PAGE containing lysates of these strains.

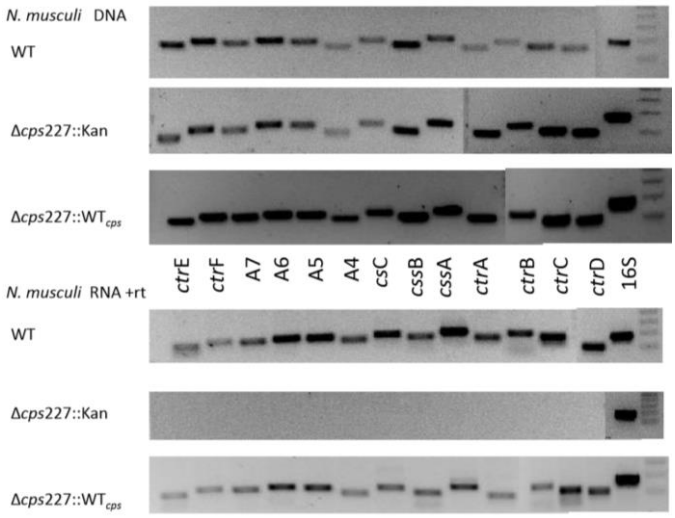

Fig. 4.4. Transcription of capsule synthesis, transport, and translocation operons is initiated from the intergenic region. Top three panels : Products from the *cps* genes, which served as positive controls. Amplicons were produced by PCR using primers listed in Table 4.1. Bottom three panels: Transcripts of the capsule synthesis, transport, and translocation operons amplified with primers listed in Table 4.1. WT: parental wild type Nmus; Δ*cps*227::Kan: *N. musculi* capsule mutant; Δ*cps*227::WT_{cps} : *N. musculi* capsule complemented strain; rt: reverse transcriptase; DNA: Nmus genomic DNA.

Capsule production inhibits biofilm formation in *N. musculi.*

The presence of the capsule can affect the overall surface properties, such as biofilm formation and growth rate, of the bacteria (370, 466, 495, 496). In Nme, CPS inhibits biofilm formation on the surfaces of plastic tubing, polystyrene plates, and on glass surfaces in flow chambers (497–500). Yi et al., 2004 also suggested that meningococcal carriage isolates, usually non-capsulated, are better at forming biofilms than the invasive isolates, which are capsulated (499). However, both capsulated and non-capsulated meningococci formed similar biofilms on monolayers of human bronchial epithelial cells (501). To test whether the presence of capsule affects the biofilm formation in Nmus, I compared biofilm formation on abiotic surface by WT, Δ*cps*227::Kan, and the complemented strain, using the crystal violet retention assay (269). I observed that both capsulated and non-capsulated Nmus were able to form biofilms on polystyrene plates. However, capsule mutant formed significantly more biofilms than WT after 6

and 24 hours of static growth (Fig 4.5A). The capsule mutant also formed morphologically distinct biofilms, compared to WT, after 24 hours of static growth (Fig 4.5B). Finally, loss of capsule production did not affect the growth in broth of Nmus, as shown in Fig. 4.6. These results suggested that capsule expression can alter the Nmus intercellular interactions.

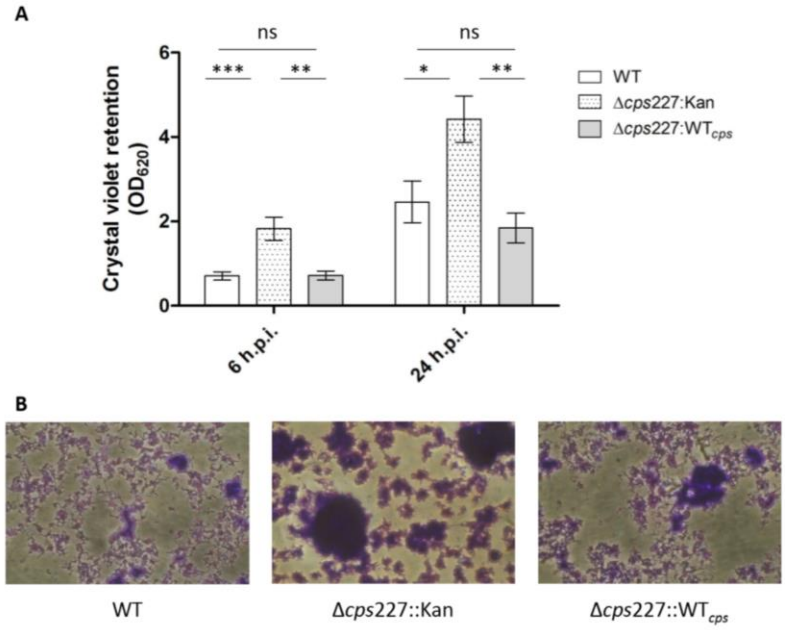

Fig. 4.5. *N. musculi* Δ*cps*227::Kan forms more robust and morphologically different biofilms than WT. A) Crystal violet staining WT, Δ*cps*227::Kan, and Δ*cps*227::WT_{cps} biofilms at 6 and 24 hours post inoculation (h.p.i.). The average and standard error of the mean from 3 independent experiments are shown. (Student's unpaired *t*-test; *$P \leq 0.05$, **$P \leq 0.005$, ***$P \leq 0.0005$, ns=not significant). B) Images of Crystal violet-stained biofilms of WT, Δ*cps*227::Kan, Δ*cps*227::WT_{cps} , 24 hours after inoculation onto on polystyrene plates (static growth).

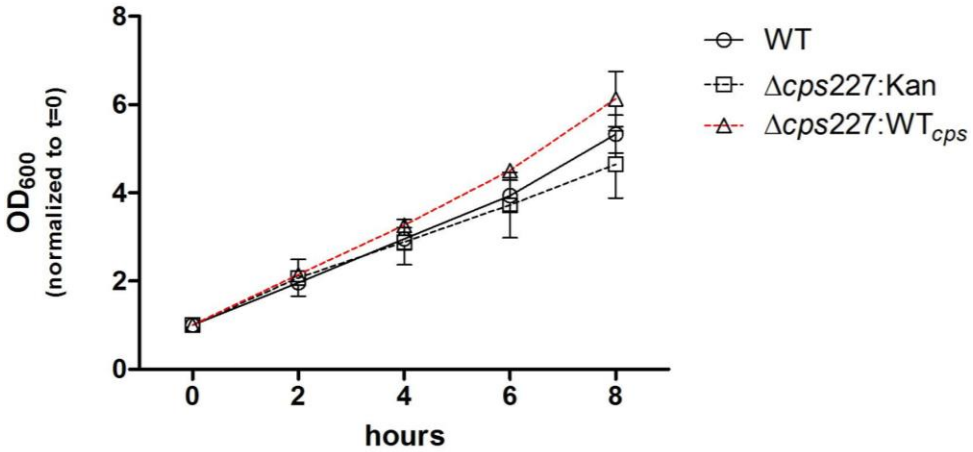

Fig. 4.6. Growth curve in broth of WT, Δ*cps*227::Kan, and Δ*cps*227::WT$_{cps}$. Optical density (OD_{600}) of cultures of *N. musculi* WT, Δ*cps*227::Kan and complemented strain Δ*cps*227::WT$_{cps}$. Values are the average of 3-6 independent experiments. Significance was determined using Student's unpaired *t*-test on the average OD_{600} per strain at each time point

Discussion

The polysaccharide capsule is thought to be a trait of Nme within the *Neisseria* genus that distinguishes this pathogen from closely related commensal spp. It is a major host interaction factor for this pathogen because it functions in protecting this pathogen inside the bloodstream against phagocytosis by macrophages and preventing complement-mediated and cationic peptide killing, as well as escaping antibody recognition through antigenic variation and immune mimicry (373–382). However, recent analyses of the genomes of 13 human and animal commensal *Neisseria* identified capsule homologues in these spp. (311). In this study, I characterized the *cps* homologues in Nmus. The Nmus classes of *cps* genes are arranged in a similar orientation as the *cps* loci in Nme, with capsule synthesis genes (*css*A-*cs*C), capsule transport genes (*ctr*A-D), and capsule translocation genes (*ctr*E-F) involved in capsule production (Fig. 4.1A). The capsule translocation and capsule synthesis genes are potentially transcribed as one single mRNA, while the capsule transport genes are divergently transcribed as a second transcript (Fig. 4.2). There are 4 additional genes that encode putative sugar modification proteins (A4-A7) downstream of the capsule synthesis genes. Their presence, and the fact that they are co-transcribed with *cps* synthesis genes, suggest that the capsule polysaccharide structure might be different from Nme (Fig. 4.1A). Capsule biosynthesis genes were recently reported in commensal *Streptococcus* spp. (*S. mitis* strains, *S. oralis*, and *S. infantis*) (502, 503). Taken in context with this finding, the assumption that the polysaccharide capsule is a trait of invasive bacteria is no longer true.

There is increasing evidence that many pathogenic *Neisseria* host interaction factors are also present in commensal *Neisseria* spp. (191, 303, 306, 311, 504). These factors include the Type IV pilus, Factor H binding proteins, and capsule. With the exception of the Tfp *pilE* (307, 308), little is known about how commensal and pathogenic *Neisseria* regulate expression of these shared factors. By replacing the Nmus IGR with a Kanamycin cassette, we showed that the production of Nmus capsule is controlled by the 227-bp IGR (Fig 4.3 and 4.4). Besides the putative housekeeping Sigma factor RpoD binding sites, there are also additional regulatory elements (a potential RpoN and activator Npa binding sites, and the poly(T) sequence) within the Nmus IGR (Fig. 4.1B). In commensals, pilin expression is regulated by Sigma factor RpoN, integration host factor, and the two component regulatory system (Npa/Nps) (307, 308). These results suggest that commensals might regulate capsule expression differently from Nme. They might utilize the RpoN/Nps/Npa system widely in order to adapt to broader niches for colonization. Future experiments will confirm the functions of these putative regulatory elements in Nmus capsule production.

Previous studies have shown that capsule expression affects biofilm formation (503, 505, 506). In Nme, capsule expression inhibits biofilm formation on abiotic surfaces (498, 499) while forming robust biofilms on HBE cells (501). In *S. pneumoniae*, non-capsulated strains also form more biofilms on polystyrene plate compared to capsulated strains (507). These findings suggest that different bacterial CPSs have different effects on biofilm formation. Therefore, I test the ability of Nmus capsule-null mutant in biofilm formation. I showed that both capsulated and non-capsulated Nmus form biofilms on abiotic surface (Fig 4.5A). I also showed that the capsule-null mutant forms more biofilms and that these biofilms have a different morphology compared to WT (Fig 4.5A and 4.5B). Unlike *S. pneumoniae* (508), the Nmus capsule-null mutant has the same growth rate as WT in broth (Fig. 4.6). At this time, it is not known whether

changes in surface charge, hydrophobicity, and autoaggregation also play a role in altering Nmus biofilm formation on abiotic surfaces. Future experiments will test whether capsules also control biofilm formation on different surfaces such as mouse epithelial cells or inside a flow chamber.

Approximately 30% of meningococcal carriage strains are non-serogroupable. The non-capsulated strains are recovered from school children at a higher rate than strains with defined serogroups (509, 510). This suggests that regulation of capsule expression play an important role in asymptomatic colonization and transmission. However, due to the strict human tropism of Nme, there is no animal model which can fully recapitulate the meningococcal pathogenesis. Identification of capsule expression in Nmus and the development of the natural mouse model of commensal *Neisseria* asymptomatic colonization and persistence (306) will allow us to understand how capsule expression affects colonization, persistence, and transmission in a natural setting. The Nme capsule also helps the pathogen to evade the immune system, by inhibiting complement-mediated and antimicrobial-peptide killing, resisting phagocytosis, and preventing antibodies recognition through molecular mimicry. Therefore, it will be interesting to investigate how different structures of commensal *Neisseria* CPS interact with the host. .

Materials and Methods

Bacterial strains and growth conditions. *Neisseria musculi* AP2365 (188) and Δ*cps*227::Kan, and Δ*cps*227::WT$_{cps}$ were used throughout this study. All strains were grown on Gonococcal Broth (GCB) agar plates supplemented with containing Kellogg's supplements I and II at 37°C with 5% CO_2 (479).

Growth curves. AP2365, Δ*cps*227::Kan, and Δ*cps*227::WT$_{cps}$ were grown for 16 h at 37 °C, 5% CO_2 on GCB agar containing Supplements I and II and the appropriate selective antibiotics. Cells were scraped from the plates, suspended in supplemented GCB, and diluted to an OD_{600} of 0.05. 2 ml of each bacterial sample was added to 60mm dishes and incubated at 37 °C, 5% CO_2. Bacterial density was measured every 2h for 8 hrs using a Beckman Coulter DU730 spectrophotometer (Brea, CA). The cell density at each time point was expressed by normalizing the OD600 value at that time of collection over the OD_{600} value at t=0.

RNA extraction, cDNA synthesis, and RT -PCR. Bacterial cells were grown to mid-log phase in GCB broth containing Supplements I and II, and total RNA was extracted using Trizol (Invitrogen) according to manufacturer's instructions. Contaminating DNA was removed using DNA -free (Ambion). The quality and amount of RNA was determined by spectrophotometry (NanoDrop, Therm Scientific). For RT -PCR, 1000 µg of RNA were used to generate the first strand using M -MLV reverse transcriptase (Promega), according to manufacturer's instructions. This was followed by a PCR reaction using GoTaq green master mix (Promega). *N. musculi cps* was amplified using primers in Table 1. Capsule transport genes were amplified using primers IM084-IM091. Capsule synthesis genes were amplified using primers IM092-IM105. Capsule translocation genes were amplified using primers IM106-IM109. *N. musculi* 16S was amplified using primers MR493 and MR494. Capsule transport operons were determined by detecting

transcripts using primers IM110-115. Capsule synthesis and translocation operons were determined by detecting transcripts using primers IM116-IM141.

TABLE 4.1. Primers used in this study.

Primer pairs	Use	Sequence	Source
MR493	Detect *16*S transcript	ATCCTGGCTCAGATTGAACG	Ma et al., 2018
MR494		CCGCTTTCCTTCTCAAAGTG	
IM084	Detect *ctr*A transcript	AGTATCCGTATGCCGCTCAC	This study
IM085		AGCTGCACCGAAATATCCTG	
IM086	Detect *ctr*B transcript	TTAAAGAAGCGGCAGGAGAG	This study
IM087		ACTTGGTCAAGCTGGGTTTG	
IM088	Detect *ctr*C transcript	ATGCGGTAGGAAAACTGTGG	This study
IM089		ACCCACAAAGCGTATTCCTG	
IM090	Detect *ctr*D transcript	CCGACTAAAGGCGAGATCAG	This study
IM091		AAAGCGCAAATTATCCATGC	
IM092	Detect *css*A transcript	CAGATGGACGTTGTGTTTGC	This study
IM093		AGGGAAAATTTCGGAGAAGG	
IM094	Detect *css*B transcript	ATTGATCTCACTGGCCAACC	This study
IM095		GGTCGGGTGTTTTATTCACG	
IM096	Detect *cs*C transcript	ACAGCGTAACCAGACCTTGG	This study
IM097		AGCCGGGTCTTCTTTATTCC	
IM098	Detect A4 transcript	TCCTGCCAATCGGATTTTAG	This study
IM099		TTTAATCAGGCTGGGTACGG	
IM100	Detect A5 transcript	TGGGGCAGTAGCTTTCTTTC	This study
IM101		AGGGCAGCAATACCTTTTCC	
IM102	Detect *A6* transcript	CGACCATAAATGGGGTGTTC	This study
IM103		ATGAACAGCGGCAGGTAATC	
IM104	Detect A7 transcript	TTCGACCGCTTTATCGATTC	This study
IM105		ACGGGAAACATCGCTACATC	
IM106	Detect *ctr*E transcript	CATAAAAAGGCAGGCCGTAG	This study
IM107		GACCAAACCGTAACCGAATG	
IM108	Detect *ctr*F transcript	AGCTGCCTTGAAAGGTGATG	This study
IM109		GTTACGCTCAACAGCACCAG	
IM110	Detect *ctr*A-*ctr*B transcript	CGCCGATTGTCAGTAGTGTG	This study
IM111		TAGCAGCTGTTGCCTGTTTC	
IM112	Detect *ctr*B-*ctr*C transcript	ATCGGTTTGATGCTTTACGG	This study
IM113		TTACCCGCCCTTGAATAACC	
IM114	Detect *ctr*C-*ctr*D transcript	CAAGTTCAGCAAAGGGGTTG	This study
IM115		AGAATGCCGATTTTCTCACC	
IM116	Detect *css*A-*css*B transcript	TACGCCGATAACTTTTACGC	This study
IM117		TGCCATTTAACGAGGAAAGC	
IM118	Detect *css*B-*cs*C transcript	TTCTCAAGCTGTTCATCATCG	This study
IM119		TGTAGATTCCGTGGTGGTTG	
IM120	Detect *cs*C-A4 transcript	CTTTAGGGAGGCAATACATGC	This study
IM121		TCGGCCAAACAATGGATTAC	
IM122		CTGAAAACAGATGGGCAATG	This study

IM123	Detect A4-A5 transcript	AACGGTCAGCGGACAAAG	
IM124	Detect A5-A6 transcript	GTTAAGGCATGGGCAGTTTG	This study
IM125		ACCGCCAATGGAACATTATC	
IM126	Detect A6-A7 transcript	GGAGGGAAAACCAACTGCTG	This study
IM127		TCTTAGAAGCAGAGATCAACAAGG	
IM128	Detect A7-*ctr*E transcript	AAGCCCGAAACTTGAGAAAAG	This study
IM129		GATTTGGAAATGGCCAAGG	
IM130	Detect *ctr*E-*ctr*F transcript	TCCGGGAATAAAGGTGTTTG	This study
IM131		AAACCGCCGTCAATATTCTG	
IM132	Detect *css*A-*cs*C transcript	GATTCCGGCGGTATTCAAG	This study
IM133		TCCTGTTGGAAATAACGTTTCA	
IM134	Detect *cs*C-A5 transcript	TGACGGAAATCGTATTGCTTT	This study
IM135		ACTCATTTCGGCCAAACAAT	
IM136	Detect *cs*C-A5 (1) transcript	CTGAAAACAGATGGGCAATG	This study
IM137		GCACAAAAATTCACCGAAGC	
IM138	Detect A5-A7 transcript	CGGCGGGTATGCAGAATC	This study
IM139		ACAAACCGGCACAGCTTACT	
IM140	Detect A7-*ctr*F transcript	AGCCATTGCGAGAAGGTTTT	This study
IM141		AAGCGGCAGCTTTATTTGAA	
IM059	Detect Δ*cps*227::Kan mutant	TGCCGAATACAATCAGCAGT	This study
IM060		TCCACATCAACCACAGCAAC	

IM132	To delete the 227-bp IGR	**TTTGATAGCCTCCGGCCGGGTGCCGAATA CAATCAGCAGTTTTTTAGCACGTGCGGGG GGAGGGGATACCGCTATGGGATTAAAAGC AAATGAATCGTTGAATGTTGTCAATGTCAG AGCACTATTATCGGCGTTATTGTTTTTTTGC ACGGCACAGCCTTTCGTATTACACGGGTA GAACTTTGTCAT**TTAAGGGATGCAGTTTAT GCATCCCTTAACTCGAGGGCTTGACACTTT ATGCTTCCGGCTCGTATAATGTGTGGATAG TGGGAGGAAAGCATGATTGAACAAGATGGA TTGCACGCAGGTTCTCCGGCCGCTTGGGT GGAGAGGCTATTCGGCTATGACTGGGCAC AACAGACAATCGGCTGCTCTGATGCCGCC GTGTTCCGGCTGTCAGCGCAGGGGCGCCC GGTTCTTTTTGTCAAGACCGACCTGTCCGG TGCCCTGAATGAACTGCAGGACGAGGCAG CGCGGCTATCGTGGCTGGCCACGACGGGC GTTCCTTGCGCAGCTGTGCTCGACGTTGTC ACTGAAGCGGGAAGGGACTGGCTGCTATT GGGCGAAGTGCCGGGGCAGGATCTCCTGT CATCCCACCTTGCTCCTGCCGAGAAAGTAT CCATCATGGCTGATGCAATGCGGCGGCTG CATACGCTTGATCCGGCTACCTGCCCATTC GACCACCAAGCGAAACATCGCATCGAGCG AGCACGTACTCGGATGGAAGCCGGTCTTG TCGATCAGGATGATCTGGACGAAGAGCATC AGGGGCTCGCGCCAGCCGAACTGTTCGCC AGGCTCAAGGCGCGCATGCCCGACGGCGA GGATCTCGTCGTGACCCATGGCGATGCCT GCTTGCCGAATATCATGGTGGAAAATGGCC GCTTTTCTGGATTCATCGACTGTGGCCGGC TGGGTGTGGCGGACCGCTATCAGGACATA GCGTTGGCTACCCGTGATATTGCTGAAGAG CTTGGCGGCGAATGGGCTGACCGCTTCCT CGTGCTTTACGGTATCGCCGCTCCCGATTC GCAGCGCATCGCCTTCTATCGCCTTCTTGA CGAGTTCTTCTGAGCCGTCTGAAGTTTAAA CATCGATAAAATGCCGTCCGAAGCTTGAGT TCAGACGGCATTTT**ATGACGCGTTTCCAGT TGCCGGCCGGATGCATTCTATTGCTGCTTT TAGGGTGTGGTGCTTTGCCTACTTCAGGG CCGAATCAAACCAAGGTGATAAGTTTGCA AAATCAAAGCCGCCAGGCGGCAGTGCCT GATGTTGCTGTGGTTGATGTGGACGAGCG GGTAATTTCCACACTGTAT**	This study
IM133	To complement Capsule mutant	ACGCCTTCACGAAACAGATT	This study
IM134		AATTATTCTGCGCCACACG	

*The regions of primers IM132 that anneal to the *css*A *and ctr*A of AP2365 are in bold.

Construction of Δ*cps*227::Kan and its complemented strain. Table 4.1 lists the primers used for these constructions. In *N. musculi* Δ*cps*227::Kan, the 227-bp intergenic region (IGR) separating the capsule transport and synthesis operons was replaced with a Kanamycin (Kan) resistance cassette. Double-stranded, liner gene fragment IM132 containing flanking sequences for the *css*A and *ctr*A genes was ligated to a pGEMT vector (Promega). The recombinant plasmid DNA was introduced into AP2365 by spot transformation. Transformants were selected on GCB containing GCB agar containing Supplements I and II and Kan (50 mg/L). The Δ*cps*227::Kan mutant was confirmed by Sanger sequencing of PCR products generated with primers IM059 and IM060.

The complemented strain Δ*cps*227::WT_{cps} was constructed as follows. Primers IM133 and IM134 were used to amplify the region between *css*A and *ctr*A from AP2365, and the amplified DNA was introduced into Δ*cps*227::Kan by spot transformation. Transformants were plated on GCB agar containing Supplements I and II first. Then the Kan sensitive isolates were selected on GCB agar containing Supplements I and II and Kan (40 mg/L). The cps complemented region was confirmed by Sanger sequencing of PCR products generated with primers IM059 and IM060.

Capsule extraction. Capsule was extracted as described (44). AP2365, Δ*cps*227::Kan, and Δ*cps*227::WT_{cps} were grown on GCB agar for 16 to 18 h at 37 °C, 5% CO_2. Cells were suspended in phosphate -buffered saline (PBS) to an OD_{600} of 0.8. 1 ml of the suspension was pelleted by centrifugation (10,000 x g, 5° C) for 2 min. The pellet cells were resuspended in 0.5 ml PBS and incubated at 55°C for 30 min to allow the release of capsular material. Bacteria were again pelleted, and the supernatants were concentrated 10 -fold in an Amicon Ultra centrifuge filter with a 10,000 molecular weight cutoff. Capsular materiaI was separated by 6%

SDS -PAGE and stained with the cationic dye Alcian Blue (0.125% alcian blue in 40% ethanol/5% acetic acid; Sigma) for 2 h, and destained overnight in 40% ethanol/5% acetic acid.

Crystal violet retention assays. OD_{600} of 0.05 bacteria were inoculated into 1 mL GCB + Kellogg's supplements I and II in a 6 well dish. After 6 or 24 hrs, the media was aspirated at the wells and were washed gently 3 times with PBS. The cells were fixed in PBS containing 1 ml of 2% methanol-free formaldehyde for 20 minutes at room temperature. The fixing media was aspirated and were washed gently 3 times with deionized water. The fixed cells were then stained with a 0.1% Crystal violet solution for 10 minutes at room temperature and then washed gently 3 times with deionized water. Crystal violet retention was measured by washing the stained biofilms with 1 mL methanol and measuring the OD_{620} of the resultant rinse.

Acknowledgments

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Chapter 5

Discussion

Overview

The genus *Neisseria* includes at least 26 species of Gram-negative bacteria, and is commonly isolated from mucosal surfaces and the oral cavity of humans and animals (186, 197). *Neisseria* is among the ten most abundant genera in all 18 body sites sampled (511, 512). In addition, *Neisseria* spp. roughly account for 10% of the bacterial population on the tongue (75). Within the genus *Neisseria*, there are only two pathogens, *Neisseria meningitidis* (Nme) and *Neisseria gonorrhoeae* (Ngo), and they cause meningitidis/septicemia and gonorrhea, respectively, only in humans.

Because these two pathogens have such a high impact on global public health, investigators have put tremendous effort into understanding how they colonize and persist in the human body (265, 513–519). These pathogens not only have the ability to elicit clinical symptoms, they are also able to colonize mucosal surfaces silently. This trait – asymptomatic colonization and persistence – is inherited from their commensal *Neisseria* ancestors (435, 520). Around 3-20% of healthy individuals carry Nme in the upper respiratory tract and up to 60% of gonococcal cervical infections are asymptomatic (14, 238, 299, 437). Despite a vast literature on the biology of the *Neisseria*, the mechanisms that allow these pathogens to colonize and not induce disease are little understood.

In my dissertation, I investigated how commensal *Neisseria* colonize and persist in its natural host in the presence of a robust immune system. As commensal *Neisseria* are closely related to pathogenic *Neisseria*, and have many genes in common with the pathogenic spp., including genes known to promote pathogenic *Neisseria* interactions with their human host, my studies may also help us understand asymptomatic colonization by Nme and Ngo.

In Chapter 2, I described my role in developing a natural mouse model to identify mechanisms of *Neisseria* colonization and persistence. This model overcomes barriers imposed by the strict tropism of pathogenic *Neisseria* for its only host, the human being. The model pairs the laboratory mouse with *Neisseria musculi* (Nmus), a species isolated recently from wild mice by Nathan Weyand in the So laboratory. Nmus colonizes laboratory mice without having to be treated with antibiotics, or hormones or invasive procedures. One oral dose of Nmus results in stable asymptomatic colonization of the entire alimentary tract for as long as 1 year. Mouse strains that are used in the Collaborative Cross were examined, this allows investigations into host genes and alleles that are permissive for Neisseria asymptomatic colonization and persistence. CAST/EiJ (CAST) and A/J mice are highly susceptible to Nmus colonization/persistence while C57BL/6J (B6) has an intermediate susceptibility. The other five mouse strains (NOD/LtJ, NZO/HILtJ, PWK/PhJ, WSB/EiJ, and 129S1/SvlmJ) are resistant to colonization/ persistence. Innate immunity also determines susceptibility to Nmus. B6 mice lacking innate immune functions (MyD88) are highly susceptible to Nmus colonization. In contrast, adaptive immunity does not affect the colonization frequency. Finally, all inoculated mice are healthy.

In Chapter 2 and 3, I described the role of a common *Neisseria* host interaction factor, the Type IV pilus (Tfp), in Nmus colonization. Both the pilin-null mutant (Δ*pilE*) and the Tfp retraction-null mutant (Δ*pilT*) failed to colonize the oral cavity and gut of CAST mice. This indicates the presence of the Tfp as well as the ability of Tfp to retract are necessary for colonization/persistence.

The natural mouse model I developed will facilitate studies of *Neisseria*-host interactions from the angle of both host and microbe. It allows the identification of mouse alleles, immune

components, and neisserial host interaction factors that determine susceptibility/resistance to Nmus colonization/ persistence.

Finally, in Chapter 2 and 4, I described my studies on the Nmus capsular polysaccharide (CPS). The capsule was previously assumed to be a unique trait of invasive Nme. I showed that Nmus has a complete set capsule biosynthesis genes and expresses a capsule. The arrangement of *cps* genes in Nmus *and* Nme is similar. Using RT-PCR and specific primer sets spanning two or more genes, I showed that the capsule transport genes (*ctr*A-D) is transcribed as one transcript while the capsule synthesis (*css*A-*cs*C), the 4 additional ORFs (A4-A7), and translocation genes (*ctr*E-F) are divergently transcribed in another transcript. I showed that the intergenic region (IGR) that separates the capsule transport and synthesis genes have potential transcriptional and post-transcriptional regulatory elements. Consistent with this finding, my results show that the IGR is required for capsule expression and production. Finally, I showed that the capsule influences biofilm formation. Taken together with published reports on Nme capsule expression, my data strongly suggest that Nmus and Nme likely use different mechanisms to regulate capsule production, and by implication, bacterial social interactions with its host.

In this chapter, I discuss how the development of our natural mouse model for Neisseria asymptomatic colonization will broaden our understanding of *Neisseria*-host interactions and the similarities of commensal and pathogenic *Neisseria.*

Models for studying *Neisseria* colonization and persistence

According to the Damage-response frame work proposed by Arturo Casadevall and Liise-anne Pirofski (313) (see Chapter 1.1, Table 1.1.3), colonization describes a state where the intensity

of damage induced by the host/microbes is not enough to affect homeostasis among the microbial flora, host immune responses, and bacterial-host interactions. This state is equivalent to the state of commensalism when damage is minimal. During either colonization or invasive disease, bacteria have evolved different strategies to interact with the host's immune system and the microflora so that they can survive inside the host.

Researchers have developed a number of *in vitro* and *in vivo* model systems for studying *Neisseria*-host interactions. Primary and established cell lines and human tissues and primary human organs are used to study attachment and invasion. As described in Chapter 1.3 (Fig. 1.1.6), these *in vitro* systems have contributed to our understanding of the *Neisseria* factors that promote adhesion and intimate attachment, the Nme and Ngo intracellular lifecycle, and the host cell response to neisseiral attachment and invasion. However, these *in vitro* systems cannot recapitulate the *in vivo* humoral systemic responses and the state of asymptomatic colonization.

Human challenge model

Since pathogenic *Neisseria* have strict tropism for humans, human volunteers have been used to understand the pathogenesis of Ngo in vivo (399–401, 473, 522). These human studies can only be performed using male subjects because gonococcal infection in women could lead to many complications. Male volunteers are inoculated intraurethrally with 10^6 CFU of gonococci through a pediatric catheter. This procedure causes infection in 90% of the subjects after 1 to 5 days post-inoculation (401). They develop urethritis with purulent exuldate or dysuria. Viable gonococci are detected from the urine and genital secretions right after inoculation and during signs of infection. Between these two time points, there is an eclipse period during which no viable gonococci can be recovered (522). Volunteers are treated with antibiotics once they develop signs of infection to terminate infection. Since Ngo is easily genetically manipulated,

human volunteer studies are used to identify the roles of gonococcal genes during early colonization (see Chapter 1.5). Induction in pro-inflammatory cytokines (IL-8, IL-6, TNFa, and IL- 1B) is observed in the urine samples of infected subjects (404). In addition, anti-gonococcal antibody production by circulating B cells is also observed after infection (522).

The human challenge model allows investigators to better understand gonococcal-host interactions in terms of host interaction factors and host early immune response to infection. It provides a model for gonococcal vaccine development and testing. However, this experimental system is limited to investigating gonococcal genes important in establishing infection in the male human urethra. Nevertheless, this system has identified iron utilization through the transferrin receptor is essential for early infection. Due to the ethical requirement for prompt treatment of infection, long term evaluation of human immune response cannot be studied. In addition, 30-50% of gonococcal infected women ,and 5-10% of infected men ,are asymptomatic (14, 258). However, asymptomatic colonization during experimental human infection with WT gonococci is rarely observed. This result indicates that the human challenge model does not fully recapitulate the pathogenesis of Ngo during natural infection.

Animal models of Neisseria colonization

The development and use of animal models began in the late 1930s. Besides humans, the chimpanzee is the only animal species which shows localized gonococcal urethral infections from 3 to 6 weeks (523). Chimpanzees have demonstrated strain-dependent acquired resistance to gonococcal infection after systemic immunization and male-to-female transmission (406, 524, 525). However, biomedical research using chimpanzees is no longer possible since 2015.

Various non-primate models including rabbits, guinea, pigs, mice, and chicken embryos, transgenic humanized mice (tg-CEACAM1/tg-CD46/tg-transferrin) are used in the study of various facets of gonococcal and meningococcal pathogenesis (407, 408, 415–417, 514, 526–528). With different model systems and inoculation routes, these models have shown significant immunity and cross-protection against different neisserial strains after systemic immunization. These models open up opportunities to understand the nature of host cell receptors/proteins that are important for neisserial attachment/invasion and survival. They also provide opportunities to test the efficacy of candidate antibiotics and vaccines. Since humans are the only natural host of these two pathogens and they are highly adapted to humans, all primate or nonprimate colonization model are heterologous systems and have their own advantages and disadvantages.

Taylor-Robinson et al. described the use of hormone (17β-estradiol) to extend gonococcal colonization in germ-free BALB/c mice (530). In 1999, Anne Jerse developed a mouse infection model which allows colonization of gonococci for an average of 10-12 days (408). This mouse gonococcal infection model involves treating the BALB/c mice with water-soluble 17β-estradiol and antibiotics to suppress the growth of commensal flora. Ngo colonizes the cervicovaginal lumen, including the lamina propria (530). Endometrial cultures are positive in 17-20% of infected mice (408), which is similar to reported rates for endometrial infections in women (484). Increased local production of pro-inflammatory cytokines and chemokines (IL6, TNFα, KC, and MIP-2) is also observed on day 5 post inoculation (531).

However, upper reproductive tract infection and asymptomatic colonization are not sustained in this BALB/c mouse model. Hormone and antibiotic treatments alter the commensal microbiota of BALB/c mice, which, in turn, might affect the host immune responses compared to natural

infection. In addition, there is no menstrual bleeding in mice which brings proteases, hemoglobin, and serum into the reproductive tract. Finally, mouse immunoglobulin A is not susceptible to gonococcal proteases. Therefore, this mouse BALB/c infection model is not suitable for studying neisserial-microflora interactions or asymptomatic colonization.

Even with the CEACAM1 mouse model (421), *N. meningitidis* only colonizes the CEACAM1 mice for a short period of time (up to 14 days with polymorphonuclear cells depletion) and does not lead to invasive disease. In humans, they can persist for as long as a year (299, 532). Therefore, all these animal models are used for studying early stage of infection.

Mouse and rhesus macaques models of commensal Neisseria colonization and persistence

There are at least 13 species of human commensal *Neisseria* which can colonize the same niches as the pathogenic species, including the nasopharynx, endocervix, and rectum of humans (186, 533, 534). Comparative genome analysis of human-adapted and animal commensal *Neisseria* (Chapter 2, Table 2.2-2.3) has shown that commensal *Neisseria* share a large repertoire of host interaction alleles with Nme and Ngo (188, 191, 303, 304, 306). However, relatively little effort has been directed at understanding the biology of commensal *Neisseria*. How do these commensal *Neisseria* interact with other microbes during colonization? Are they part of a stable microbiomes or is their presence in the microbiome dynamic? Do they use similar attachment mechanisms? How do they establish asymptomatic colonization and persistence under a robust immune system?

The natural mouse model of commensal *Neisseria* colonization and persistence together with other studies allow us to start answering some of these questions. The *N. macacae*- rhesus

macaques model (304) (RM) shows that horizontal gene transfer (HGT) and transmission of RM *Neisseria* derivatives (AP206 and AP678 are rifampicin resistance derivatives; AP312 is streptomycin derivative) happens within the hosts and between cage mates. These RM *Neisseria* derivatives also migrates to uninoculated sites- distal site of the nasal cavity. In contrast, there is no horizontal transmission of Nmus between cage mates in our natural mouse model of *Neisseria* colonization and persistence.

There could be several reasons to explain this horizontal transmission difference between these two animal models. First, this might due to the natural strain differences between *N. macacae* derivatives and Nmus. The number of *N. macacae* derivatives required to overcome the colonization bottleneck might be different from the number of Nmus. The lowest CFU of Nmus to establish colonization in mice is $2.3x10^6$ (data not shown) and we do not know the lowest CFU of *N. macacae* derivatives to establish colonization in rhesus macaques. Second, the grooming patterns between rhesus macaques are different from mice. Even though mice are coprophagic, we cannot detect the CFU of Nmus from the fecal samples that haven been eaten.

Whether *in vivo* horizontal gene transfer also happens with Nmus is yet to be determined. Both animal models can study asymptomatic colonization and/or persistence by commensal *Neisseria* species. However, the availability of genetic tools/ modifications for laboratory mice is more than rhesus macaques. In addition, the logistic and expense of the RM model as well as the relative inaccessibility of nonhuman primate facilities make it less favorable among investigators.

Neisseria-microbial interactions *in vivo*

Using spectral imaging fluorescence in situ hybridization (FISH) and metagenomics sequence analysis, Welch et al. identified a unique multi-genus organization of microbial consortium from supragingival dental plaque (535). This structure is comprised of nine taxa. Each taxon has segregated into distinct zones within the structure. *Neisseriaceae* is located at the perimeter of the structure. Due to its periphery location within the hedgehog structure, *Neisseriacece*, together with *Streptococcus*, *Haemophilus/Aggregatibacter*, and *Porphyromonas,* may contribute to aerobic metabolism (Fig 5.1) (535).

Since Nmus persists along the alimentary tract for at least 1-year post inoculation (Chapter 2, Fig. S3B), we can combine FISH, Laser Capture Microdissection, and whole genome sequencing to analyze these different micron-scale biogeographies. They will further our understanding of *Neisseria*-host/ *Neisseria*-*Neisseria*/ *Neisseria*-microbes interactions and the ecology among different communities. Different colonization niches have varieties in microbiome composition, pH and oxygen level, temperature, and immune profile, therefore, I hypothesize that Nmus segregates differently among these consortia while having diverse transcription profiles for adaptation. Our laboratory has generated a Nmus transposon mutant bank (Kate Rhodes, unpublished data) and will collaborate with the Mark Welch laboratory to identify colonization and persistence genes.

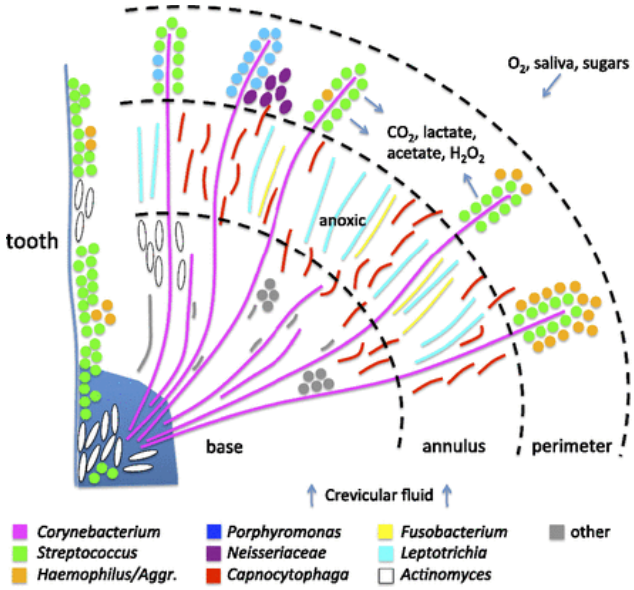

Fig. 5.1. Summary hypothesis for interpretation of hedgehog structures (535). *Corynebacterium* filaments bind to an existing biofilm containing *Streptococcus and Actinomyces.* At the distal tips of the *Corynebacterium* filaments, corncob structures form in which the filaments are surrounded by cocci, including *Streptococcus* and *Porphyromonas*, in direct contact with the *Corynebacterium* filament as well as *Haemophilus*/*Aggregatibacter* in contact with *Streptococcus*. Clusters of *Neisseriaceae* also occupy the periphery of the hedgehog. The *Streptococcus* cells create a microenvironment rich in CO_2, lactate, and acetate, containing peroxide, and low in oxygen. Elongated filaments of *Fusobacterium* and *Leptotrichia* proliferate in this low-oxygen, high-CO_2 environment in an annulus just proximal to the corncob-containing peripheral shell of the hedgehog. The CO_2-requiring *Capnocytophaga* also proliferates abundantly in and around this annulus. The base of the hedgehog is dominated by *Corynebacterium* filaments and thinly populated by additional rods, filaments, and/or cocci. [Reproduced from ref (535)]

Host interaction factors of pathogenic and commensal *Neisseria*

Based on *in vitro* studies, PilE, the structural subunit of the Tfp fiber, is essential for cell attachment (536) and PilT, which forms the motor for pilus retraction, is important for intimate attachment, invasion, and host cell signaling (267–269, 285, 353, 354, 537). A recent *in vivo* study using CD46-humanized mice showed a 50% reduction in meningococcal virulence when mice are infected with *pilT*-null mutant (477). The only *in vivo* neisserial Tfp pilin study was performed with human subjects. Based on the *in vitro* data, investigators were surprised to find that FA1090 *pilE*-null mutant was able to colonize most subjects while causing mild urethritis in

sub-portion of those colonized subjects (Cannon JG, unpublished data). This human study suggests that Tfp pilin is not important for colonization of male urethra.

Since the *in vivo* functions of Tfp pilin and retraction have never been tested for commensal *Neisseria*, I generated Nmus *pilE*- and *pilT*-null mutants, respectively. As expected, both mutants are non-transformable (Chapter 2, Table S1; Chapter 3, Table 3.1) and are less effective in attaching to cell when compared with WT (Chapter 2, Fig 2.3; Kate Rhodes unpublished data). In Chapter 2 and 3, I described using our natural mouse model of commensal *Neisseria* colonization and persistence to investigate whether the Tfp fiber and Tfp retraction are necessary for Nmus colonization and persistence. Both *pilE*- and *pilT*-null mutants are defective in colonizing the oral cavity and gut of CAST mice, demonstrating the importance of Tfp pilin and Tfp retraction in establishing colonization. These results also support this model in studying *Neisseria*-host interactions and asymptomatic colonization of *Neisseria*.

The discrepancy between the Tfp pilin human study and mouse model might be due to the difference(s) in the host cell attachment mechanism. Human-adapted commensal *Neisseria* (*N. subflava, N. mucosa, N. sicca, N. flava,* and *N. lactamic*) encode one to two *opa* genes (309, 310, 538) while Nmus encodes only one *opa* gene (*opa*D with 32% amino acid identity, 69% query coverage) (Chapter 2, Table 2.2). Whether *opa*D is functional is yet to be tested. In contrast, Ngo encodes at least 9-12 variant *opa* genes (539) and pilus-negative/Opa-positive gonococci can still adhere to epithelial cells *in vitro* (540). In addition, human studies have shown that there is a strong selection for Opa expression *in vivo* (541, 542). Therefore, I speculate that Opa proteins can facilitate host cell adhesion even in the absence of pilin during colonization of male urethra.

Other studies together with what I have presented in Chapter 2 and 4 have demonstrated that both human-adapted and animal commensal *Neisseria* are closely related to the pathogenic *Neisseria*. Among the human-adapted commensal *Neisseria*, *N. lactamica* has the largest set of host interaction genes shared with the pathogenic *Neisseria*. Although commensal *Neisseria* share similar host interaction factors with the pathogenic *Neisseria*, they may have different mechanisms in regulating the expression of these host interaction factors. Expression of Ngo *pilE* is dependent on housekeeping Sigma factor, RpoD (543–545), and activated by integration host factor (IHF) (546), and repressed by RegF (547). *N. elongata pilE* transcription is dependent on Sigma factor RpoN, and a two-component regulatory systems, Nps and Nps (308). The gonococcal *pilE* is expressed constitutively while the commensal *pilE* expression is regulated upon detection of an unknown signal(s).

When I compared the Nme and Nmus intergenic region (IGR; Chapter 4), I also found potential binding sites for RpoN and Npa within the IGR of Nmus. There are also other interesting regulatory elements, including the 20 bases of thymidines, the MisR-binding site, and the 17-bp direct repeat within the IGR and the coding region of the capsule genes. Even though the role of these sequences in regulating capsule expression still has to be tested, these findings suggest that different mechanisms may regulate capsule expression (transcriptionally and/or post-translationally) in pathogen Nme and commensal Nmus. Future promoter site mutagenesis experiments will identify the mechanisms that regulate capsule production in Nmus. The mouse model will facilitate these studies, allowing in depth analysis of the *in vivo* signals that influence capsule expression.

Nmus and RM *Neisseria* spp. also encode genes products that are components of current meningococcal vaccines (rMenB-OMV, Bexsero, and Trumemba) such as genome-derived

Neisseria antigens (GNA) 1220/33/1946/1030/2091, PorA P1, LptD, fHbp, and capsule (detailed in Chapter 2, Table 2.2; ref. 189). Capsule genes are also identified in human and animal commensal *Neisseria*, including *N. subflava, N. elongata, ,N. mucosa, N. dentiae, N. zoodegmatis, and N. weaveri* (311). In addition, serum raised against Bexsero has bactericidal activity against *N. cinerea* expressing fHbp (504). This demonstrates that antibodies generated against the meningococcal fHbp can cross-react with the *N. cinerea* fHbp. These data raise questions about unintended consequences from vaccination, for example, whether vaccines may influence the host-bacterial interactions of both commensals and the pathogenic *Neisseria*.

Several studies have indicated that commensal *Neisseria* spp. can protect the host against pathogenic *Neisseria*. For example, colonization of *N. lactamica* in the nasal cavity during early childhood is correlated with a reduced incidence of meningococcal infection (147, 150, 194, 548, 549). A recent study by Kim et al., 2019 (551) showed that commensal *Neisseria* spp. can kill the two pathogenic *Neisseria* due to differences in their DNA methylation pattern. *In vivo* experiments are consistent with this finding. *N. elongata* accelerates the clearance of Ngo from mice and a gonococcal mutant that cannot take up DNA resists *N. elongata* mediated clearance. In addition, the presence and abundance of commensal *Neisseria* are correlated with the progression of some diseases. For example, obese individuals have a sixfold higher amount of *N. mucosa* in their oral cavity when compared to persons of normal weight (550). *Neisseria* spp. are significantly more abundant in adult celiac patients and *N. flavescens* can be isolated from the duodenum of adult celiac patients (192). On the other hand, oral samples from inflammatory bowel disease patients have reduced Proteobacteria, mainly due to a twofold reduction in *N. mucosa* (551). In light of these observations, it is tempting to speculate that vaccination with either conjugate vaccine/rMenB-OMV/Bexsero/Trumemba might affect the

balance of *Neisseria* spp. inside the host. Therefore, it will be essential to monitor the impact of meningococcal vaccination on the carriage of commensal *Neisseria*.

Final Remarks

The work presented in this dissertation mainly focuses on identifying and characterizing components of commensal *Neisseria* that play a role in colonization of their natural host, using Nmus as a model commensal *Neisseria*. Mouse commensal Nmus have genes that are known or strongly suspected to promote interactions of pathogenic *Neisseria* spp. with their human host. By developing a natural mouse model which combines Nmus with the mouse, its natural host, I showed that host genetics, host immune status, and neisserial host interaction factors determine the status of colonization and/or persistence (Fig 5.4). Within the Collaborative Cross mouse strains, only CAST and A/J are highly susceptible to Nmus colonization while B6 has intermediate susceptibility, indicating host genetics play an important role in determining *Neisseria* colonization. Host innate immune system also determine the susceptibility of Nmus. B6 mice without innate immune system is now 100% colonized with Nmus. I showed that Nmus with a null mutation in either PilE or PilT is defective in colonizing its natural host. I also presented evidence that Nmus produces a capsule, and that the capsule influences biofilm formation. Finally, my sequence analysis strongly suggests that Nme and Nmus regulate expression of capsule genes using overlapping or entirely different mechanisms. Collectively, these observations support the value of the natural small animal model for understanding neisserial and host genes that are critical for asymptomatic colonization and persistence by commensal and pathogenic *Neisseria*.

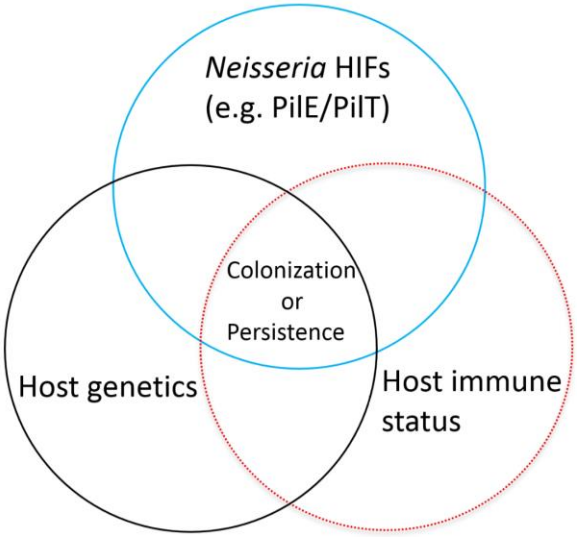

Fig. 5.4. Colonization determinants of *Neisseria*. The interaction between *Neisseria* host interaction factors, host immune status, and host genetics determine host colonization or persistence.

Future studies will focus on using this mouse model of commensal *Neisseria* colonization and persistence to identify new/unknown host interaction factors that are essential for colonization and/or persistence; investigating how Nmus adapts to different colonization niches; examining the local and systemic immune responses towards colonization of Nmus; and discovering the changes and interactions between Nmus and the microflora. These studies will further our understanding of *Neisseria*-host interactions.

Lastly, there are a variety of animal models for studying the biology of *Neisseria*. Each has its own advantages and disadvantages. Investigators should select the most appropriate model based on the questions/conditions (i.e. colonization status/ route of inoculation) they want to address. Results obtained using different animal models will support the development of new antibiotics and vaccine candidates for Nme and Ngo.

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