Influences of peptidoglycan on *Neisseria*-host interactions in an *in vivo* mouse model using *Neisseria musculi*

by

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Abstract

Neisseria gonorrhoeae (Gc) is a human-specific pathogen that is the etiological agent of the sexually transmitted infection gonorrhea. Gc can infect many mucosal epithelial surfaces resulting in pharyngitis, conjunctivitis, salpingitis, proctitis, cervicitis, and urethritis. A consequence of gonococcal infection in the urogenital tract is sterility in both males and females. During growth Gc releases significant amounts of peptidoglycan (PG) fragments which have been associated with inflammation and ciliated cell death. PG is a dynamic mesh-like polymer that is constantly being degraded and rebuilt to allow for cell growth and separation. Several different proteins are involved in the degradation of PG. The combined action of all these peptidoglycanases results in the production of PG fragments which are either recycled back into the cytoplasm or released from the cell. Due to the damaging effects of these fragments, understanding the proteins that produce them can provide insight into the pathology of gonorrhea. One such method to study gonococcal infection and the proteins involved in PG breakdown is through a mouse model. Since Gc is a human specific pathogen, we used a homologous species, *Neisseria musculi* (Nmus), to mimic gonococcal infection and study the importance of peptidoglycanases in infection. In addition to many *in vitro* methods, the lytic transglycosylase, MltG, was characterized to elucidate its role in PG synthesis and degradation. The amidase, AmiC, was also characterized, specifically in Nmus, to determine its role in cell separation and in systemic infection. Finally, nonpathogenic *Neisseria*, including Nmus, were analyzed for PG fragment release and NOD1 activation to compare these results to the pathogenic Gc to identify differences that may contribute to its pathogenesis.

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Chapter 1: Introduction

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Neisseria of humans and animals

Most members of the genus *Neisseria* have a commensal relationship with humans or animals. While there are two pathogenic species, *Neisseria gonorrhoeae* and *Neisseria meningitidis*, most other identified species of *Neisseria* live a mainly commensal lifestyle in the mucosal microbiota within their respective host(1). Commensal *Neisseria* have been characterized from multiple mammalian species as well as birds and reptiles and are found in the oral cavity, nasopharynx, and digestive tract. These bacteria are almost all harmless, apart from a sporadic reports of bite wound infections or odd infections in birds or bird eggs. In humans, there are eight commensal species that colonize oral, nasopharyngeal, or vaginal tissue. Occasionally these non-pathogenic *Neisseria* will cause opportunistic infections in humans, however, they have also been shown to be a reservoir for antibiotic resistance to their pathogenic counterparts(2–4). Non-pathogenic *Neisseria* can also antagonize infections with pathogenic *Neisseria* by direct killing through a DNA-dependent mechanism(5). In addition, *Neisseria lactamica* serves to prevent colonization by *N. meningitidis* in children(6). Therefore, non-pathogenic *Neisseria* play an important role in the development but also prevention of infection with pathogenic *Neisseria.*

Neisseria gonorrhoeae

Neisseria gonorrhoeae (Gc) is a human specific bacterial pathogen that causes the sexually-transmitted infection (STI) gonorrhea. Gc is a Gram-negative diplococcus that is a member of the beta-proteobacteria family. Gonorrhea is the second most reported bacterial transmittable disease and has developed resistance to all the antibiotics that have been used for treatment previously(7–9). Those ages 15-24 carry the largest burden of disease and from 2017 to 2021 there was a 25% increase in cases of gonorrhea(10). Infections with *N. gonorrhoeae*

increase the risk of contracting HIV and can also result in sterility in both males and in females(10, 11).

Gonococci can infect mucosal epithelia throughout the body resulting in pharyngitis, salpingitis, proctitis, ophthalmia, and can even disseminate into the blood causing disseminated gonococcal infection(12). Females are more likely to have asymptomatic infections(13). Females can transmit gonorrhea to neonates born vaginally, causing neonatal ophthalmia which can result in blindness(13, 14). Pelvic inflammatory disease (PID) can develop after cervical infections ascend into the fallopian tubes leading to an increase in the risk of ectopic pregnancy, infertility, and chronic pelvic pain(15). Males are more likely to develop symptomatic infections, presenting as urethritis with the main symptom being purulent discharge caused by the large inflammatory response generated against Gc(13).

Disseminated gonococcal infection (DGI) occurs in 1-3% of gonorrhea cases(16). DGI occurs when a mucosal infection disseminates into the bloodstream causing bacteremia, and DGI is more likely to proceed from asymptomatic and thus untreated infections(7, 16). While this type of infection is rare, it is more likely to occur in patients with complement deficiencies(16, 17). Most DGI patients don't have complement deficiencies, but patients with complement deficiencies are more likely to get DGI or meningococcemia than the general population. Female sex, menstruation, pharyngeal gonorrhea, and pregnancy can increase the risk of DGI(16). DGI presents most commonly as an arthritis-dermatitis syndrome where joint pain is experienced primarily in the knees, elbows, and other distal joints as well as the presence of papules and pustules on the skin(7, 16). Serious complications, such as gonococcal endocarditis and meningitis can occur, but this is extremely rare. Endocarditis occurs in 1-2% of DGI cases(18).

Mouse models of gonococcal infection

Due to the human specificity of Gc, modeling the infection in animals is difficult. There are models using male humans and chimpanzees were previously used, but financial and clinical limitations make mouse models more favorable. Common problems associated with current mouse models include differences in infection location, inability to cause continuous infection, lack of necessary receptors, inability of the bacteria to bind mouse complement inhibitors or mouse transferrin or lactoferrin, and the necessity to use chemicals to promote infection.

The 17β-estradiol (17E) mouse model is perhaps the most well-studied mouse model for gonorrhea. In this model, female BALB/c mice are intravaginally inoculated with Gc. Estradiol is administered to facilitate continuous colonization with Gc by extending the estrus phase of the reproductive cycle(12, 19). Antibiotics are also administered to reduce the number of commensal bacteria that increase with estrogen treatment(19). It is still unknown why estradiol promotes colonization with Gc. In this model, Gc localized in the vaginal lumen, and more neutrophils were found in vaginal smears from infected mice(19). Interestingly, C57BL/6 mice can be successfully colonized but do not show an innate inflammatory response during infection.

Despite the advances in knowledge about gonococcal infection that were gained using the 17E model, there are several limitations of this model. A potential issue with the 17E model is the necessity to administer both estradiol and antibiotics and the effects both may have on the reproductive cycle or infection environment. Additionally, not knowing the specific mechanisms by which estradiol promotes colonization acknowledges that there may be uncontrollable and unknown effects to the immune system, bacteria, or general health of the mouse upon treatment. Furthermore, the reduction of commensal bacteria in the mouse by antibiotics could alter interactions that occur between *Neisseria* and other bacteria or their products that could be

important to colonization. Bacteria were also found to be localized to the vaginal lumen which does not commonly occur in humans, where localization occurs primarily in the cervix(13, 19). Finally, the infection will only last 4-6 days during the proestrus stage of the estrous cycle without the addition of estradiol(19).

To combat issues with a lack of necessary receptors that Gc interacts with during adherence and infection in mice, several transgenic mouse strains were created that express human proteins. These include carcinoembryonic antigen cell adhesin molecule 1 (CEACAM1), CEACAM5, transferrin, CD46, and C4b-binding protein (C4bp) transgenic mice. Certain opacity proteins bind to CEACAM1, and this interaction is important for cell adhesion of the bacteria. Gc obtains iron for survival from human transferrin. The type IV pilus can recruit CD46 which is a complement regulator. Porin binds C4bp to reduce complement system activation. All these host receptors are important for successful infection with Gc, and all are lacking in mice. While transgenic mouse models are useful for studying specific interactions that occur with the transgenic receptor, they fail to allow the study of the complexity of the many different interactions that occur during gonococcal infection.

Neisseria musculi

Neisseria musculi (Nmus) is a commensal of wild mice that has oropharyngeal colonization. Nmus was isolated from wild mice and is not naturally found in laboratory mice. Although isolated from the oropharynx, Nmus can also colonize the entire gastrointestinal tract and can be isolated from feces(20). Nmus is a Gram-negative coccobacillus that exists as two morphotypes: a smooth morphotype and a rough morphotype(21). The primary difference between these two morphotypes is colony morphology on solid agar medium. It is currently unknown if there are any genetic differences between the morphotypes, but the smooth variant

shows increased expression of type IV pili, and the increase in pili may explain both the increased colonization by this variant and its colony morphology (22). Comparable to Gc, Nmus is naturally transformable and relies on a *Neisseria* DNA uptake sequence (DUS) and the presence of a Type IV pilus (Tfp) for transformation(21). The host specificity of Nmus as well as the ease in genetic manipulation, make mouse models using this bacterium better suited to study specific *Neisseria*-host interactions that occur during colonization and infection. The use of animal-specific pathogens in their natural hosts to mimic infection by related human-specific pathogens is not a new concept. Species of *Chlamydia* and *Mycobacterium* that naturally infect mice or fish, respectively, have been used to study chlamydia and tuberculosis, respectively(23, 24).

During a study looking at colonization of Nmus in several different mouse strains, it was found that a single dose of bacteria could facilitate colonization of the oral cavity and gastrointestinal tract for at least a year(20). Mouse strain was important for the rate of colonization of the mice. AJ and CAST mice were found to have the highest rates of colonization, while C57BL/6 mice had an intermediate rate of colonization(20). Additional strains tested had low or even no colonization (NOD/LtJ, NZO/HILtJ, PWK/PhJ, WSB/EiJ, 129S1/SvImJ)(20). It was also found that innate immunity, but not adaptive immunity, was important for susceptibility to colonization using experiments with immunodeficient mice(20).

Nmus was also found to modulate innate host responses to increase oral colonization(25). The IL-6 response was different between mice colonized with live or killed Nmus, and IL-6 knockout mice had higher colonization burdens and rates(25). Additionally, colonization produced an antibody response, and Nmus specific IgG was only produced in mice successfully colonized(25). This result indicates that although there is an immune response to Nmus, it does

not result in bacterial clearance(25). These few studies highlight the breadth of research that can be done using Nmus and suggest that models using this *Neisseria* species may be useful for the study of pathogenic *Neisseria*.

Immune responses to gonococci

An influx of neutrophils (PMNs) plays a major role in the immune response to gonococcal infection(26). The recruitment of PMNs in addition to the activation of Nod-like receptors (NLRs) and Toll-like receptors (TLRs) results in large amounts of inflammation. Despite this immune activity, Gc will still survive and persist in the host(26). While the large inflammatory response may seem disadvantageous to the survival of the bacteria, Gc manages to thrive in this environment and will infect and grow intracellularly in the recruited PMNs.

Initiation of infection requires initial adherence to epithelial cells which is primarily mediated through binding of Type IV pili and Opacity (Opa) proteins(27). Type IV pili are appendages that extend from the bacterial cells(28). They are important for motility, DNA transformation, and biofilm formation(26, 28). Opa proteins are outer membrane proteins that alter the opaqueness of a gonococcal bacterial colony(26, 29). These structures are critical for adherence and invasion of host cells(27).

There are several different structures of Gc that the innate immune system responds to. These include lipooligosaccharide (LOS), components of outer membrane vesicles (OMVs), heptose-1,7-bisphosphate (HBP), and peptidoglycan (PG). All of these products are involved in activating innate immune receptors such as TLRs and NLRs(27, 30). *N. gonorrhoeae* infections do not result in a protective immune response and so no memory immunity is produced; therefore, it is possible to contract gonorrhea multiple times(26).

LOS is a component of the outer membrane of a gonococcal cell. It differs from the analogous molecule associated with other Gram-negatives, lipopolysaccharide (LPS), by the absence of a repeating O-antigen as well as a limit of approximately 10 saccharide units on the oligosaccharide structure(31, 32). LOS of pathogenic *Neisseria* can also act as a ligand to promote invasion of host cells(31). LOS can also activate TLR4 resulting in the production and release of proinflammatory cytokines(33, 34).

OMVs are derivatives of the outer membrane of Gram-negative bacteria that contain outer membrane proteins and lipids that envelope periplasmic contents(35, 36). These OMVs are then released from the bacterial cell where they can deliver virulence factors or products to the host cells(37, 38). Once inside a host cell, these products can then activate immune receptors. OMVs are currently being studied for use as a component for a vaccine for gonorrhea(12, 39). Because of the lack of memory immunity generated against gonorrhea and the ability of several structures of Gc to undergo phase and antigenic variation, the development of a gonococcal vaccine has been difficult(39).

Heptose-1,7-bisphosphate (HBP) is a sugar that is an intermediate in LPS biosynthesis(30). It is unique to Gram-negative bacteria and absent from mammalian cells(30). HBP is sensed in the cytosol of host cells resulting in a proinflammatory immune response(30).

Peptidoglycan

Peptidoglycan (PG) is a dynamic mesh-like polymer that is important for maintaining bacterial cell shape and turgor pressure(40). The polymer is constantly degraded and rebuilt to allow for changes in cell size as well as for cell division. PG is found in both Gram-negative and -positive bacteria. The structure of PG consists of repeating subunits of *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc). There is a peptide stem of 2-5 amino acids

attached to the MurNAc residue where adjacent strands of PG can be crosslinked. *Neisseria* peptidoglycan is of chemotype 1, as in many Gram-negative bacteria such as in *Escherichia coli* and in Gram-positive rods, such as *Bacillus subtilis*. Thus, the amino acids of the peptide stem occur in the order L-alanine, D-glutamate, *meso*-diaminopimelic acid (DAP), terminating in two D-alanine residues.

Although most of the PG fragments generated during growth and division are recycled and reincorporated back into the PG layer, a portion of these fragments will be released from the cell. Compared to other Gram-negative species of bacteria, Gc and Nmus release a larger percentage of their PG: Gc releases about 15%, Nmus releases about 12% (Chapter 4), and *Escherichia coli* only releases about 5%(41–43). Additionally, *E. coli* releases most of its PG fragments as free disaccharide and DAP-D-ala dipeptide, while Gc releases mostly PG monomers and free tri- and tetra- peptides(41, 44). PG fragments, specifically PG monomers, are inflammatory and cause cell death induction and tissue damage(45, 46). Fragments are also known to be arthropathic and may play a key role in gonococcal arthritis that occurs during DGI(47). PG fragments are recognized by the host innate immune system through NLRs. Monomers and free peptides are agonists of NOD1, whereas muramyl-dipeptide and PG dimers are NOD2 agonists(48, 49). PG can be cleaved at multiple sites by different PG-degrading enzymes. Peptidoglycanases include lytic transglycosylases, carboxypeptidases, amidases, and endopeptidases.

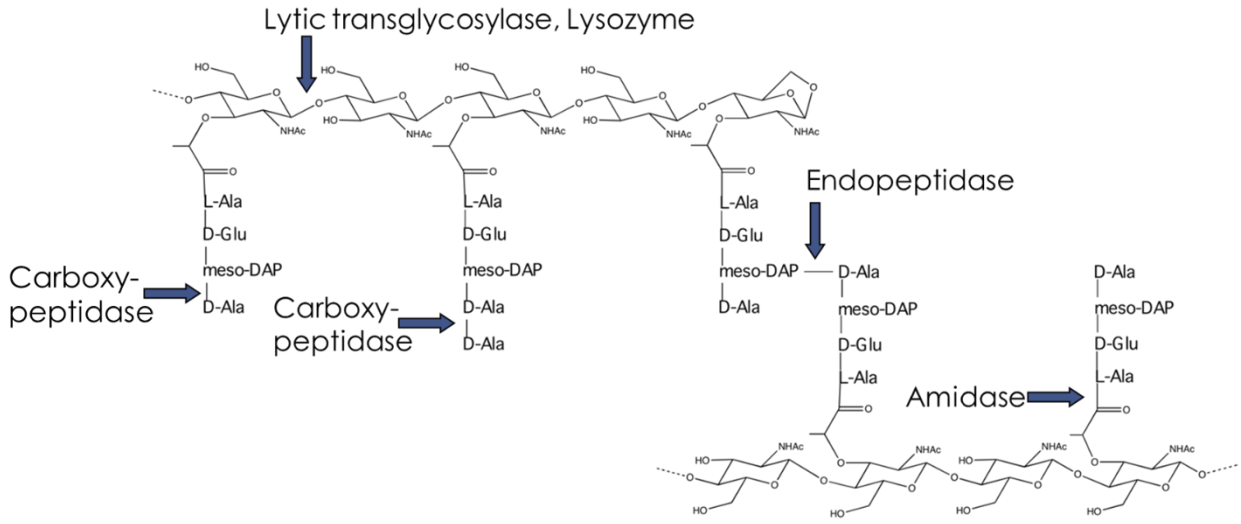

Figure 1. Structure of peptidoglycan and sites of enzyme activity. Peptidoglycan (PG) consists of repeating subunits of *N*-acetylmuramic acid (NAM) and *N*-acetylglucosamine (NAG). Peptide stems extend from the NAM which also serve to crosslink adjacent strands. Several proteins are involved in the degradation of PG. Lytic transglycosylases cleave the bond between the NAM and NAG. Amidases cleave the linkage between the peptide stem and NAM. Endopeptidases cleave the crosslinks and carboxypeptidases cleave bonds between the amino acids of the peptide stem.

Production of inflammatory PG fragments by lytic transglycosylases and amidases

Lytic transglycosylases (LTs) cleave the β-1,4 linkage between the MurNAc and GlcNAc residues producing PG monomer and dimer fragments(50). As mentioned previously, monomers are agonists of NOD1. Specifically, tripeptide monomers are human NOD1 agonists and tetrapeptide monomers are mouse NOD1 agonists(51). The L,D-carboxypeptidase LdcA is responsible for converting a tetrapeptide to a tripeptide(43). There are several LTs that are present in *Neisseria*. It was originally recognized that there are 5 LTs that are encoded in the core

genome of Gc with an additional one or two present on the gonococcal genetic island (GGI)(52). Looking specifically at the LTs of the core genome, there are three with distinct roles in the cell. LtgA and LtgD are primarily responsible for generating the monomer fragments that are released from the cell; however, more of the monomers produced by LtgD are released as compared to LtgA where more are recycled(53, 54). LtgC is involved in cell separation(55). Two additional putative LTs were identified by genomic sequence, RlpA and MltG, and characterization of MltG function is found in chapter 2 of this thesis.

Free peptides (and free disaccharides) are produced by amidases that remove the peptide stem from the MurNAc residue(56). Like monomers, free peptides are also agonists of NOD1. The periplasmic amidase in Gc is AmiC which is activated by the protein NlpD. Both AmiC and NlpD are required for free peptide release and cell separation(56, 57).

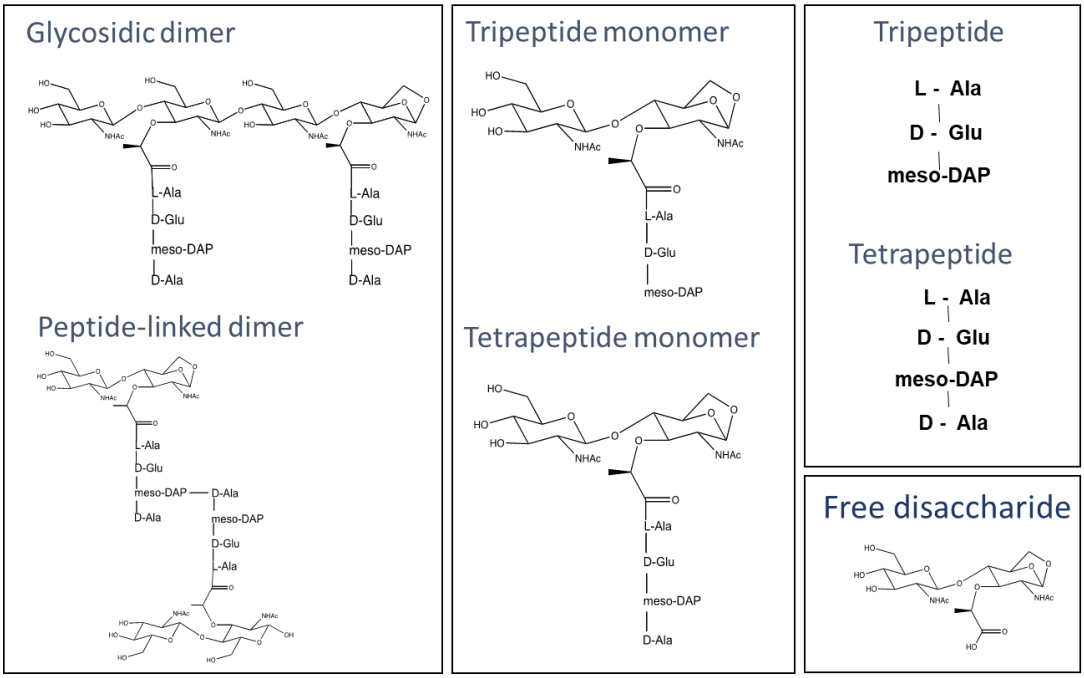

Figure 2. Small peptidoglycan fragments released by *Neisseria.* Chemical structures of released small peptidoglycan (PG) fragments. Including dimers, monomers, peptides, and free disaccharide.

Current work

The thesis project described here aims to elucidate the effects PG fragments have on infection through the development of a mouse model. This model mimics gonococcal infection in mice using the commensal Nmus. To address these aims, I used my developed model to look at infection of the blood and immune responses to this infection through quantification of changes in bacterial load and cytokine release. These changes were observed during infections where PG fragment release is altered. Additionally, I characterized the newly identified lytic transglycosylase, MltG, using various methods to identify effects the protein has on PG fragment release, antibiotic resistance, autolysis, NOD1 activation, and colonization and infection in a mouse model. I analyzed the cell separation amidase AmiC in Nmus, specifically determining the protein's effect on NOD1 activation, fragment release, and infection. Finally, I examined the activation of NOD1 by non-pathogenic *Neisseria.* This work highlights the usefulness a mouse model using Nmus can have in further characterization of peptidoglycanases as well as their possible importance in infection with pathogenic *Neisseria*.

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Chapter 2: Mutation of *mltG* increases peptidoglycan fragment release, cell size, and antibiotic susceptibility in *Neisseria gonorrhoeae*

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Author contributions: JPD conceived of study. KPM performed BACTH assays. KTH performed enzyme assays, some fragment releases, built point mutant and double mutant strains and corresponding plasmids, and conducted TEMs. MAS built strains and plasmids for single mutant. TNH performed remaining experiments. TNH and JPD wrote the manuscript.

Abstract

Infection with the Gram-negative species *Neisseria gonorrhoeae* leads to inflammatory responses that are responsible for the disease symptoms of gonococcal urethritis, cervicitis, and pelvic inflammatory disease. During growth and infection these bacteria release significant amounts of peptidoglycan (PG) fragments which elicit inflammatory responses in the human host. These PG fragments are produced through degradation of the cell wall by a variety of peptidoglycanases. One such group of peptidoglycanases are the lytic transglycosylases which are responsible for producing PG monomers, the most abundant PG fragments released by gonococci. The PG monomers are agonists of the immune receptor NOD1 and are also associated with death of ciliated Fallopian tube cells. The exact mechanisms by which PG breakdown and synthesis are coordinated in *Neisseria* are currently unknown. Based on previous research, an inner membrane bound LT, MltG, was identified as being a terminase that stops PG elongation in *Escherichia coli*. This protein was also identified in *N. gonorrhoeae,* and in this study, we have characterized the role of MltG in PG metabolism in gonococci. *In vitro* methods to determine enzymatic function, PG fragment release, PG turnover, cell autolysis, and antibiotic resistance were conducted. From these experiments it was confirmed that MltG is a lytic transglycosylase and that its function is to terminate the elongation of PG strands.

Introduction

Neisseria gonorrhoeae (Gc) is a Gram-negative diplococcus that is the causative agent of the sexually transmitted infection gonorrhea. Treatment of this infection has become increasingly challenging due to antibiotic resistance to all previously used antibiotic therapies, highlighting the need for new treatments and new drug targets (1). Currently, ceftriaxone is the only antibiotic therapy that is recommended for treatment of gonorrhea (2). Serious consequences of gonorrhea include infertility, pelvic inflammatory disease, ectopic pregnancy, neonatal blindness, and disseminated gonococcal infection (3). Symptoms and pathology of the infection are derived from the large inflammatory response that occurs in most gonococcal infections (3). Bacterial products released by Gc, including lipooligosaccharide, outer membrane vesicles, heptose-containing metabolites, and peptidoglycan, contribute to this inflammatory response (3). During growth, Gc releases significant amounts of peptidoglycan (PG) fragments that are known inflammatory products (4). These fragments are sufficient to cause the death of ciliated cells of the human Fallopian tube tissue, recapitulating the damage that occurs during gonococcal pelvic inflammatory disease (5–7).

PG consists of repeating subunits of *N*-acetylmuramic acid (MurNAc) and *N*-acetylglucosamine (GlcNAc). A short peptide chain is attached to the MurNAc sugar, which also serves to crosslink adjacent strands of PG. Several enzymes are involved in the breakdown of PG in the cell wall, including lytic transglycosylases, carboxypeptidases and endopeptidases, and an *N*-acetylmuramyl-L-alanine amidase. The combined action of these enzymes results in the production of small PG fragments. Monomer PG fragments are produced by lytic transglycosylases (LTs), which cleave the MurNAc-β-(1,4)-GlcNAc linkage (8). Gc releases monomers in the form of 1,6-anhydro disaccharide tetrapeptide and 1,6-anhydro disaccharide

tripeptide (9). The most abundant fragments released by gonococci are the tripeptide monomer (GlcNAc-anhydroMurNAc-Ala-Glu-Dap) and the tetrapeptide monomer GlcNAc-anhydroMurNAc-Ala-Glu-Dap-Ala). Seven LTs have thus far been identified in Gc (10, 11). Some LTs have specialized roles in the cell, such as LtgC, which is involved in cell separation (12), while others have significant effects on PG fragment release, such as LtgA and LtgD, where monomer release is abolished upon loss of both of these proteins (8).

MltG is an LT that was previously characterized in *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Streptococcus* species (13–18). Some work has also been done on MltG in *Vibrio cholerae* (19). In *E. coli*, MltG was suggested to be a terminase, stopping the elongation of glycan strands (14). In *B. subtilis* and *E. coli*, MltG was found to interact with both classes of penicillin binding proteins (PBPs) which are involved in PG synthesis (16, 17). Studies in *P. aeruginosa* show an *mltG* deletion can potentiate susceptibility to antibiotics (13, 20). The role of MltG has not been characterized in Gc; however, we hypothesize that MltG plays a role in PG biosynthesis similar to that seen in *E. coli* and *B. subtilis* (14, 17). We characterized the role of MltG in PG fragment release, protein-protein interactions, antibiotic sensitivity, PG turnover, and cell autolysis.

Materials and Methods

Bacterial strains and growth

All *N. gonorrhoeae* strains used in this study are derivatives of strain MS11. Piliated strains of MS11 were used for all transformations, whereas non-piliated strains were used for all other experiments. All *N. musculi* strains used in this study are derivatives of strain AP2031. Smooth morphotypes of AP2031 were used for all transformations and experiments. *N. gonorrhoeae* and *N. musculi* strains were grown at 37˚C and 5% CO_2 on GCB agar plates (Difco) with Kellogg's supplements(21). Strains were also grown in gonococcal base liquid medium (GCBL) containing 0.042% $NaHCO_3$ and Kellogg's supplements with aeration(22, 23). *E. coli* was grown in Luria broth or on Luria agar plates. Antibiotics were used at the following concentrations for *E. coli*: erythromycin at 500 mg/mL, chloramphenicol at 25 mg/mL, and ampicillin at 100 mg/mL. For *N. gonorrhoeae*, chloramphenicol was used at 10 mg/mL, tetracycline was used at 1.5 mg/mL, erythromycin was used at 1.5 mg/mL, ceftriaxone was used at 0.25 mg/mL, fosfomycin was used at 10 mg/mL, penicillin was used at 8 mg/mL, and vancomycin was used at 1.5 mg/mL. For *N. musculi*, rifampicin was used at 40 mg/mL.

Plasmid and strain construction

The plasmids used in this study are listed in Table 1. Chromosomal DNA from *Neisseria gonorrhoeae* MS11 was used as a PCR template for Gc unless otherwise noted. Chromosomal DNA from *Neisseria musculi* AP2031 was used as PCR template for Nmus unless otherwise noted. The priners used in this study are listed in Table 2.

Table 1.

Plasmid or Strain	Description	Reference
pIDN1/3	Insertion-duplication plasmid (Erm^R)	Hamilton et al (2001) (24)
pKH37	Complementation vector (Cm^R)	Kohler et al (2007) (25)
pKH52	*pacA* point mutation (H329Q)	Dillard and Hackett (2005)
pMRS1	*mltG* deletion constructed in pIDN3, Gibson cloning	This study
pKH189	*mltG* in pTEV5; 6x HIS tag	This study
pKH193	*mltG* in pKLD116; 6x HIS tag + MBP	This study
pKH198	*mltG* complementation; containing *mltG* in pKH37 (Cm^R)	This study
pKH209	*mltG* gene block in pIDN3; E213Q point mutant	This study
pTEV5	Vector for synthesis of recombinant protein with a N-terminal hexahistidine, removable by tobacco etch virus (TEV) protease	Rocco et al (2008) (26)
pKLD116	Vector for synthesis of recombinant protein with a N-terminal hexahistidine and maltose binding protein tag in tandem, removable by TEV.	Rocco et al (2008) (26)
pTNH3	*mltG* deletion constructed in pIDN1 (Kan^R)	This study
pRS91	*ltgA* point mutant constructed in pIDN1	Schaub et al (2016) (28)
pRS92	*ltgD* point mutant constructed in pIDN1	Schaub et al (2016) (28)
MS11	Wildtype *Neisseria gonorrhoeae*	Segal et al (1985) (27)
MRS500	MS11 transformed with pMRS1; *mltG* deletion mutant	This study
KH530	MS11 transformed with pKH52; *pacA* point mutation H329Q	Dillard and Hackett (2005)
KH624	KH530 transformed with 1291ΔmsbB chromosomal DNA; *pacA* point mutation H329Q, msbB mutation (Kan^R)	
KH651	MRS500 transformed with pKH198; *mltG* complemented (Cm^R)	This study
KH658	MS11 transformed with pKH209; *mltG* E213Q point mutant	This study
KH673	KH658 transformed with pKH209 and pRS91; *mltG* and *ltgA* double point mutant	This study
KH674	KH658 transformed with pKH209 and pRS92; *mltG* and *ltgD* double point mutant	This study
RS555	MS11 transformed with pRS91; *ltgA* point mutant (E481A) (Erm^R)	Schaub et al. 2016 (28)

RS557	MS11 transformed with pRS92; *ltgD* point mutant (E158A) (Erm^R)	Schaub et al. 2016 (28)
AP2031	Wildtype *Neisseria musculi*	This study
TNH2002	Wildtype *Neisseria musculi* with natural rifampicin resistance (Rif^R)	This study
TNH2003	TNH2002 transformed with pTNH3; *mltG* deletion mutant (Kan^R, Rif^R)	This study
Escherichia coli		
Plasmids for BACTH		
pKT25	Cloning vector to add N-terminal T25 fragment for BACTH assays	(Euromedex kit)
pUT18C	Cloning vector to add N-terminal T18 fragment for BACTH assays	(Euromedex kit)
pKT25-zip	Positive control for BACTH assays	(Euromedex kit)
pUT18C-zip	Negative control for BACTH assays	(Euromedex kit)

Table 2.

Primer	Sequence	Plasmid or DNA
yceG start F Gib	GTGGCGGCCGCTCTAGAAGAGCGAACGGTATTGCGCTTC	pMRS1
yceG start R Gib	GCATGGTTTACATGGTAGGATTCCCAGCATTC	pMRS1
yceG stop F Gib	TCCTACCATGTAAACCATGCCGTCTGAAAAGTTTG	pMRS1
yceG stop R Gib	CTGCAGCCCGGGGGATCCAGTCCGATACGCGCCATCGAT	pMRS1
yceG pTEV5 F Nhe	AGAGCTAGCCCTAAAGACAACGGCAGGGC	pKH189 pKH193
yceG pTEV5 R Xho	GGTACTCGAGGCTTGCAGTTCCGGTAAGGG	pKH189
yceGFcomp Spe	TTTACTAGTCCTGTCCGGCGGGCAATTTG	pKH198
new yceG R Hind	TGC AAG CTT CAT CCA AGC TGC GGT TAC TG	pKH193
UF mltG	GAC TGA GCT CCG CGA TGT ATT GGT G	pTNH3
UR mltG	GAC TAC TAG TGG TAG GAT TCG TGG	pTNH3
DF mltG	GAC TGG ATC CAA CAT GAT GCC GCC G	pTNH3
DR mltG	GAC TCT GCA GAA AGC GGC TTC TTC C	pTNH3

ITS_R2	CAA GAA CTT CTT TGA CTT CG	*Neisseria musculi* gDNA
ile_tRNA_F	CCG GGT TTG TAG CTC AGC T	*Neisseria musculi* gDNA

MltG protein purification

Purification of *mltG* was adapted from Rocco *et al*. (doi: 10.1016/j.plasmid.2008.01.001). An *E. coli* strain expressing pKH193 was induced with 2 mM IPTG at 30˚C for 2 hours. Cultures were harvested by centrifugation and washed with column buffer (20 mM $NaPO_4$ [pH 7.35], 300 mM NaCl, 20 mM imidazole), and resuspended in 25 mL of column buffer with 0.5% TritonX. Resuspended cells were processed with a French press two times to lyse cells. The lysate was then centrifuged for 15 minutes at 20,000 x g. The clarified supernatant was mixed with 250 µL of prewashed nickel resin and mixed by rotation for 1 hour at 4˚C. The supernatant-bead mixture was poured into a column and washed with 25 mL column buffer with 20 mM imidazole. The protein was eluted with 500 µL of increasing concentrations of imidazole, 20mM $NaPO_4$ pH 7.35, and 300 mM NaCl. Most of protein eluted at 250 mM and 500 mM imidazole. Elution fractions were combined and dialyzed overnight into 50 mM $NaPO_4$ pH 7.5, 100 mM NaCl, and 10% glycerol.

Sacculi labeling and purification

For analysis of PG sacculi, PG from a strain of *N. gonorrhoeae,* with a *pacA* point mutation resulting in a lack of PG acetylation (KH624), was labeled with [6,^{3}H]-glucosamine for an hour. The bacterial cells were then harvested by centrifugation and washed once with PBS before resuspension in 500 µL of NaOAc at pH 5. 500 µL of hot 8% SDS was added, and the suspension was boiled for 30 minutes. Sacculi were pelleted by centrifugation, washed with 50

mM sodium phosphate buffer at pH 7.5, and then resuspended in 50 mM sodium phosphate buffer. Isolated sacculi were treated with 100 µg/mL Pronase overnight, to remove any protein from the sacculi. After Pronase treatment, sacculi were boiled in 8% SDS for 30 minutes, then washed twice, before resuspension in water.

MltG enzyme activity

To test MltG activity, 50 µL of [6-^{3}H]-glucosamine labeled, purified sacculi were digested with 25 µL of purified MltG in 25 mM $NaPO_4$ buffer pH 6 (total reaction volume was 500 µL) at 37˚C overnight. After digestion, MltG was heat inactivated by placing the reaction in a boiling water bath for 10 minutes. To remove insoluble PG, the mixture was centrifuged for 10 minutes at 13,000 rpm. The PG fragments in the sample were subsequently separated using a size-exclusion column. Monomer and dimer PG fragments from the column were collected, concentrated by speed vac, and then desalted prior to HPLC analysis. Tri- and tetrapeptides were separated using a Grace Prevail C18 reversed phase column (250 mm X 4.6 mm) and run over a 4-13% acetonitrile (with 0.05% TFA) gradient for 30 minutes at a 0.5 mL/minute flow rate.

Disk diffusion assays for antibiotic resistance

N. gonorrhoeae was grown overnight on GCB agar. Gc cells were suspended in GCBL medium to an OD_{540} of 0.2. This culture was spread onto GCB plates and incubated for 15 minutes to dry. Diffusion disks were then placed onto the agar, and antibiotics were pipetted onto the disks. Plates were incubated overnight at 37˚C in 5% CO_2. The length, in millimeters, between the edge of the disk and the point where bacterial growth begins was measured. Antibiotics were used at the following concentrations: erythromycin, tetracycline, and vancomycin at 1.5 mg/mL; penicillin at 8 mg/mL; ceftriaxone at 0.25 mg/mL; and fosfomycin at 10 mg/mL. This experiment was repeated three times in triplicate.

Thin-section electron microscopy (TEM)

For visualization of bacteria by thin-section electron microscopy, strains were grown on GCB plates overnight and then grown in GCBL medium for 3 hours. Cells were harvested by centrifugation, washed once with PBS, and then resuspended in Karnovsky's fixative. TEM was performed at the University of Wisconsin Medical School Electron Microscope Facility with the assistance of Ben August.

Autolysis

To measure autolysis of in buffer, gonococci were grown in GCBL medium for 4 hours from an initial OD_{540} of 0.2. Cultures were centrifuged and cells resuspended in 3 mL of 50 mM TrisHCl buffer pH of 6 to wash pellet. Culture was then added to 3 mL TrisHCl buffer pH of 8 in a conical tube at OD_{540} adjusted to 0.3. Cultures were rotated at room temperature and OD_{540} measurements taken every 20 minutes for 2 hours. This experiment was repeated 3 times.

Turnover

To assess peptidoglycan turnover during growth, cultures of GCBL medium at an OD_{540} of 0.2 were grown into log phase (3-4 hours). Cultures were then diluted back to an OD_{540} of 0.2 and labeled with 10 µCi/mL of [6,^{3}H]-glucosamine for 30 minutes. After labeling, cells were washed once and resuspended into 4 mL GCBL medium. At each timepoint, 1 mL of culture was removed, and 200 µL of *E. coli* culture were added as a carrier. Cells were then centrifuged at 13,000 rpm for 5 minutes. Pellets were at stored at -20˚C until analysis. To purify PG, the cell pellets were thawed, resuspended in 0.5 mL NaOAc at pH5, and added to 0.5 mL of hot 8% SDS. The mixture was placed into a boiling water bath for 30 minutes and centrifuged for 30 minutes at 43,000 x g. Supernatants were extracted, resuspended in water and scintillation fluid, and counted for radiation using a Packard Tri-Carb 2100TR liquid scintillation counter.

Characterization of PG fragment release

Characterization of released gonococcal PG was conducted as described by Rosenthal and Dziarski (Isolation of PG and soluble PG fragments, Method Enzymol 1994) and adapted by Cloud and Dillard (A LT of NG is involved in PG-derived cytotoxin production, Inf Imm 2002). For labeling the glycan chain, gonococcal cultures were grown in GCBL medium containing 0.4% pyruvate without glucose with 10 µCi/mL of [6-^{3}H]-glucosamine. Cultures were labeled for 30-45 minutes. Cultures were then centrifuged, and pellets resuspended in GCBL medium, allowing PG release for 2.5 hours. Cells were removed by centrifugation, and the supernatant was passed through a 0.22 µM filter. Radioactive counts per minute were normalized by taking 60 µL aliquots from each culture and adjusting the culture volumes. This procedure is performed so there are equivalent amounts of radioactivity in the cells in each culture, allowing quantitative comparisons of PG fragment release(29). Supernatants were passed through a size-exclusion column and eluted with 0.1 M LiCl. Fractions were collected consisting of 500 µL of the fraction mixed into 3 mL of scintillation fluid and counted with a Packard Tri-Carb 2100TR liquid scintillation counter or a Perkin Elmer Tri-Carb 4910TR scintillation counter.

Bacterial Adenylate Cyclase Two Hybrid (BACTH) System

Bacterial Adenylate Cyclase Two Hybrid System experiments were adapted from Battesti and Bouveret (The bacterial two-hybrid system based on adenylate cyclase reconstitution in *Escherichia coli*, Methods 2012). Plasmids were created that expressed the protein of interest fused to T25 or T18 fragments. A microliter of each plasmid was added to 20 µL of BTH101 competent cells in an ice-cold tube and incubated on ice for 30 minutes. Cells were heat shocked at 42˚C for 45 seconds followed by a 1-minute incubation on ice. Cells were added to 200 µL of LB and incubated in a rotator at 37˚C for 1 hour. Following incubation, 100 µL of the

transformation mixture was plated onto LB agar plates with 40 µg/mL of kanamycin and 100 µg/mL of ampicillin. Plates were incubated for 2 days at 30˚C. For the colorimetric assay, following the 2-day incubation, 3 mL of LB containing 40 µg/mL of kanamycin, 100 µg/mL of ampicillin, 40 µg/mL of X-Gal, and 0.5 mM of IPTG. Cultures were grown overnight at 30˚C in a tube rotator. The following day, 2 µL of each culture was spotted on LB agar containing 40 µg/mL of kanamycin, 100 µg/mL of ampicillin, 40 µg/mL of X-Gal, and 0.5 mM of IPTG.

Infection of mice and cytokine analysis

All mice used were C57BL/6J from the Jackson Laboratory. Animal protocols were approved by the University of Wisconsin-Madison Institutional Animal Care and Use Committee. All mice were held in a room that was specific pathogen free 1 for two weeks prior to inoculation. On the day of infection, TNH2002 (WT) and TNH2003 (Δ*mltG*) were swabbed from GCB agar incubated ON and suspended in cGCBL to an OD_{540} of 0.2. Cells were harvested by centrifugation and resuspended in Dulbecco's PBS. All mice were manually restrained and 100 µL was injected intra-peritoneally. Blood was collected retro-orbitally using heparinized and non-heparinized capillary tubes at 4-, 12-, and 24-hours post injection. Blood from heparinized capillary tubes was diluted in cGCBL, plated onto GCB rifampicin 40 µg/mL, and incubated for 48 hours at 37℃ and 5% CO_2. Blood collected in non-heparinized capillary tubes was aliquoted to a microcentrifuge tube and allowed to clot at room temperature for approximately 30 minutes before being placed on ice. Clotted blood was centrifuged at 3,000 RPM at 4℃ to separate serum. Serum was extracted and stored at -80℃ until cytokine analysis. Isolates obtained from GCB rifampicin agar plates were verified as *N. musculi* by colony lysis PCR using ITS primers specific to Nmus (Ma et al., 2018)(12). Amplified sequences were screened by PCR and comparing to the wildtype strain with rifampicin resistance, TNH2002, and found to be identical

(Sequences of TNH2002 and AP2013 (wild-type strain) were found to be identical in unpublished data). Serum was analyzed using the BD CBA Mouse Inflammation Kit (Becton Dickinson) using the manufacturer's instructions.

Results

MltG enzymatic function

To determine what enzymatic role gonococcal MltG has in PG breakdown, digestion products of MltG were analyzed using high performance liquid chromatography (HPLC). A soluble form of MltG was expressed in *E. coli* and purified. *N. gonorrhoeae* sacculi were purified from a Gc strain lacking PG acetylation, to allow cleavage by lytic transglycosylases. The PG was metabolically labeled with [6,^{3}H]-glucosamine, which is incorporated into both sugars of PG. Separation of the soluble PG fragments resulting from MltG digestion by size-exclusion chromatography showed MltG generated large PG fragments, PG dimers, and PG monomers (Fig. 1A). Dimers from the MltG-digested sacculi were further analyzed by HPLC after digestion by LtgD and it was found that the dimers were glycosidically linked (Fig. 1B), suggesting that MltG may prefer PG that is not crosslinked. LtgD will only cleave glycosidically linked dimers and the products from this digestion are PG monomers. This preference was also observed in *E. coli* (14). These data are consistent with MltG acting as an endo-lytic transglycosylase.

Cultures of an mltG *deletion mutant show more large cells*

To understand the role of MltG in *N. gonorrhoeae*, we made an in-frame deletion of the *mltG* coding sequence in the chromosome of strain MS11. A complement was created by placing a wild-type copy of *mltG* on the gonococcal chromosome at an unlinked locus, between *aspC* and *lctP*, using constructs previously described(23, 31). Mutations affecting cell wall degradation can affect the size of the bacterial cells. In *N. meningitidis*, a mutant lacking the PG deacetylase Ape1 produced larger bacterial cells. As Ape1 activity is necessary to allow lytic transglycosylase function, the *apeI* mutant would be unable to degrade PG strands. MltG is

thought to act to terminate PG chain synthesis(14). Thus, *mltG* mutants may be deficient in terminating the biosynthetic transglycosylation reaction and may produce glycan strands that are much longer than those of the WT. To determine if *mltG* cells were altered in cell size, we performed thin-section transmission electron microscopy. The *mltG* cells appeared larger than those of the WT or complement (Fig. 2a). To quantify the apparent differences, we measured cell size for over 1000 cells greater than 0.5µ for WT, *mltG*, and complement (Fig. 2b). The number of cells counted were 1004, 1041, and 1136 for WT, mutant, and complement, respectively. The number of mutant cells that were 0.5-0.6 or 0.6-0.7 were lower than those of the WT and complement, but this apparent difference did not rise to the level of significance. However, for the largest category of cells, those 1.0 or larger, the percentage of those cells in the population was much larger for the *mltG* mutant (14.5%) than that of the WT (3.1%) or complement strain (3.5%) populations. These results indicate that *mltG* mutant cells have altered cell wall morphology, making bigger gonococcal cells.

Mutants of mltG *release more peptidoglycan fragments*

During growth, Gc releases PG fragments, including dimers, monomers, free peptides, free disaccharide, and anhydro-MurNAc (4). Compared with other Gram-negative species, such as *E. coli* or *N. meningitidis*, Gc releases a larger portion of its PG (29, 32). The fragments that are released can be characterized through metabolic labeling with [6,^{3}H]-glucosamine, followed by fragment separation using a size exclusion column (33). The *mltG* deletion mutant was found to release more monomer, dimer, and multimer fragments compared to the wild-type strain (Fig. 3A). Complementation restored the mutant to near wild-type levels of PG fragment release. Increased release of PG fragments was not what we predicted, and this phenotype stands in contrast with those obtained with other lytic transglycosylase defective strains. Mutants lacking

ltgA or *ltgD* have reduced PG fragment release, and mutants lacking other lytic transglycosylase genes show little to no effect on PG fragment release(8). To determine if the increased PG fragment release in the mltG mutant resulted from loss of MltG function or loss of MltG protein, we made a point mutation affecting the predicted MltG catalytic site, *mltG* E213Q. Analysis of PG fragment release showed that the point mutant behaved like the deletion mutant, also releasing increased amounts of large PG fragments, PG dimers, tetrasaccharide-peptide, and monomers (Fig. 3B).

Monomer fragment release is decreased in double mutants lacking mltG *and other lytic transglycosylases*

Both the in-frame deletion and point mutation of *mltG* resulted in a large increase in PG monomer and PG dimer release (Fig. 3). It seems possible that in the *mltG* mutants, PG strands are being created that are longer than normal and these too-long strands are being degraded by one of the lytic transglycosylases active in creating released PG fragments, i.e., LtgA or LtgD(8, 28). To determine which lytic transglycosylase is responsible for producing the excess of PG fragments released in the *mltG* mutants, we created double mutants with point mutations of the catalytic glutamate for either *ltgA* or *ltgD* plus the point mutation in *mltG*. We found that PG monomer and dimer release was decreased in both the *ltgA mltG* and *ltgD mltG* double mutants compared to the *mltG* single mutant (Fig. 4). In fact, the characterizations of the PG fragments released were similar to those of the single *ltgA* and *ltgD* mutants. Since both double mutants were reduced in amounts of PG monomer fragments released, we cannot assign PG degradation in the *mltG* mutant to just LtgA or LtgD.

MltG interacts with PBPs

Synthesis of the cell wall requires the coordination of several proteins to incorporate PG monomers into the existing PG layer (34). In Gc, there are two penicillin-binding proteins (PBPs) involved in synthesis, PBP1 and PBP2, where PBP1 has transglycosylation and transpeptidation activity and PBP2 has only transpeptidation activity (34). PBP1 is responsible for increasing the PG strand length. As PG is assembled in the periplasm, PBPs bind peptide stems of the newly incorporated PG subunits and crosslink them to the existing cell wall (34). If MltG acts in terminating addition of new PG subunits to the growing PG strand, as has been proposed for other bacterial species (14, 17), then MltG might interact directly with one or both of the gonococcal PBPs. Using bacterial adenylate cyclase-based two-hybrid assays (BACTH) we determined that MltG interacts with both PBP1 and PBP2 (Fig. 5). MltG preventing continued addition of cell wall subunits by PBP1 and PBP2 would stop constant addition of PG monomers to the PG layer. Deletion of *mltG* would be expected to affect PG synthesis by allowing strand synthesis to continue beyond the normal length which can affect cell size, as was seen with some mutant cells(35).

During our BACTH assays we also determined that MltG interacts with PBP4 (Fig. 5): an endopeptidase and D,D-carboxypeptidase. PBP4 can cleave bonds crosslinking adjacent PG monomers and cleave the terminal alanine residue on the peptide converting a pentapeptide to a tetrapeptide(36). The other low molecular weight PBP, PBP3, can also perform these functions, and one of these two enzymes must be present for normal separation of PG strands for PG degradation by Ltg's and AmiC(37).

Despite obtaining positive results in the colorimetric assay for some proteins, we determined that when *mltG* is attached to the T18 plasmid there is no evidence of a protein-protein interactions for any protein. This result could indicate that the fusion in pT18 affects

MltG protein folding or covers an interaction domain, preventing interaction with partners that were interacting when *mltG* was in pT25.

MltG mutants are more sensitive to antibiotics that target later processes in PG biosynthesis

Changes in sensitivity to antibiotics can indicate a protein's importance in a cellular process, and alterations of the cell wall can lead to cell wall-specific antibiotic sensitivities or general defects in permeability. We tested antibiotic resistance to erythromycin, tetracycline, vancomycin, ceftriaxone, fosfomycin, and penicillin using disk diffusion assays, where the zones of clearing were measured as a representation of growth inhibition. For most antibiotics, the *mltG* deletion mutant had comparable susceptibilities to the wildtype. However, an *mltG* deletion mutant was more susceptible to penicillin, vancomycin, and ceftriaxone, all of which are antibiotics that target the cell wall crosslinking (Fig. 6). This result may indicate that an altered cell wall structure in the *mltG* mutant allows more antibiotic to reach its target or that the mutation results in a more permeable outer membrane.

Although fosfomycin targets cell wall synthesis, there was not a significant change in susceptibility in the *mltG* mutant. A lack of change in fosfomycin resistance was also observed in a *P. aeruginosa* strain with a deletion of *mltG* (20). Fosfomycin does not target processes involving transpeptidation and PBPs, and this could explain the lack of change in susceptibility, as MltG interacts with PBPs. Also, fosfomycin targets a cytoplasmic enzyme. The effects of an *mltG* mutation on antibiotic susceptibility may all be on factors that act at the periplasm or outer membrane.

mltG *mutation affects PG recycling and autolysis*

The cell wall is an important structure in bacteria for maintaining cell shape and protecting against osmotic stress (38). During growth, PG is constantly being broken down and

rebuilt to allow changes in cell size and to allow cell separation (39). A constant balance between degradation and synthesis of the PG must occur to prevent thickening or weakening of the cell wall and subsequent cell lysis. During PG degradation in *N. gonorrhoeae*, most of the PG fragments are recycled back into the cell to be reincorporated into the PG layer (29, 40, 41). We measured PG turnover using metabolic labeling with [6-^{3}H]-glucosamine in a pulse-chase experiment. The amount of labeled PG remaining in the sacculus over time was determined. The *mltG* deletion mutant showed a higher rate of turnover, nearly twice that of the wild-type strain (Fig. 7). Complementation restored a wild-type level of PG turnover. Our turnover results are consistent with the larger amount of PG fragments release into the medium by the *mltG* deletion mutant demonstrated in Fig. 3.

N. gonorrhoeae undergoes autolysis when in conditions not favorable to growth(42). To examine the effects of the *mltG* deletion on autolysis, we suspended log-phase Gc in TrisHCl buffer (pH 8) and measured OD_{540} to follow cell lysis. Both the point mutant and deletion mutant were significantly less autolytic than the WT in buffer (Fig. 8). Complementation of *mltG* restored the wild-type phenotype. This significant difference in autolysis might be due to a change in cell wall structure or effects on other peptidoglycanase proteins in the periplasm.

Mutation of mltG *does not affect colonization or infection*

We used the *Neisseria musculi* mouse infection model to examine possible defects in *Neisseria* infection or colonization due to *mltG* mutation(30). The entire *mltG* coding region except for a single base, was replaced with a kanamycin-resistance cassette in *N. musculi* strain AP2031. Oral colonization and blood infection were measured for the wildtype and *mltG* mutant strains. Mice were either orally inoculated or injected intra-peritoneally with either strain. For oral colonization, swabs of the oral cavity were taken at 7-, 14-, and 17-days. For blood

infection, blood was collected retro-orbitally at 4-, 12- and 24-hours. There was no significant difference in oral colonization or blood infection between the mutant and the wildtype (Figure 9). Similarly, an analysis of inflammatory cytokine production showed no differences between the wild type and *mltG* mutant (Fig. 10).

Discussion

LTs play critical roles in several processes in Gc including in PG breakdown, cell division, and in the Type 4 Secretion System (10–12). MltG is a newly identified LT in Gc that has been characterized in other bacterial species. Our goal was to identify the role of MltG in PG metabolism of *N. gonorrhoeae*. In Gc, MltG is a LT that may primarily act on uncrosslinked strands of PG. When it is deleted or mutated, more PG fragments are released. This increase indicates that MltG is not involved in monomer production for release. However, the increase in released PG fragments suggests another LT is involved in producing monomer fragments. To identify this LT, released fragments from double mutants of *ltgA* and *mltG* and *ltgD* and *mltG* were characterized. Characterizations of released PG fragments for the double mutants were similar to those of single mutants of *ltgA* and *ltgD* which show decreased PG monomer fragment released, indicating both LtgA and LtgD may be producing the monomers released in the single *mltG* mutant or the process resulting in the large monomer release does not occur in the double mutant.

Just as more PG is released, more PG of the cell wall is also turned over. The continued synthesis of PG in the *mltG* mutant may be driving the increased release. The continued PG synthesis might require increased PG degradation for the bacteria to maintain normal cell size. This hypothesis is supported by the increased number of cells of large cell size. The higher turnover could also indicate a deficiency in recycling, as we have noted previously that certain PG recycling mutants alter their PG fragment uptake in a way that suggests PG fragment monitoring and regulation(7, 8, 29). Looking further at the cell wall, we also determined that *mltG* mutant cells were less autolytic in non-growth conditions. The process of autolysis is poorly understood in *N. gonorrhoeae*, but mutants lacking various PG degradation enzymes or

lacking a phospholipase are more autolysis-resistant, suggesting that cell wall breakdown or membrane degradation act in this process (43–46). The increased resistance to autolysis in the *mltG* mutant might indicate an altered cell wall structure or decreased autolysis activity of PG-degrading enzymes in the absence of MltG function.

Studying MltG can have importance in combating antibiotic resistance. We see in *P. aeruginosa* that deleting *mltG* results in decreased MIC or increased sensitivity to ß-lactam antibiotics (20). We also see with Gc that an *mltG* deletion changes the sensitivity to this class of antibiotics. Mutants are more susceptible to penicillin, vancomycin, and ceftriaxone, which are all antibiotics that target the cell wall and involve the transpeptidation reaction. Targeting this enzyme could allow the use of antibiotics that were previously off limits due to high levels of resistance. This could allow the treatment of multi-drug resistant cases and allow time for the development of new antibiotics for treatment of gonorrhea. Using this strategy would have large implications on our current struggles to treat Gc infection and prevent long term consequences associated with untreatable gonorrhea. Currently there is a compound, bulgecin A, that was shown to target MltG in *P. aeruginosa* (13). Further investigation into this compound and others like it could be beneficial for future drug development.

The bacterial two-hybrid analysis suggests that MltG interacts with both PBP1 and PBP2. Interactions between MltG and PBPs have also been observed in *E. coli* and *B. subtilis* (14, 17). This result is consistent with a role for MltG in cell wall synthesis. Additionally, MltG was shown to interact with PBP4 which is an endopeptidase in Gc, similar to PbpG(36, 44). The endopeptidase activities of PBP4 and PBP3 in *N. gonorrhoeae* are critical to normal growth of the bacteria and for function of the amidase AmiC in PG degradation(44, 47). Thus, MltG

binding to PBP4 may couple PG synthesis machinery to the PG degradation enzymes that open a space for newly synthesized PG strands.

Through the interaction with PBPs, MltG can terminate elongation through cleavage of PG. The resulting anhydro-muropeptide that caps the strand would prevent further elongation and crosslinking by PBP1 and PBP2. Without this cleavage, PBPs would continue to extend the glycan chain. As elongation of the glycan chain continues in the absence of MltG, lytic transglycosylases such as LtgA, LtgD, and/or another LT may attempt to maintain normal cell size by cleaving off excess subunits of PG. As they cleave off excess PG, monomers are released leading to more PG monomer fragment release compared to wildtype. Without MltG, the cell is unable to coordinate synthesis to maintain the structure of the PG layer in the cell.

The lack of significant differences seen in oral colonization and blood infection indicate that the large increase in PG monomer may not be sufficient to significantly increase the activation of the immune response in such a way that colonization and infection are affected. Specifically for oral colonization, there are many other microbes present and the host may have developed methods to decrease the response to commensals to prevent unnecessary activation of the immune response. The cytokine data from the blood infection showed that there were no significant differences between the wildtype and the mutant in the concentrations of any of the tested cytokines. However, the slightly larger amount of blood infection at 4-hours contributed to a significant decrease in IL-6, MCP-1, TNF, and IL-10 between either 4- and 12-hours, 4- and 24-hours, or both. This indicates that a larger immune response correlates with higher infection.

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Figures

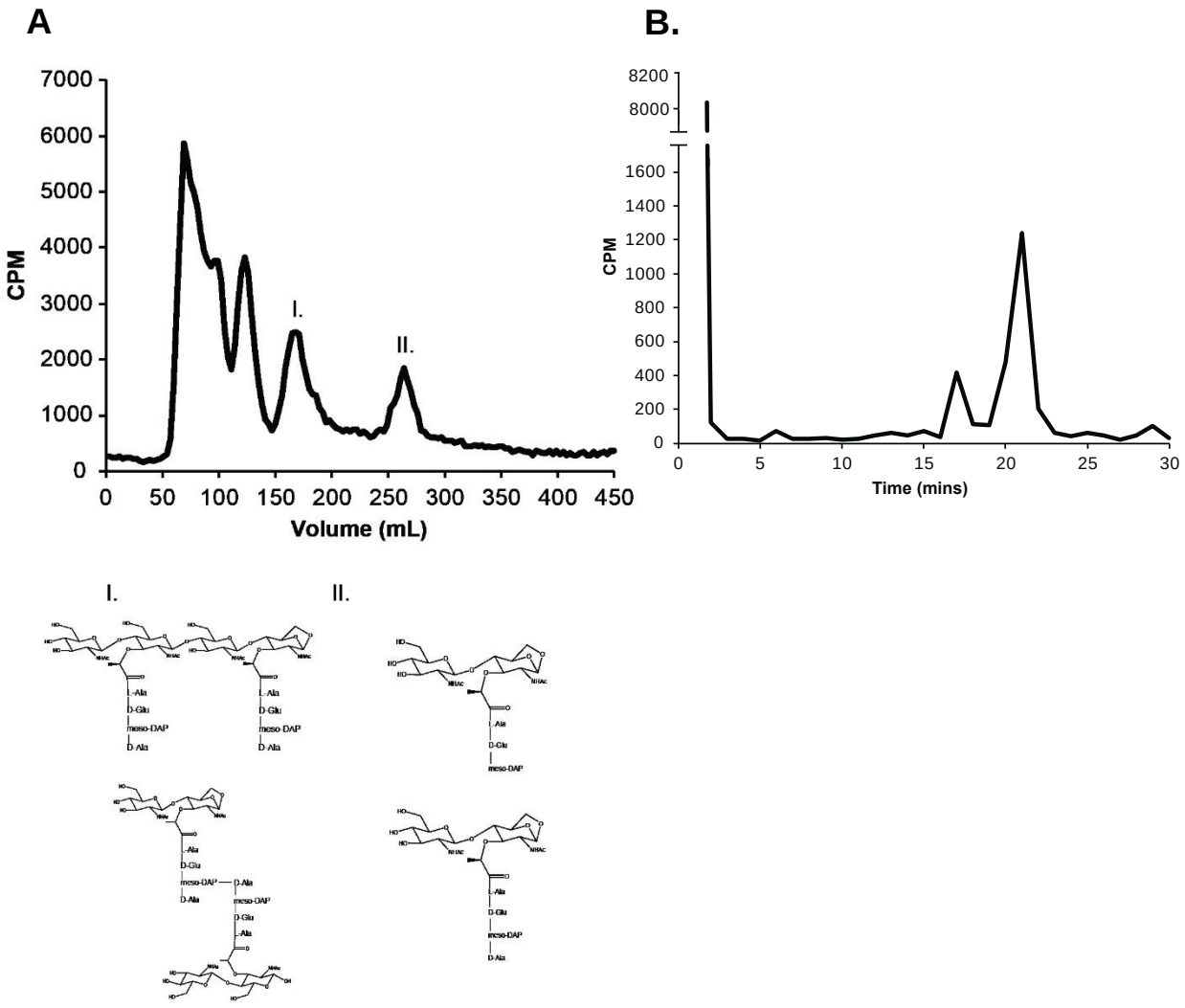


Figure 1. Digestion products of MltG. *a)* A soluble form of MltG was purified and used to digest whole sacculi metabolically labeled with [6, ^{3}H]-glucosamine. The soluble PG fragments generated from the digestion by MltG were separated by size-exclusion chromatography. MltG generated large PG fragments, PG monomers (II.), and PG dimers (I.). *b)* Dimers from the digested sacculi were further analyzed by HPLC after digestion with LtgD. The peaks represent tri- and tetrapeptide PG monomers which can only be produced by LtgD if there are PG dimers present that are glycosidically linked.

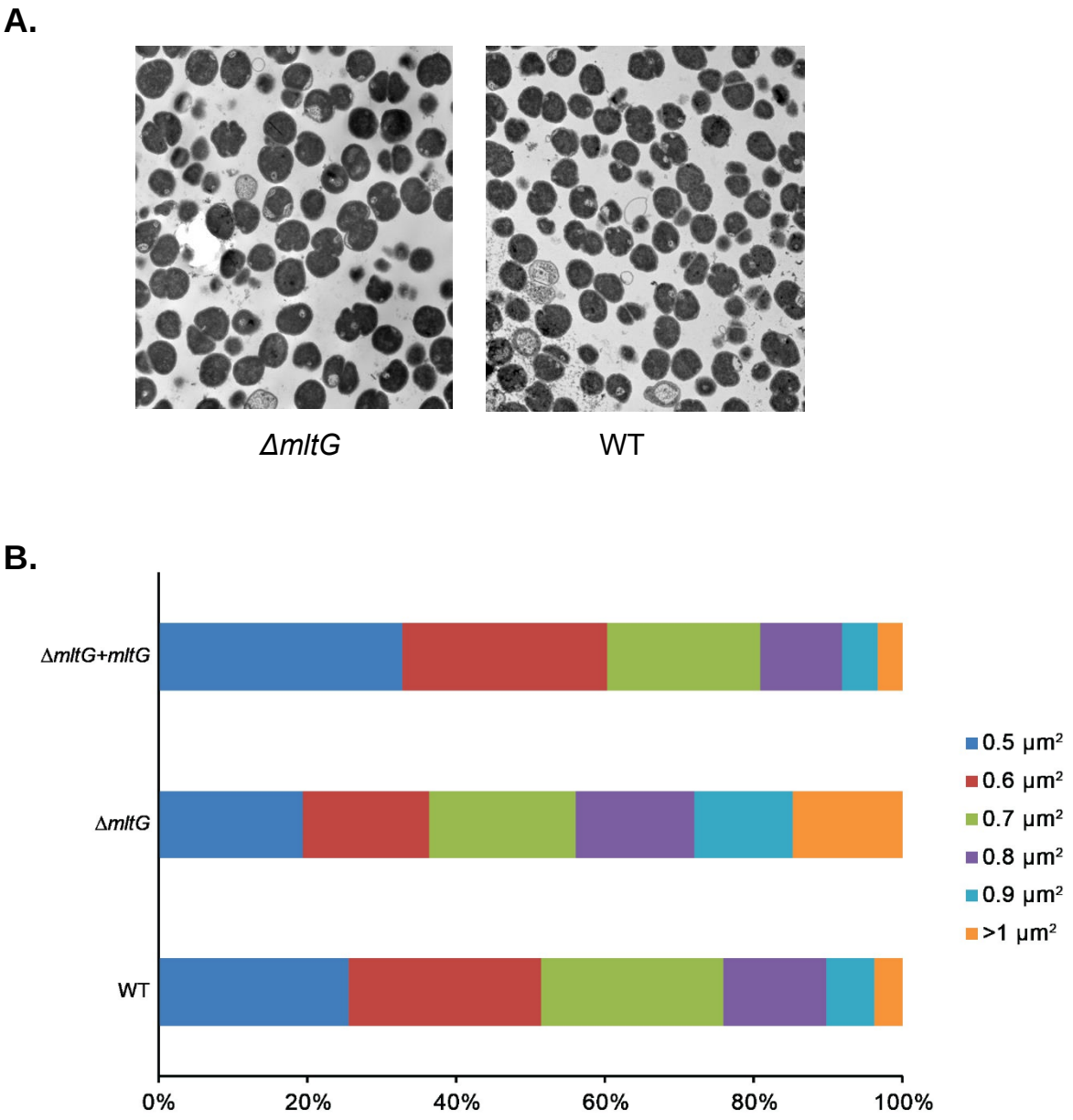


Figure 2. Thin-section microscopy of *mltG* mutants. *a)* Cells of both the WT and deletion (Δ*mltG*) have both mono- and diplococcal cell morphologies. There is not a significant change in cell shape. *b)* Looking at the percentage of cells of different areas, the *mltG* deletion (Δ*mltG*) has a higher percentage of cells that are larger than 0.7 µm^2.

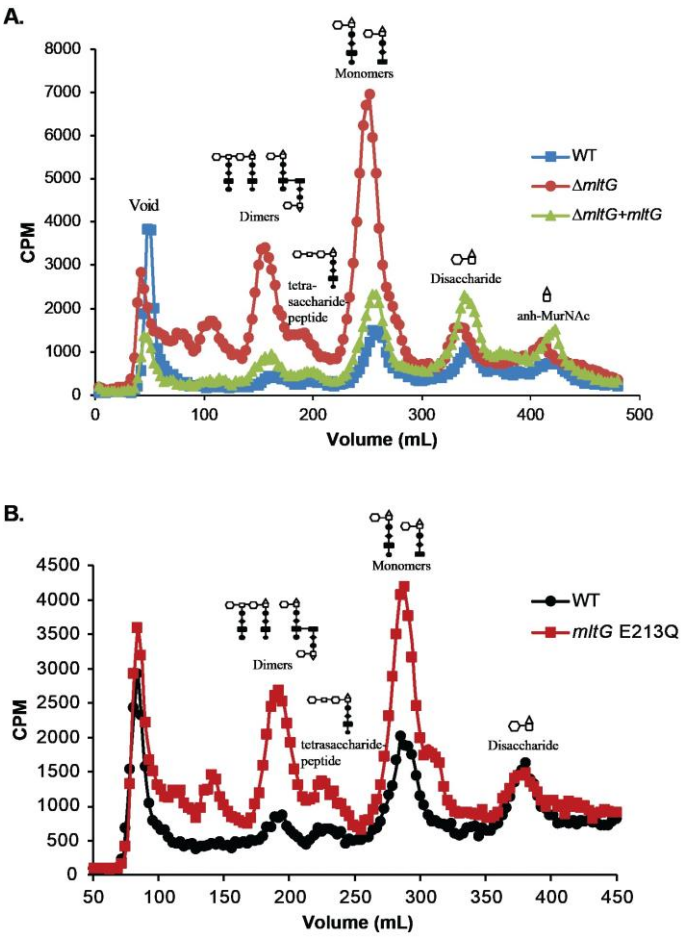


Figure 3. An *mltG* mutant releases more multimer, dimer, and monomer PG fragments. Characterization of released PG fragments using [6,^{3}H]-glucosamine was conducted. Briefly, cells were incubated with the labeled glucosamine and allowed to incorporate the molecule into their PG. This was followed by a period of incubation allowing the bacteria to release the labeled PG. Cells were pelleted out and the supernatant was run on a size-exclusion column. The fragments were separated and collected into fractions which were measured for counts per minute (CPM). The strains tested include a strain with an *mltG* deletion (Δ*mltG*) (A.), a strain complemented with *mltG* at an ectopic site (Δ*mltG*+*mltG*), and an *mltG* point mutant (*mltG* E213Q) (B.), and wildtype (WT). For both *mltG* mutants, multimer, dimer, and monomer PG fragments were increased as compared to wildtype. The complement strain (Δ*mltG*+*mltG*) had similar released PG fragments as the wildtype (WT).

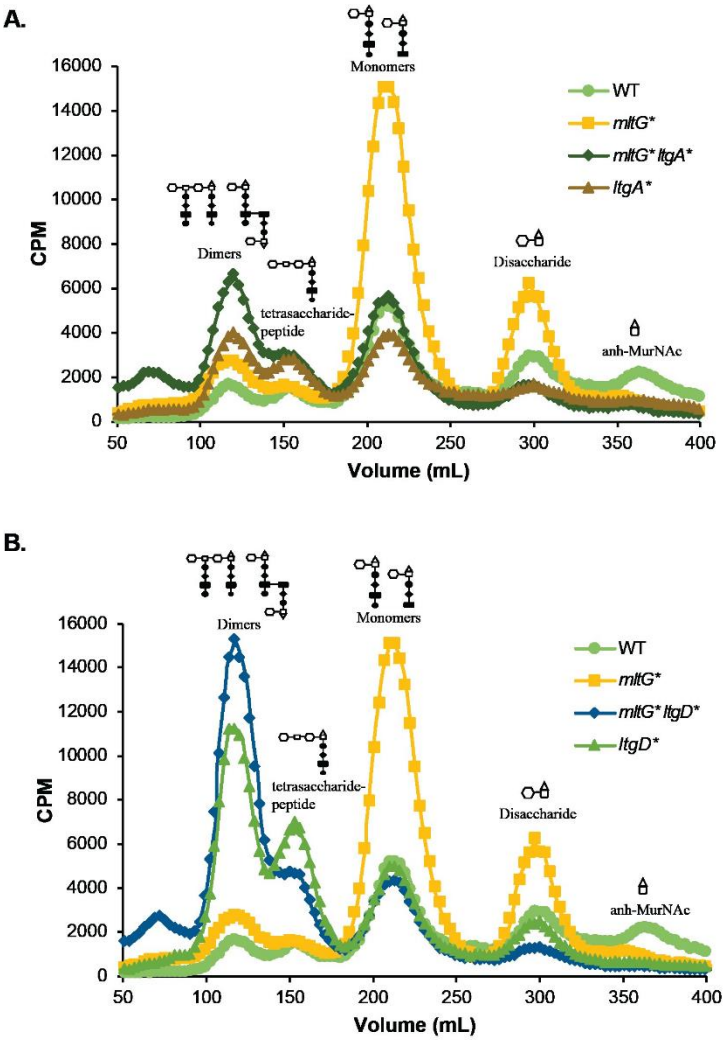


Figure 4. LtgA and LtgD may produce the large amount of PG monomer fragments that are released in the *mltG* mutant. Characterization of released PG fragments was conducted as previously described. Double mutants of *mltG* and either *ltgA* or *ltgD*, released similar PG fragments and amounts as the respective single mutants of *ltgA* or *ltgD*. For the single and double mutants of *ltgA* (*ltgA** and *mltG *ltgA**) (A.) and *ltgD* (*ltgD** and *mltG * ltgD**) (B.), there were more dimer and tetrasaccharide peptide PG fragments released as compared to WT. Furthermore, for the single and double mutants of *ltgD*, there were more of the dimer and tetrasaccharide peptide PG fragments released compared to the single and double *ltgA* mutants.

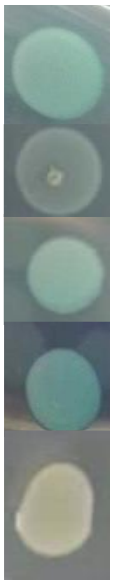


Figure 5. MltG interacts with PBP1, PBP2, and PBP4. Using bacterial adenylate two-hybrid assays proteins that MltG interacts with were elucidated. White colonies indicate no interaction, whilst blue colonies indicate the two proteins interact. MltG was found to interact with PBP1, PBP2, and PBP4. PBP1 is a transglycosylase and a transpeptidase. PBP2 is just a transpeptidase. PBP4 is endopeptidase and a D,D carboxypeptidase. The presence of an interaction between these proteins indicates a possible role for MltG in PG synthesis.

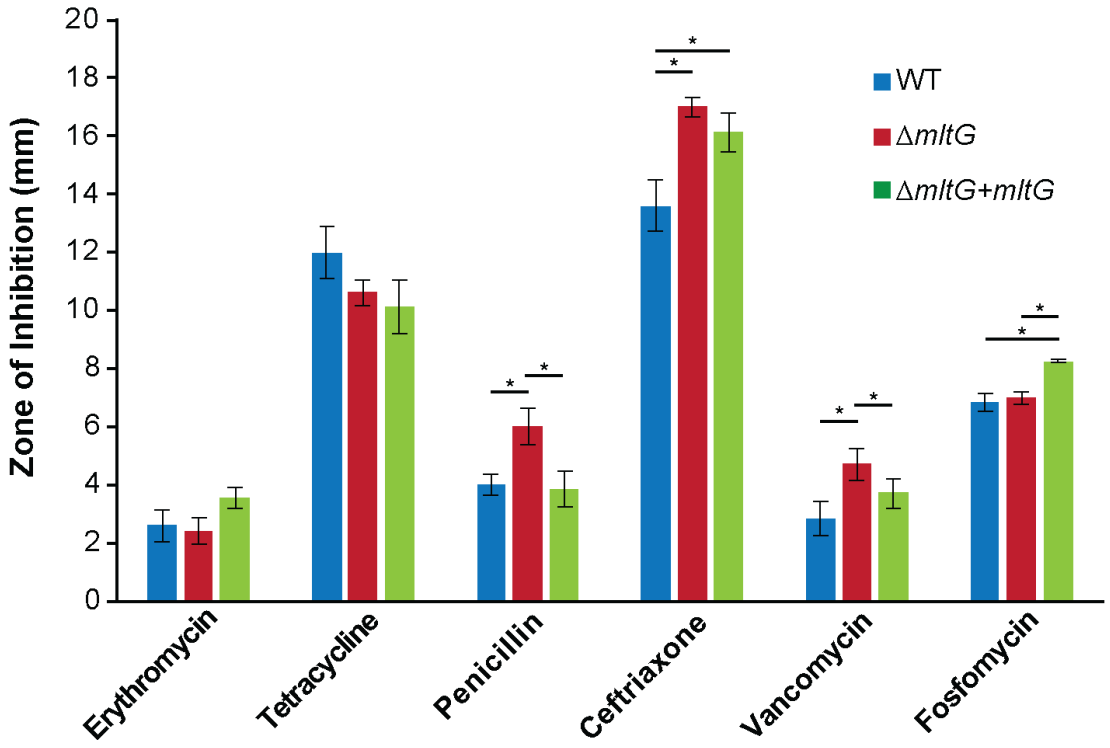


Figure 6. Mutants of *mltG* are more sensitive to penicillin, vancomycin, and ceftriaxone, but not fosfomycin. Using disk diffusion assays, the zones of inhibition representing the susceptibility or resistance of a strain to a certain antibiotic were measured. There were significant increases in susceptibility to most antibiotics that target the cell wall between the WT and *mltG* deletion (Δ*mltG*). These antibiotics include the beta-lactams penicillin and ceftriaxone, and vancomycin, but exclude Fosfomycin. The lack of a significant difference between the WT and *mltG* deletion (Δ*mltG*) is most likely because Fosfomycin targets a cytoplasmic protein involved in the early synthesis of PG. *$p<0.05$

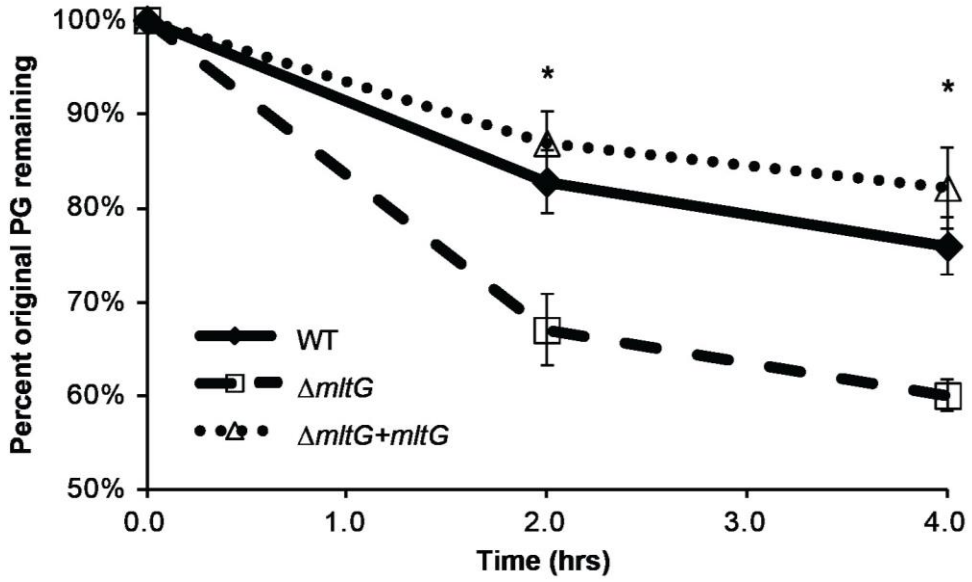


Figure 7. Mutants of *mltG* show a higher rate of PG turnover. *N. gonorrhoeae* strains were labeled with [6,^{3}H]-glucosamine and grown in log phase for 4h. At each time-point, cells were removed, the PG in the sacculi was extracted, and radiation measured to determine the amount of original PG remaining in the cell wall. Observations revealed that the *mltG* deletion (Δ*mltG*) had significantly less original PG remaining as compared with WT. This result indicates that there is more PG turnover in the *mltG* deletion (Δ*mltG*). * $p<0.05$

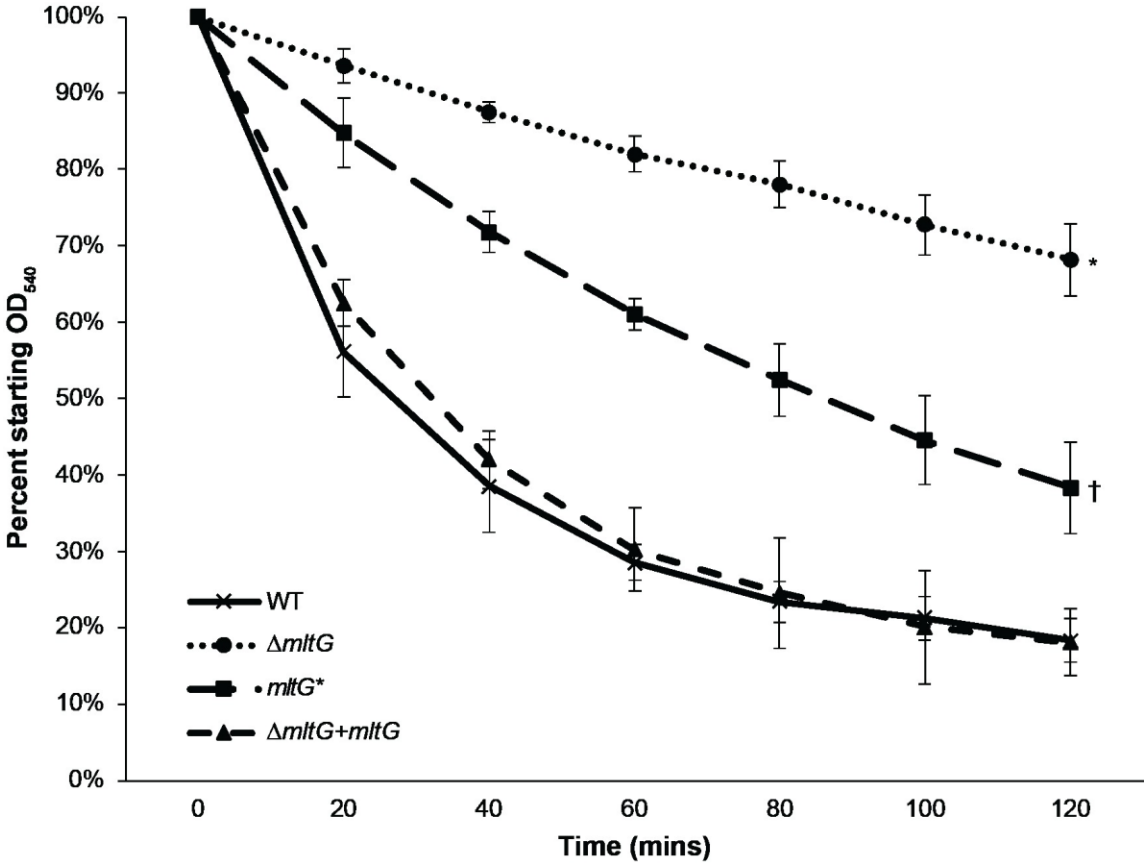


Figure 8. *mltG* mutations result in less autolysis in buffer. Strains were grown to log phase and then resuspended in TrisHCl buffer pH 8. Absorbance values at an OD_{540} were taken every 20 minutes for two hours. Both the deletion mutant (Δ*mltG*) (* $p<0.01$) and point (*mltG**) mutant († $p<0.05$) had significantly less lysis compared with wildtype (WT). However, the deletion mutant and point mutant were also significantly different at all timepoints except at 20 minutes ($p<0.05$). There was no difference in lysis between the wildtype and complement (Δ*mltG +mltG*) strains.

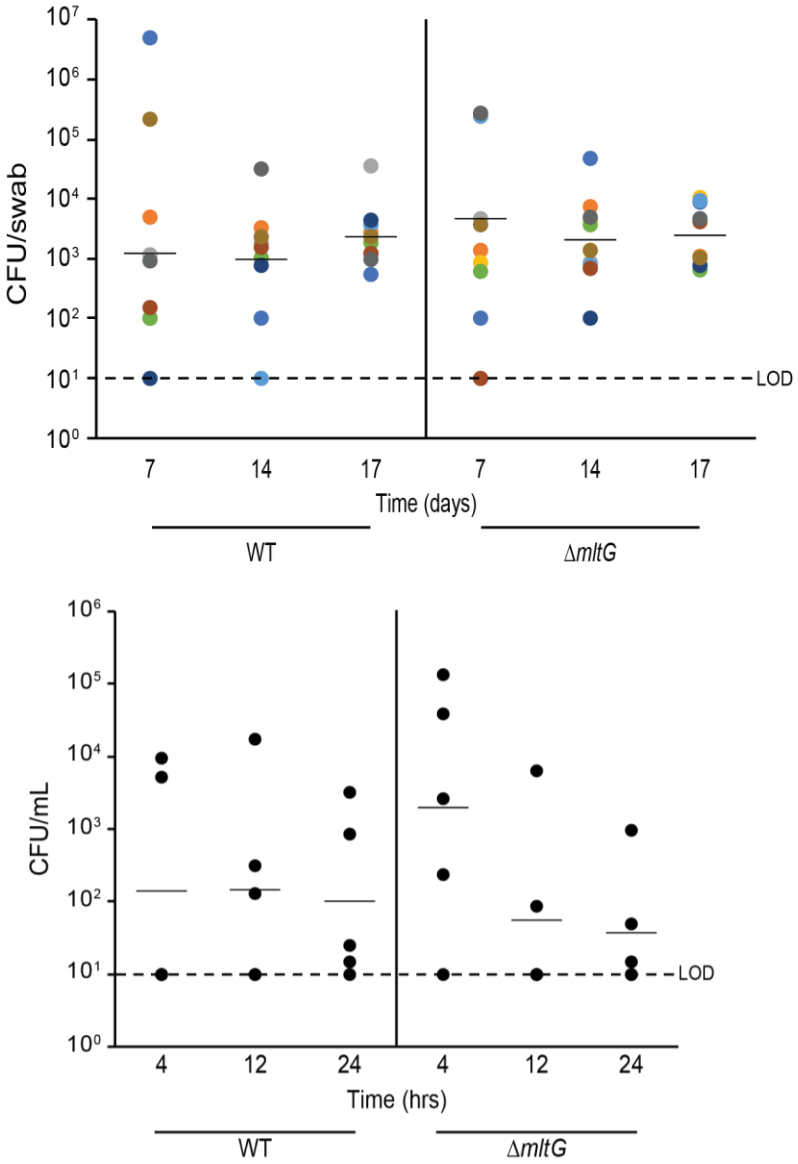
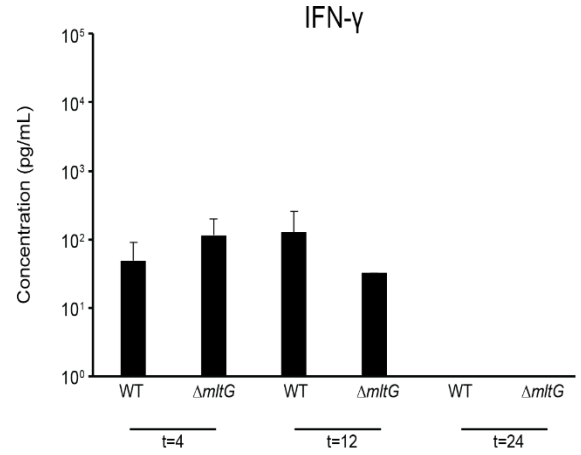
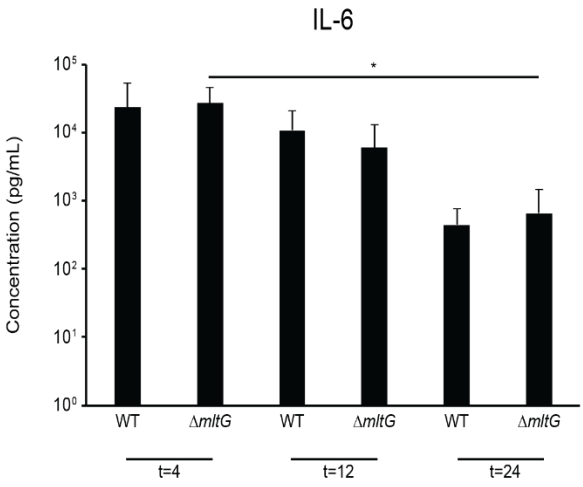
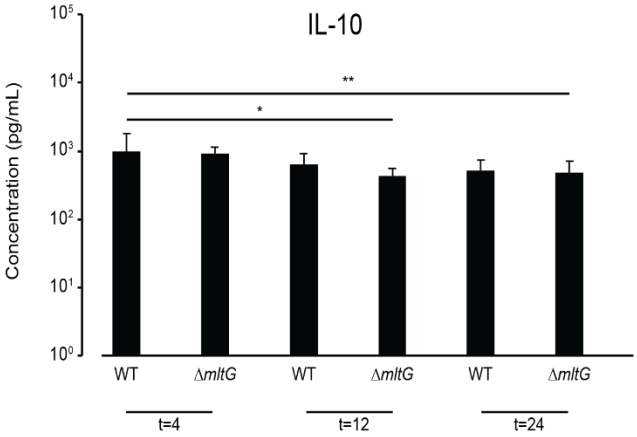
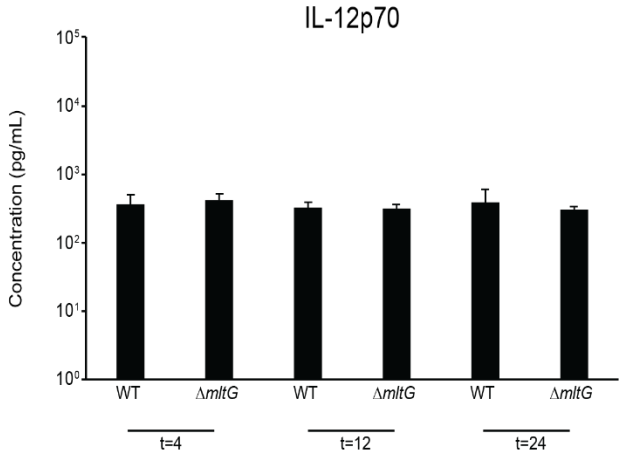


Figure 9. Oral colonization and blood infection are unchanged in the *mltG* mutant. C57BL/6J mice were either orally inoculated or intraperitoneally (IP) injected with Nmus. After taking samples at 7-, 14-, and 17-days post oral inoculation and 4-, 12-, and 24-hours post IP injection it was determined that an *mltG* deletion (Δ*mltG*) did not significantly affect oral colonization or blood infection. Despite the lack of significant difference, there were higher CFU/mL values in the *mltG* deletion (Δ*mltG*) at 4-hours compared to 12- and 24-hours during blood infection.


IFN-γ
Concentration (pg/mL)
WT
ΔmltG
t=4
t=12
t=24


IL-6
Concentration (pg/mL)
*
WT
ΔmltG
t=4
t=12
t=24


IL-10
Concentration (pg/mL)
**
*
WT
ΔmltG
t=4
t=12
t=24


IL-12p70
Concentration (pg/mL)
WT
ΔmltG
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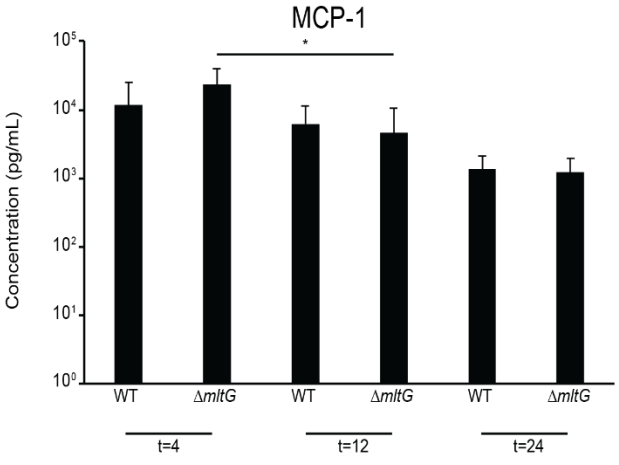
t=12
t=24


MCP-1
Concentration (pg/mL)
*
WT
ΔmltG
t=4
t=12
t=24

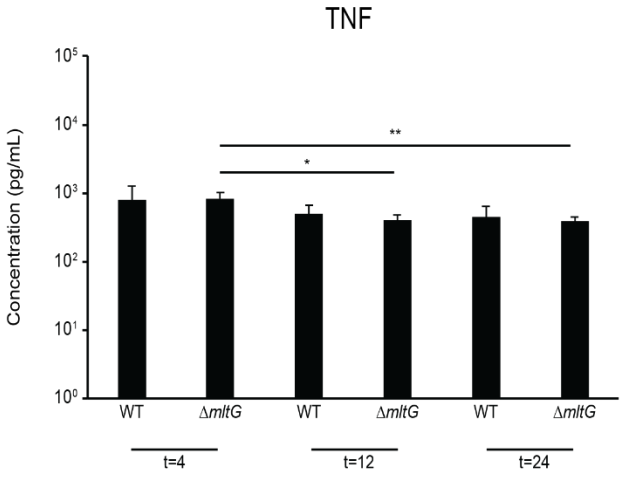

TNF
Concentration (pg/mL)
**
*
WT
ΔmltG
t=4
t=12
t=24

Figure 10. Decreasing blood infection correlates with decreasing cytokine concentrations in serum. Correlating to the lack of significant difference in oral colonization and blood infection (Fig 9), There was also not a significant difference in cytokine concentration between the WT and the *mltG* deletion (Δ*mltG*). However, the higher CFU/mL observed at 4-hours correlated with significantly higher cytokine concentrations of IL-6, IL-10, MCP-1, and TNF between certain timepoints. IL-6 had higher concentrations at 4-hours compared with 24-hours. IL-10 had higher concentrations at 4-hours than at both 12- and 24-hours. MCP-1 had higher concentrations at 4-hours compared with 12-hours. TNF had higher concentrations at 4-hours than at both 12- and 24-hours * $p<0.05$, ** $p<0.05$

Chapter 3: Effect of a cell separation defect on infection and peptidoglycan fragment release in *Neisseria musculi*

Tiffany N. Harris-Jones and Joseph P. Dillard

TN Harris-Jones and JP Dillard wrote the manuscript

Abstract

Cell separation is an important cellular process in the growth and multiplication of bacteria. This event must be properly coordinated to ensure daughter cells contain all necessary components for viability. Peptidoglycan (PG) is a macromolecule that surrounds the bacterial cell allowing it to maintain shape. This rigid structure is constantly being degraded and resynthesized to allow for bacterial growth and separation. In *Neisseria gonorrhoeae* (Gc), the amidase AmiC is required for proper cell separation. Mutants of *amiC*, in Gc, have severe defects in cell separation and grow in clusters. In 2016, a mouse commensal of the genus *Neisseria* was identified. *Neisseria musculi* (Nmus) was isolated from wild mice in Arizona. There is homology between the human pathogen Gc and the mouse commensal, including the presence of this amidase. This homology allows the development of a useful mouse model to mimic infection with Gc in mice. In this study, we characterize AmiC in Nmus and investigate its effects on PG fragment release, mNOD1 activation, and infection. Our study results show that although Nmus has a cell separation defect like Gc, its mNOD1 activation and PG fragment release differ. Additionally, systemic infection is significantly decreased. Despite the differences, our model has shown usefulness to provide additional insight into the importance of AmiC in infection but possibly also successful cell separation.

Introduction

Neisseria are found in many different species of animals primarily as oral mucosal commensals (1, 2). However, there are two pathogenic *Neisseria* species, *Neisseria gonorrhoeae* (Gc) and *Neisseria meningitidis*, that are human-specific pathogens. Due to the human specificity of Gc, it is difficult to model these pathogens in *in vivo* animal models. Human models for Gc are restricted to early infections in males and are not ethical in females or in late stages of the infection (source). There are no human models of infection for *N. meningitidis.*

While there are mouse models for Gc, current models are insufficient because they do not allow the study of specific host-*Neisseria* interactions. These models are useful and have allowed the discovery of important aspects of gonococcal infection but are lacking in their ability to provide insight into the many interactions that are host specific(3). These interactions in Gc infection include infection functions as basic as adherence, iron acquisition, and serum resistance. For many other human-specific pathogens, mouse-specific bacteria that are highly similar to their pathogenic counterparts are used to model infection in mice. These include *Chlamydia muridarum* for *Chlamydia trachomatis* infection and *Mycobaterium marinum* for *Mycobacterium tuberculosis* infection (4, 5). In 2016, the mouse oral commensal *Neisseria musculi* (Nmus) was isolated from wild mice in Arizona (6). This species has high genetic homology to Gc as well as similarities in transformation ability (6). These features make Nmus highly favorable for adaptation into a mouse model to allow the study of specific host-*Neisseria* interactions since the species is the natural host for mice.

During gonococcal infection, significant amounts of peptidoglycan (PG) fragments are released from the cell. These fragments are inflammatory and are known agonists of the pattern recognition receptors NOD1 and NOD2 (7–9). PG is a dynamic mesh-like polymer that consists

of repeating subunits of *N*-acetylmuramic acid (NAM) and *N*-acetylglucosamine (NAG). There is a peptide stem of 2-5 amino acids attached to the NAM, and adjacent strands of PG are crosslinked at this peptide stem. The amino acids occur in the order L-alanine, D-glutamate, *meso*-diaminopimelic acid, D-alanine, and D-alanine. There are several proteins involved in the breakdown of PG to produce the fragments that are either released from the cell or recycled back into the cell. These proteins include amidases, lytic transglycosylases, endopeptidases, and carboxypeptidases.

Amidases cleave the bond between the peptide stem and the L-alanine of NAM, freeing the peptide stem. The periplasmic amidase in *Neisseria* is AmiC, and its presence is necessary for successful cell separation(10, 11). Our study uses an Nmus mouse model to evaluate the effect an *amiC* mutation has on blood infection. Additionally, analyses of NOD1 activation, PG fragment release, and cell morphology of *amiC* mutants were conducted.

Materials and Methods

Bacterial strains and growth

All *N. musculi* strains were derivatives of the smooth variant of strain AP2031. *N. musculi* strains were grown at 37°C and 5% CO_2 on GCB agar plates (Difco) with Kellogg's supplements. Strains were also grown in gonococcal base liquid medium (GCBL) containing 0.042% $NaHCO_3$ and Kellogg's supplements with aeration(12, 13). Antibiotics were used at the following concentrations: kanamycin at 80 mg/mL and rifampicin at 40 mg/mL.

Plasmid and strain construction

The plasmids used in this study are listed in Table 1. Chromosomal DNA from *Neisseria musculi* AP2031 was used as a PCR template unless otherwise noted.

Table 1.

Plasmid or Strain	Description	Reference
pIDN3	Insertion-duplication plasmid (Erm^R)	Hamilton et al (2001) (14)
pEC175	*amiC* interruption constructed in pIDN3 (Kan^R)	This study
pEC176	*amiC* interruption constructed in pIDN3 (Cm^R)	This study
AP2031	Wildtype *Neisseria musculi*	Weyand et al (2016) (6)
TNH2002	Wildtype *Neisseria musculi* with natural resistance to rifampicin (Rif^R)	Chapter 2
TNH2006	TNH2002 transformed with pEC175 (Rif^R, Kan^R)	This study
EC2023	AP2031 transformed with pEC176 (Cm^R)	This study

Thin-section electron microscopy (TEM)

Thin-section electron microscopy was conducted by initially growing Nmus strains on GCB plates overnight. Cells from plates were suspended in PBS, pelleted, washed once in PBS, and then resuspended in Karnovsky's fixative. TEM was performed at the University of

Wisconsin-Madison Medical School Electron Microscope Facility with the assistance of Randall Massey.

Characterization of PG fragment release

Characterization of released PG fragments from Nmus was conducted as described in Rosenthal and Dziarski (Isolation of PG and soluble PG fragments, Method Enzymol 1994) and adapted by Cloud and Dillard (A LT of NG is involved in PG-derived cytotoxin production, Inf Imm 2002). To label the glycan chain, Nmus cultures were grown in GCBL medium containing 0.4% pyruvate without glucose and 10 µCi/mL of [6-^{3}H]-glucosamine. Cultures were labeled for 45 minutes and then centrifuged. Pellets were resuspended in GCBL medium with Kellogg's supplements (containing glucose) and allowed to release PG during 2.5 hours of growth. After incubation, cells were pelleted, and supernatant passed through a 0.2 µM filter. Normalization of radioactive counts per minute in each culture was done by aliquoting 60 µL from each supernatant culture and adjusting the culture volume. This methodology allows equivalent amounts of radioactivity between cultures allowing quantitative comparison(15). Samples were passed through a size exclusion column eluted with 0.1 M LiCl. Fractions collected consist of 500 µL of the fraction which is then resuspended in 3 mL of scintillation fluid. Fractions were counted with a Perkin Elmer Tri-Carb 4910TR scintillation counter.

Analysis of HEK293-blue NOD1 activation

Strains of Nmus were grown for 3 hours in GCBL from an initial OD_{540} of 0.2. Cultures were centrifuged and supernatants passed through a 0.2 µM filter. Supernatants were normalized by dilution with GCBL, based on total protein content of cultures, and added to HEK293-reporter cells overexpressing NOD1 as described in (16). NF-kB activation was measured at

OD_{650} after incubation of cell supernatants in QUANTI-blue medium as described in the manufacturer's instructions (InvivoGen).

Infection of mice and cytokine analysis

All mice used were C57BL/6J from the Jackson Laboratory. Animal protocols were approved by the University of Wisconsin-Madison Institutional Animal Care and Use Committee. All mice were held in a room that was specific pathogen free 1 for two weeks prior to inoculation. The day of infection, TNH2002 and TNH2006 were swabbed from GCB agar, suspended in cGCBL at an OD_{540} of 0.2, centrifuged to pellet cells, and resuspended in Dulbecco's PBS. All mice were manually restrained and 100 µL was injected intra-peritoneally. Blood was collected retro-orbitally using heparinized or non-heparinized capillary tubes at 4-, 12-, and 24-hours post injection. Blood from heparinized capillary tubes was diluted in cGCBL, plated onto GCB rifampicin 40 mg/mL, and incubated for 48 hours at 37℃ and 5% CO_2. Blood collected in non-heparinized capillary tubes was aliquoted to a microcentrifuge tube and allowed to clot at room temperature for approximately 30 minutes before being placed on ice. Clotted blood was centrifuged at 3,000 RPM at 4℃. Serum was extracted and stored at -80℃ until cytokine analysis. Isolates obtained from GCB rifampicin agar plates were verified as *N. musculi* by colony lysis PCR using ITS primers specific to Nmus (17). Amplified sequences were screened by gel electrophoresis by comparison to the wild-type strain with rifampicin resistance, TNH2002, and found to be identical. Sequences of TNH2002 and AP2013 (wild-type strain) were found to be identical in unpublished data. Serum was analyzed using the BD CBA Mouse Inflammation Kit (Becton Dickinson) using the manufacturer's instructions.

Results

Mutation of amiC *causes a cell separation defect*

To identify if mutating *amiC* in Nmus has a similar cell separation defect to what occurs in Gc, thin-section electron microscopy (TEM) was conducted. Nmus strains were grown overnight on GCB agar, fixed, and subjected to TEM. The WT strain showed the bacteria growing as short rods, and no unseparated cells were seen. By contrast, the *amiC* mutant fields contained many elongated cells, and multiple cells were seen growing as a chain of unseparated cells. Thus, there is indeed a cell separation defect in the *amiC* mutant causing chaining of these rod-shaped cells (Figure 1).

Mutants of amiC *release less peptidoglycan fragments*

In *N. gonorrhoeae*, mutation of *amiC* eliminates bacterial release of certain soluble PG fragments including free disaccharide and tetrasaccharide-peptide, and alters the release of others including increasing release of PG dimers(10). We sought to determine if the *amiC* mutation in Nmus also affected PG fragment release. Pulse-chase metabolic labeling of PG was used to evaluate release of PG fragments into the culture supernatant. For the pulse phase each strain was labeled with ^{3}H-glucosamine followed by a chase phase allowing release of labeled PG fragments. Labeled PG fragments were separated by size-exclusion chromatography, and counts per minute (CPM) were measured by scintillation counting. Figure 2 shows the characterization of released PG fragments for each strain. Labeling efficiency in Nmus was not as high as that in previous studies of *N. gonorrhoeae*, making detection of the released PG fragments more difficult(10). For the WT Nmus strain, we noted release of PG dimer, monomers, free disaccharide, and free anhydro-NAM. The *amiC* mutant released lower amounts of PG fragments as compared with the wildtype. The amounts of monomer were reduced, and it is unclear if any

PG dimer was released by the mutant. There was no release of free disaccharide from *amiC*, as expected. Amounts of anhydro-*N*-acetyl muramic acid were similar for both strains.

Mutants of amiC *have decreased activation of mNOD1*

NOD1 is an intracellular pattern recognition receptor that recognizes the presence of PG monomer fragments. As the mutant has fewer monomer fragments released compared with the wildtype, analysis of mNOD1 activation was conducted to investigate the effects the difference in released monomer fragments have on NF-kB mediated inflammatory responses. mNOD1 activation was significantly decreased in the *amiC* mutant as compared to the wildtype strain (Figure 3). This observation follows the results for the PG fragment release which shows decreased monomers.

Mutants of amiC *have reduced infection in a mouse model*

Using our Nmus bacteremia mouse model we infected mice with either wildtype Nmus or a mutant of *amiC.* After measuring infection at 3 timepoints (4-, 12-, and 24-hours) by measuring CFUs in blood, we observed that the mutant had decreased infection at all timepoints (Figure 4). This result indicates that decreased immune activation from mNOD1 in the *amiC* mutant is not sufficient to cause increased bacterial survival and growth in the blood. It was possible that decreased immune activation would have resulted in more infection by the *amiC* mutant since the immune system is responsible for clearing the infection. However, our results do not suggest that. Instead, we can infer that the cells are unsuccessful at establishing an infection due to their inability to properly undergo separation.

Discussion

AmiC was previously identified in Gc as an *N*-acetylmuramyl-L-alanine amidase. It was found that when this protein was mutated there was a cell separation defect and increased outer membrane permeability as well as changes in PG fragment release (10). Herein we describe the changes that occur with mutation of *amiC* in the mouse commensal Nmus. Similarly to Gc, there is a cell separation defect that also occurs upon mutation of *amiC* in Nmus. The morphology of Nmus is short rods, so the defect produces long chains of cells instead of the cell clumping observed in Gc (Fig 1).

Mutation of *amiC* in Gc causes an increase in the release of PG dimer fragments and the abolishment of release of PG disaccharide fragments(10). For Nmus, the mutant released fewer PG fragments overall compared to wildtype, and showed a large decrease in PG monomer fragments (Fig 2). There were also no free disaccharide PG fragments released by the *amiC* mutant, consistent with a role for AmiC in removing peptides from PG strands prior to degradation by lytic transglycosylases(18). Consistent with the PG fragment release results, mNOD1 activation was reduced in the mutant compared to WT (Fig 3). PG monomers are the major PG fragments released by gonococci, and our results show that PG monomers are also released in significant amounts by Nmus. The lower amount of PG monomers released by the *amiC* mutant is likely responsible for the reduced mNOD1 activation, though AmiC also acts in free peptide release in *Neisseria* species and those molecules would also be expected to contribute to mNOD1 activation(18, 19).

Although mNOD1 activation was reduced in the mutant which would suggest lower immune activation against this strain, infection was also decreased (Fig 4). We predicted in our model that infection would be altered due to differences in immune activation resulting from

released PG fragments. The cytokine data indicates that after 4 hours, the mutant had significantly lower concentrations of cytokines (MCP-1 and TNF) or no detectable levels (IL-6, IL-10). So the immune system was activated by the mutant, but only for a short period. However, it seems most likely that the cell separation defect seen in the *amiC* mutant results in the decreased infection compared to wildtype. In *N. meningitidis*, mutation of *nlpD* (encoding the AmiC activator) results in loss of cell separation and inability of the bacteria to survive in human blood(18). Similarly, *ltgC* mutants of *N. gonorrhoeae* have a similar cell separation defect and exhibit reduced survival and growth in blood(20). The difference in immune activation and PG fragment release between Gc and Nmus does not negate the usefulness of our mouse model. Although the changes in infection were not immune driven and most likely due to the cell separation defect, the ability of our model to determine this deficiency in the mutant suggests that the model will be useful for identifying additional genes required for *Neisseria* infections.

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Figures

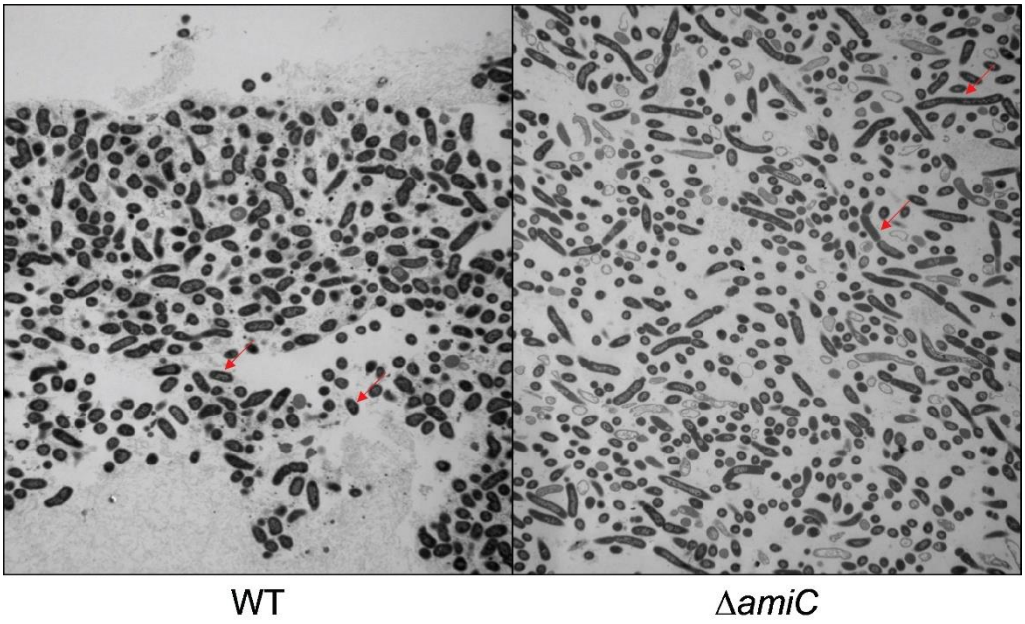

Figure 1. Mutation of *amiC* causes a cell separation defect. Thin-section electron microscopy (TEM) was done for the wildtype strain and the *amiC* mutation strain. The mutant strain had more elongated cells and more unseparated cells present as compared to the wildtype.

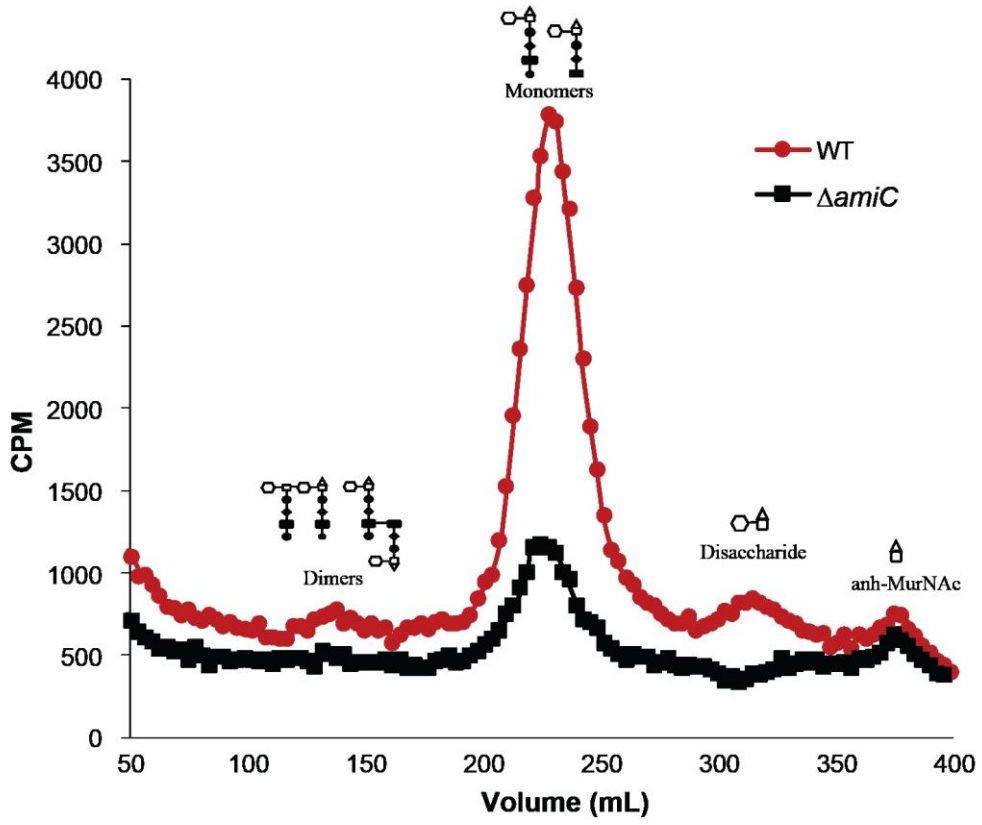

Figure 2. A mutant of *amiC* releases lower amounts of peptidoglycan (PG) fragments. PG fragments were separated using size-exclusion chromatography and collected into fractions. Counts per minute (CPM) were measured for each fraction. The mutant strain released less PG monomer fragments than the wildtype.

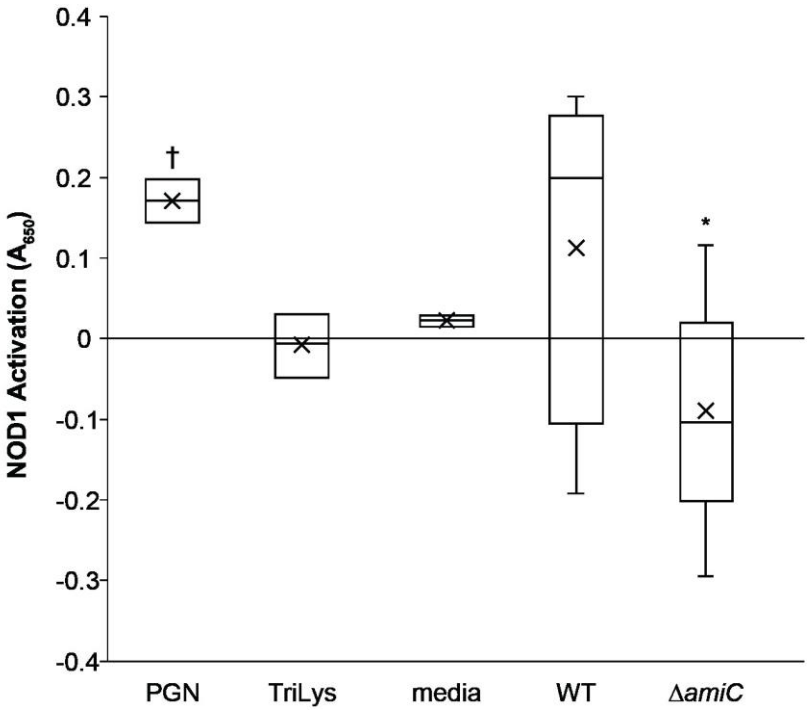

Figure 3. mNOD1 activation is decreased in the *amiC* mutant. HEK293 reporter cells overexpressing mNOD1 were used to measure mNOD1 activation between strains. The WT strain has significantly increased mNOD1 activation as compared with the mutant. The *amiC* mutant is significantly different than WT and media (* $p<0.05$). PGN is significantly different than the *amiC* mutant, TriLys, and media († $p<0.01$).

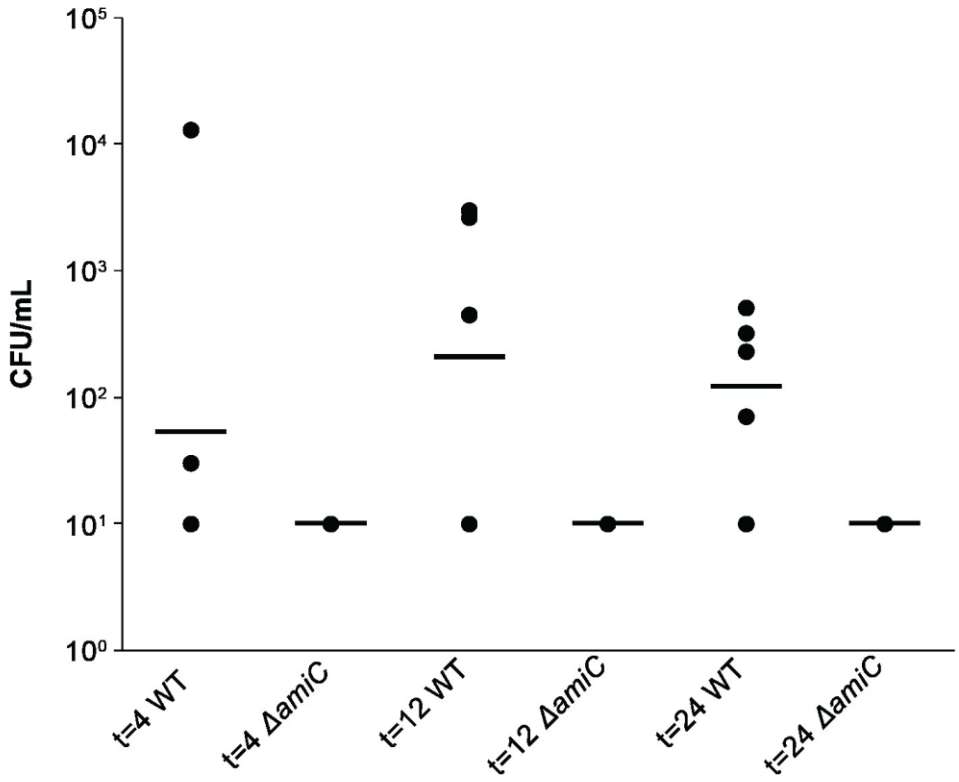

Figure 4. Mice infected with mutants of *amiC* have less infection compared to those infected with wildtype. C57BL/6J mice were intraperitoneally (IP) injected with Nmus. Samples were taken 4-, 12-, and 24-hours post IP injection and it was determined that an *amiC* mutation (Δ*amiC*) had decreased infection compared with WT.

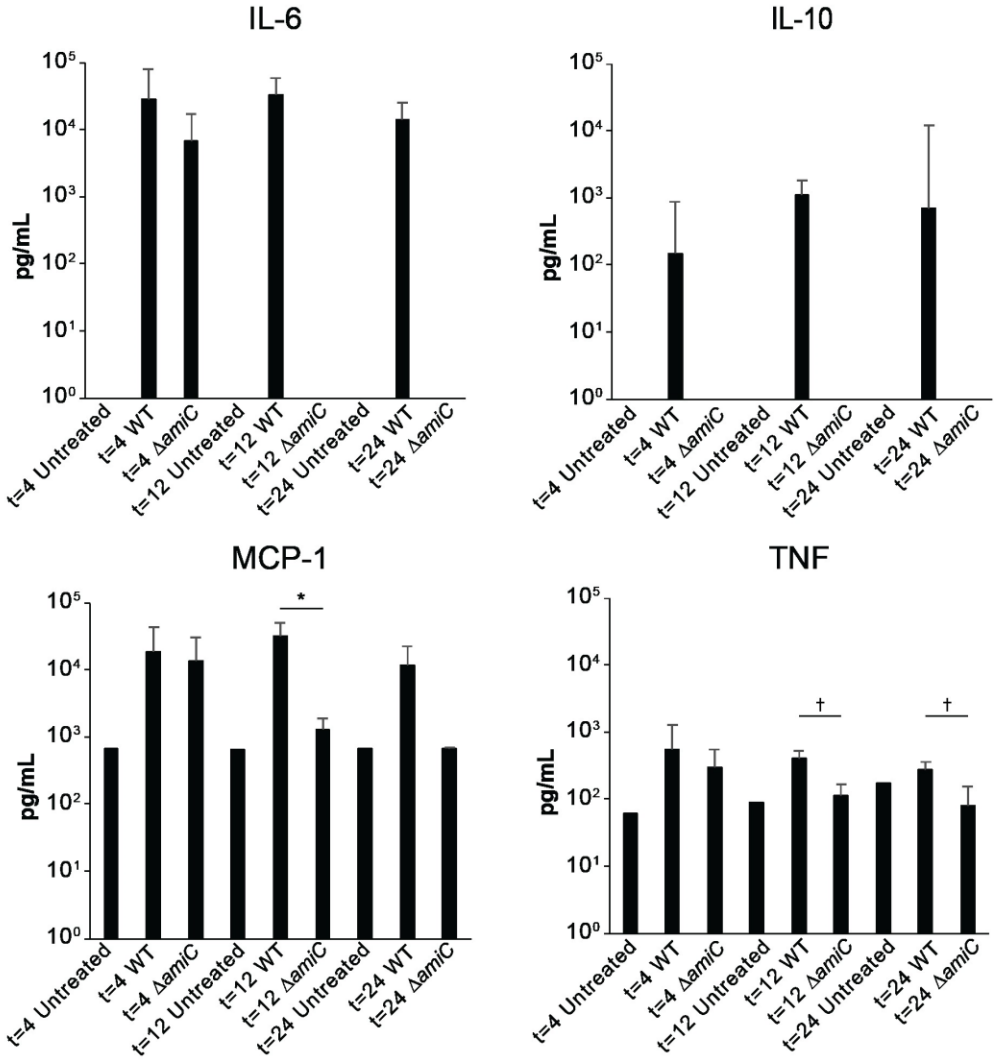

Figure 5. Decreased infection in the *amiC* mutant correlates with decreased cytokines. Mice were infected with either an *amiC* mutant (*ΔamiC*) or WT *Neisseria musculi*. Blood was collected at 4-, 12-, and 24-hours. Serum was extracted by centrifugation of blood. Cytokines were quantified using flow cytometry. IL-6 was not detected in untreated mice at any timepoint or in the mutant mice at 12- and 24-hours. IL-10 was only detected in serum from WT infected mice. There was a significant decrease in MCP-1 between mutant and WT infected mice at 12-hours. There was a significant decrease in TNF between the mutant and WT infected mice at 12- and 24-hours. *$p<0.05$, †$p<0.01$

Chapter 4: Peptidoglycan fragment release and NOD activation of nonpathogenic *Neisseria* of humans and animals

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Manuscript in preparation for submission

Author contributions: JPD conceived of study. JMC completed characterizations of PG fragment releases and hNOD1 and hNOD2 activation experiments. TNH completed mNOD1 activation experiments. JMC, TNH, and JPD wrote manuscript.

Abstract

Peptidoglycan (PG) is a structural macromolecule that is important for bacterial survival. Multicellular organisms, such as humans, have evolved ways to detect PG and respond to the presence of bacteria. Human pattern-recognition receptors NOD1 and NOD2 recognize PG intracellularly and induce inflammatory responses upon detection of specific types of PG. A limited number of species of Gram-negative bacteria release PG fragments during normal growth. *Neisseria gonorrhoeae* (Gc), a human restricted pathogen and the etiological agent of gonorrhea, releases inflammatory PG fragments that contribute to the pathophysiology of pelvic inflammatory disease (PID). PID is a complication of ascending gonococcal infection in women. In addition to the pathogenic species, the genus *Neisseria* is also home to multiple species of human- or animal-associated *Neisseria* that form part of the normal microbiota of their cognate host organisms. The amounts or types of PG fragments released by these species may influence the inflammatory responses to these symbionts and their abilities to asymptomatically colonize their hosts. In this chapter, we study the human-associated nonpathogenic species *N. lactamica* and *N. mucosa*, and animal-associated *Neisseria* isolated from macaques and wild mice through investigation into PG recycling and release in these species as well as in NOD1 and NOD2 activation. Our studies determined that even nonpathogenic species of *Neisseria* release large amounts of PG fragments and also activate NOD receptors.

Introduction

The genus *Neisseria* contains multiple species of Gram-negative bacteria with varying cell morphology and niches. *Neisseria* species are almost always found associated with higher order host organisms, which includes multiple species of insects, birds and waterfowl, land and marine mammals, as well as the rhinoceros iguana and the marsupial quokka (1). The most well-studied of the *Neisseria* species are *Neisseria gonorrhoeae* and *Neisseria meningitidis*, which are human-restricted pathogens and etiological agents for the sexually transmitted infection gonorrhea and invasive meningococcal disease, respectively. Most species in this genus are nonpathogenic colonizers of mucosal surfaces of their respective host organism. In fact, *N. meningitidis* has a nonpathogenic lifestyle as a colonizer of the naso- and oropharyngeal space of healthy adults (2). Like *N. meningitidis*, the major niche occupied by commensal mammal-associated *Neisseria* is the oral and nasopharyngeal space. *Neisseria* are considered part of the core oral microbiome of healthy humans and constitute up to 8% of the oral microbiota of humans (3–8).

The four species of *Neisseria* used in this study are all nonpathogenic colonizers of mammals. *Neisseria lactamica* and *N. mucosa* are associated with humans. The newly recognized species, *N. musculi*, was isolated from wild house mice. The macaque isolates AP312 and AP678 were isolated from macaques housed in a zoological garden (9, 10). In terms of cell morphology, *N. lactamica*, *N. mucosa*, and macaque isolates AP312 and AP678 are all diplococci, while *N. musculi* is a rod-shaped bacterium (1, 10). There are temporal and tissue specific colonization patterns in different species of nonpathogenic *Neisseria*. For example, *N. lactamica* carriage rates are higher in children compared to adults, and nasopharyngeal colonization by *N. lactamica* protects against colonization by *N. meningitidis* (11–13). In

contrast, *N. meningitidis* is commonly found in the throat of adults (11, 14). *N. mucosa* is mostly found in the gingival plaque and tooth surfaces of children and adults but does not appear to contribute to exacerbating or preventing tooth decay (14, 15). *N. musculi* was isolated from the mouths of wild mice but is able to colonize both the oral cavity and the gastrointestinal tract of CAST mice in the laboratory (10, 16). Although the macaque isolates colonize the nasopharyngeal and oral cavities of rhesus macaques, AP312 was initially isolated from a bite wound (9).

Despite their roles as part of the core oral microbiome, nonpathogenic human-associated *Neisseria* encode several virulence-associated factors and in rare cases will cause disease in people who are immunocompromised, have pre-existing risk factors, or post-trauma (1, 17, 18). Some diseases caused by commensal *Neisseria* spp. include high fatality diseases like meningitis, septicemia, and endocarditis, as well as conjunctivitis, respiratory tract infections and pneumonia (1, 17). Multiple cases of endocarditis by commensal *Neisseria* spp. have occurred after dental procedures most likely due to oral wounds sustained from these procedures that provided a direct route for the bacteria to travel from the mouth to the bloodstream (17). Factors that favor colonization by these nonpathogenic *Neisseria* species while not promoting inflammation and disease are not clear.

Investigations into the release of peptidoglycan fragments and their activation of the pattern recognition receptors NOD1 and NOD2 were conducted. Peptidoglycan (PG) is a macromolecular structure that confers cell shape and protection against osmotic shock. PG consists of a glycan backbone made of repeating subunits of *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc), with peptide stems extending off MurNAc that can be crosslinked to adjacent peptide stems forming a mesh-like structure. Because of PG remodeling

to allow for cell enlargement and cell separation, small PG fragments are liberated from the sacculus during growth. In Gram-negative bacteria, these PG fragments are usually taken back into the cytoplasm to be recycled for cellular metabolism. A limited number of Gram-negative bacteria, including the human pathogens *N. gonorrhoeae* and *N. meningitidis*, release sufficient amounts of pro-inflammatory PG fragments to stimulate production of pro-inflammatory cytokines in tissue explants (19–21). NOD1 and NOD2 are immune receptors found in host cells that are activated by small PG fragments(22, 23). In this study, we characterized the PG fragments released by nonpathogenic human- and animal-associated *Neisseria* and their ability to activate NOD1 and/or NOD2.

Materials and methods

Bacterial strains and growth conditions.

All strains used are listed in Table 1. All *Neisseria* strains are grown at 37°C either on gonococcal base medium (Difco) agar plates (GCB) with 5% CO_2 or in gonococcal base liquid medium with 0.042% $NaHCO_3$ and Kellogg's supplements (cGCBL) (24). When necessary, 80 μg/ml kanamycin was added to the growth medium. *Escherichia coli* strains were grown on LB agar or in LB broth (Difco) at 37°C. The growth medium was supplemented with 40 μg/ml kanamycin, 500 μg/ml erythromycin or 25 μg/ml chloramphenicol as needed.

Strain and plasmid construction.

All strains and plasmids used in this study are listed in Table 1, and primers are listed in Table 2. Spot transformation was used to generate *Neisseria* mutants (25). Briefly, around 0.5 - 1 μg plasmid DNA was digested with PciI with a subsequent heat-inactivation step to linearize the plasmid, and the digest reaction was spotted onto a pre-warmed GCB agar plate. Five to ten piliated colonies were then streaked over the DNA spots, and the plate was incubated at 37°C overnight. Colonies growing on the spots were restreaked onto fresh GCB plates for selection or screening. All transformants were screened by PCR and sequencing.

To construct pEC012 (AP312 *ampG*::cat in pIDN3), AP312 *ampG* was first amplified from macaque isolate AP312 chromosomal DNA with primers AP312 *ampG* SacI F and AP312 *ampG* BamHI R, and subsequently digested with SacI, BamHI, DpnI and EarI, resulting in two DNA fragments. The chloramphenicol resistance gene cat was excised from pKH6 by digestion with DpnI and EarI. The cloning vector pIDN3 was digested with SacI and BamHI. All four digest

products were ligated together to form pEC012. Macaque isolate AP312 was transformed with pEC012 to make EC2000.

The insert for pEC088 (P*ampG*$_{GC}$-*ampG*$_{NL49142}$ in pIDN3) was generated via overlap-extension-PCR (OE-PCR). First, *ampG*$_{NL49142}$ was amplified from *N. lactamica* ATCC 49142 chromosomal DNA using primers SOE-NL *ampG* F and SOE-NL *ampG* R. Approximately 1kb *ampG*$_{GC}$ upstream and downstream regions, which include the native *ampG*$_{GC}$ promoter and transcriptional terminator, were amplified from *N. gonorrhoeae* MS11 chromosomal DNA using primer pairs MC *ampG* SacI F3/NL SOE-MS11 *ampG* 5' flank R and NL SOE-MS11 *ampG* 3' flank F/*ampG* 3' flank R BamHI, respectively. The three PCR products were used as templates in OE-PCR with primes MC *ampG* SacI F3 and *ampG* 3' flank R BamHI. The final PCR product was digested with SacI and BamHI and ligated into similarly digested pIDN3 to form pEC088. pEC089 (P*ampG*GC-*ampG*$_{NL23970}$ in pIDN3) was constructed almost exactly as pEC088; the only exception is that *ampG*$_{NL23970}$ was amplified from *N. lactamica* ATCC 23970 chromosomal DNA. Transformation of *N. gonorrhoeae* MS11 with pEC088 or pEC089 yielded EC544 and EC540, respectively.

To generate pEC095 (*ampG*$_{Nmus}$ in pIDN3), *ampG*$_{Nmus}$ was amplified from *N. musculi* AP2031 chromosomal DNA using primers AP2031 *ampG* SacI F and AP2031 *ampG* BamHI R, digested with SacI and BamHI and ligated into similarly digested pIDN3. Plasmid pEC095 was digested with BtsBI and the 5' overhanging DNA ends were filled in with T4 Polymerase (NEB). A kanamycin resistance marker, kan, was excised from pHSS6 by digestion with NheI and BamHI, treated with T4 Polymerase and blunt-ligated with the BtsBI digested pEC095 to form pEC096 (*ampG*$_{Nmus}$::kan in pIDN3). Plasmid pEC096 was transformed into *N. musculi* AP2031 to generate EC2005.

Plasmid pEC088 was used as template in two PCR reactions with primer pairs MC *ampG* SacI F3/NL49142 *ampG* T272A F and NL49142 *ampG* T272A R/*ampG* 3' flank R BamHI. The primer sequences of NL49142 *ampG* T272A F and NL49142 *ampG* T272A R contain a missense mutation (ACA->GCA) that would result in substitution of residue 272 from a threonine to an alanine in the final gene product. The two PCR products were used in OE-PCR with primers MC *ampG* SacI F3 and *ampG* 3' flank R BamHI, and the final OE-PCR product was digested with SacI and BamHI and ligated into similarly digested pIDN3 to form pEC113 (P*ampG*GC-*ampG*$_{NL49142}$T272A in pIDN3). pEC116 (P*ampG*GC-*ampG*$_{NL23970}$A272T in pIDN3) was built with a similar strategy as pEC113, with two variations during the initial PCR reaction. Chromosomal DNA from EC540 was used as template in the PCR reaction, with primer pairs MC *ampG* SacI F3/NL23970 *ampG* A272T F and NL23970 *ampG* A272T R/*ampG* 3' flank R BamHI instead. *N. gonorrhoeae* MS11 was transformed with pEC113 or pEC116 to form EC561 and EC564, respectively.

Metabolic labeling of peptidoglycan with [^{3}H] glucosamine or [^{3}H] DAP and quantitative fragment release.

Metabolic labeling of PG with [^{3}H] glucosamine or [^{3}H] DAP and quantitative fragment release was performed as described previously for *N. gonorrhoeae* (26–28). For [^{3}H] glucosamine labeling, strains were grown in cGCBL to mid-log phase, diluted to OD540 of 0.2 and pulse labeled with 10 μCi/ml [^{3}H] glucosamine in GCBL supplemented with 0.042% $NaHCO_3$ and modified Kellogg's supplements containing pyruvate instead of glucose for 30 minutes. The cells were washed to remove unincorporated label and resuspended in GCBL containing supplements and glucose for the chase period. The cells were grown for 2.5 hours,

after which cell-free supernatant was harvested by centrifugation at 3,000 x g for 10 minutes and filtration using a 0.22 μm filter. For quantitative fragment release, the amount of radiation (counts per minute, CPM) in the cell pellets were determined by liquid scintillation counting prior to the chase period and normalized to each other. Labeling with [^{3}H] DAP was performed in a similar way as labeling with [^{3}H] glucosamine, with variations in the pulse labeling phase. Strains were grown in DMEM lacking cysteine supplemented with 25 μCi/ml [^{3}H] DAP, 100 μg/ml threonine and 100 μg/ml methionine for 60 minutes for the labeling phase. Released radiolabeled PG fragments are separated by tandem size-exclusion chromatography and detected by liquid scintillation counting.

NOD1 and NOD2 activation with HEK293-reporter cells.

NOD1 and NOD2 activation were measured using HEK293-reporter cells. Briefly, bacterial strains were grown to mid-log phase in cGCBL from an initial OD_{540} of 0.2. Cells were pelleted and supernatants passed through a 0.2 μM filter. Supernatants were normalized by dilution with GCBL, based on total protein content of cultures, and added to HEK293-reporter cells overexpressing NOD1 or NOD2 as described in (29). NF-kB activation was measured at OD_{650} after incubation of cell supernatants in QUANTI-blue medium as described in manufacturer's instructions (InvivoGen). Tridap, PGN, and MDP are positive controls

Results

Nonpathogenic Neisseria release PG fragments.

PG fragments released by growing *N. gonorrhoeae* and *N. meningitidis* have been previously characterized (20, 22, 23) through the method of metabolic pulse-chase labeling of the PG with [^{3}H] glucosamine and [^{3}H] diaminopimelic acid (DAP), which labels the glycan backbone and peptide stems of PG, respectively. [^{3}H] glucosamine labeled PG fragments released by WT cells are PG dimers, tetrasaccharide-peptide, PG monomers, and free disaccharide. A fifth peak, consisting of anhydro-*N*-acetylmuramic acid (anhMurNAc), is sometimes present, dependent on growth conditions. [^{3}H] DAP labeled PG fragments are PG dimers, tetrasaccharide-peptide, PG monomers, free tetrapeptide and free tripeptide (in one peak), and free dipeptide (in a second free peptide peak). Any DAP that was unincorporated will appear as a shoulder on the fifth peak. The known structures of the PG fragments are summarized in Figure 1H. The major inflammatory molecules released are the PG dimers, PG monomers, and free tetra- and tri-peptide(32).

We performed metabolic pulse-chase labeling of the PG of *N. mucosa*, *N. musculi,* and macaque isolates AP312 and AP678 with [^{3}H] glucosamine. Consistent with previous observations, *N. mucosa* released PG monomers and disaccharide, with no discernable PG dimer peak (33) (Figure 1G). Like *N. mucosa*, macaque isolate AP312 released PG monomers and disaccharide but little if any PG dimers (Figure 1A). In contrast, *N. musculi* and macaque isolate AP678 released PG monomers, disaccharide, and PG dimers (Figure 1B-C). Attempts to metabolically label PG of the two *N. lactamica* isolates, *N. lactamica* ATCC 23970 and *N. lactamica* ATCC 49142, with [^{3}H] glucosamine were not successful.

To characterize the PG fragments released by *N. lactamica* ATCC 23970 and *N. lactamica* ATCC 49142, the two isolates were labeled with [^{3}H] DAP. Interestingly, while both isolates released PG dimers, PG monomers, and PG peptides, the relative amounts of PG fragments released by the two isolates differ (Figure 1D-F). *N. lactamica* ATCC 49142 released similar amounts of PG monomer and PG-derived peptides (Figure 1E), while *N. lactamica* ATCC 23970 released lower levels of PG monomer compared to PG-derived peptides (Figure 1F). Metabolic labeling of *N. musculi* PG with [^{3}H] DAP revealed that in contrast to both *N. lactamica* isolates, *N. musculi* released much lower levels of PG-derived peptides compared to PG monomers (Figure 1D). Similar analyses with *N. mucosa* and quantitative fragment release comparisons with *N. gonorrhoeae* showed that *N. mucosa* released more PG-derived peptides but lower levels of PG monomer compared to the pathogenic *N. gonorrhoeae* (Figure 2).

Analysis of PG recycling by animal-associated N. musculi and macaque isolate AP312 using mutation of ampG.

AmpG is an inner membrane permease that transports PG monomers and anhydro-disaccharide from the periplasm to the cytoplasm for recycling in Gram-negative bacteria (34–36). Both *N. gonorrhoeae* and *N. meningitidis* recycle PG fragments in an AmpG-dependent manner, and deletion of *ampG* in either species results in a large increase in PG monomer and anhydro-disaccharide release (20, 28). By comparing the amount of PG released by *ampG* mutants compared to the amount released by WT strains, the normal amounts of glycan-containing PG fragments recycled and released can be calculated. We previously demonstrated that the human-associated species *N. mucosa* encodes a functional AmpG permease and recycles 95% of PG monomers liberated during growth (33).

We mutated *ampG* in *N. musculi* and macaque isolate AP312 by insertional inactivation with an antibiotic resistance marker and determined the PG fragment release profile of the *ampG* mutants. Mutation of *ampG* in both *N. musculi* and macaque isolate AP312 resulted in an 8.7-fold and 10.8-fold increase in PG monomer release, respectively, with modest changes to anhydro-disaccharide release (Figure 3A-B). *N. musculi* releases around 12% of PG monomer generated during growth, while macaque isolate AP312 releases approximately 10% of PG monomers. Like other *Neisseria* species, mutation of *ampG* did not alter PG dimer release in *N. musculi* or in macaque isolate AP312. This observation is consistent with the reported inability of *E. coli* AmpG to transport PG dimers (36).

The difference in PG monomer release by N. lactamica ATCC 23970 and N. lactamica ATCC 49142 is partially due to a single nucleotide polymorphism in AmpG.

We were unable to genetically manipulate both *N. lactamica* isolates to study PG recycling in *N. lactamica*. As an alternative strategy, we expressed *N. lactamica* ATCC 23970 *ampG* (*ampG*$_{NL23970}$) or *N. lactamica* ATCC 49142 *ampG* (*ampG*$_{NL49142}$) in *N. gonorrhoeae* in lieu of the native gonococcal *ampG* (*ampG*$_{GC}$). Expression of *N. lactamica ampG* in this background is driven by the native *ampG*$_{GC}$ promoter. Determination of the amount of PG monomers released by the allelic replacement mutants relative to the amount released by WT *N. gonorrhoeae* will allow us to determine if *N. lactamica* AmpG is more, less, or equally efficient as AmpG$_{GC}$. Expression of *ampG*$_{NL23970}$, but not of *ampG*$_{NL49142}$ in *N. gonorrhoeae* resulted in lower levels of PG monomer release compared to WT *N. gonorrhoeae* (Figure 4). The gonococcal strain expressing *ampG*$_{NL49142}$ released similar levels of PG monomer as WT *N. gonorrhoeae* (Figure 3). These results suggest that AmpG$_{NL49142}$ transports similar amounts of

PG monomer as $AmpG_{GC}$, while $AmpG_{NL23970}$ is the most efficient at recycling of the three. *N. gonorrhoeae* has a known deficiency in PG recycling due to reduced AmpG function compared to that of other characterized *Neisseria* species(37).

$AmpG_{NL49142}$ and $AmpG_{NL23970}$ differ only by five residues. Previous work established that natural polymorphisms of gonococcal and meningococcal AmpG at residues 391, 398 and 402 contribute to differences in PG monomer release in these two pathogenic *Neisseria* (33). Both isolates of *N. lactamica* encode for the same amino acids at AmpG positions 391, 398 and 402. We compared AmpG sequences from the two *N. lactamica* isolates, *N. gonorrhoeae,* and *N. meningitidis*, and one of the five aforementioned residues is only found in $AmpG_{NL49142}$. AmpG residue 272 is a threonine in *N. lactamica* ATCC 49142 and is an alanine in the other three species. We performed site-directed mutagenesis to determine if AmpG residue 272 plays a role in modulating AmpG efficiency in *N. lactamica*.

Expression of $ampG_{NL49142}$T272A in *N. gonorrhoeae* lacking $ampG_{GC}$ resulted in lower levels of PG monomer release compared to WT *N. gonorrhoeae* (Figure 5A), mimicking the phenotype seen when $ampG_{NL23970}$ is expressed by *N. gonorrhoeae*. Additionally, expression of $ampG_{NL23970}$A272T in *N. gonorrhoeae* resulted in WT-like levels of PG monomer release, which phenocopied the gonococcal strain expressing $ampG_{NL49142}$. We conclude that *N. lactamica* ATCC 23970 is better at recycling PG monomers compared to *N. lactamica* ATCC 49142 due to a polymorphism at AmpG site 272, which contributes in part to differences in the amount of PG fragments released by the two *N. lactamica* isolates.

PG fragments released by nonpathogenic human-associated Neisseria *stimulate hNOD1 and hNOD2 activation in HEK293-reporter cells.*

Recognition of bacterial products by the immune system is important to prevent or resolve infections, as well as to attune the immune response (38). In humans, PG fragments are recognized by two intracellular receptors, human NOD1 (hNOD1) and NOD2 (hNOD2). hNOD1 responds to small PG fragments with a terminal DAP moiety (39–41). hNOD2 binds to muramyl-dipeptide (MDP) and also responds to reducing-end PG monomers, which are generated from PG dimers or longer strands by the action of host lysozyme (42–45). PG monomers released by *Neisseria* have anhydro-ends instead of reducing-ends due to the enzymatic activity of lytic transglycosylases that cleave the glycan backbone (46, 47). The NOD receptors signal through two major pathways to induce an immune response, and one of pathways results in activation of NF-kB(48). We sought to determine if PG fragments released by nonpathogenic *Neisseria* are capable of stimulating an hNOD and NF-kB dependent response.

To answer this question, we utilized HEK293 reporter cells that express a secreted alkaline phosphatase (SEAP) under the control of an NF-kB promoter and measured the amount of SEAP in the supernatant using a colorimetric assay. We treated the reporter cell lines with cell-free supernatant from *N. mucosa* and both *N. lactamica* isolates, as well as supernatant from *N. gonorrhoeae* for comparison. Our initial hypothesis was that nonpathogenic *Neisseria* would induce lower levels of NOD activation. However, most of our nonpathogenic strains induced similar levels of hNOD1 and hNOD2 activation as *N. gonorrhoeae* (Figure 6A-B). *N. lactamica* ATCC 23970 also induced higher levels of hNOD2 activation than *N. gonorrhoeae* in this assay (Figure 6B). The value for *N. mucosa* stimulation of hNOD1 was lower than that for *N. gonorrhoeae*, though the values were not statistically different. A lower level of stimulation would be consistent with the decreased PG monomer release seen in *N. mucosa*. In addition to PG dimers and large PG fragments stimulating NOD2, disaccharide PG monomers stimulate

NOD2, just as muramyl dipeptide stimulates NOD2(49). The dipeptide monomer peak is seen on the PG release profile as a shoulder on the right side of the PG monomer peak. A substantial disaccharide monomer peak is seen in the *N. mucosa* profile, and a noticible shoulder is detected on the monomer peak for *N. lactamica* ATCC 23970.

Since mutation of *ampG* increases the amount of PG monomers released, and tripeptide monomers are known agonists of hNOD1, we hypothesized that supernatant from *ampG* mutants of *N. mucosa* would induce higher levels of hNOD1 activation compared to WT. As expected, supernatant from the *ampG* mutants activated hNOD1, but not hNOD2, to a greater degree compared to their respective WT (Figure 6C-D). Thus, we conclude that *N. mucosa* and *N. lactamica* release hNOD-activating PG fragments, and that PG recycling modulates the amount of hNOD1 agonists released by the bacteria.

PG fragments released by N. musculi stimulate mNOD1 activation in HEK293-reporter cells.

Similar to hNOD1 expressed in humans, mice also express a NOD1 receptor. While hNOD1 is activated by tripeptide monomer and tripeptides, mNOD1 is activated by tetrapeptide monomer and tetrapeptides(50). mNOD1 activation in HEK293-reporter cells was analyzed for *ampG* and *ldcA* mutant strains. mNOD1 activation by *N. musculi* supernatants were surprisingly low (Figure 7). Mutation of *ampG* in *N. musculi* resulted in slightly higher mNOD1 activation. We mutated *ldcA* in *N. musculi* since LdcA is the enzyme which converts tetrapeptides into tripeptides in the periplasm(29). Since free tetrapeptides and tetrapeptide monomers are the mNOD1 agonists, and tripeptides versions of those molecules are poor stimulatory molecules for mNOD1, the mutation would make all the released PG monomers and free peptides into mNOD1 agonists. Supernatant from the *ldcA* mutant stimulated mNOD1 to a much higher degree than

WT or *ampG* supernatants. Complementation with *ldcA* elsewhere on the chromosome partially reduced this effect. Overexpression of *ldcA* in *N. musculi* resulted in a background level of mNOD1 stimulation.

Discussion

Due to the role of PG monomers in causing damage to mucosal epithelium by inducing death and sloughing of ciliated cells during *N. gonorrhoeae* and *Bordetella pertussis* infections, we hypothesized that nonpathogenic *Neisseria* would release very little to no inflammatory PG fragments. The first characterization of PG fragments released by *N. mucosa*, performed as quantitative fragment release in comparison to *N. gonorrhoeae*, showed that this species releases lower levels of proinflammatory PG monomers compared to *N. gonorrhoeae* and no PG dimers (33). It was logical to infer that the reduced PG monomer release by *N. mucosa* means that nonpathogenic *Neisseria* are less inflammatory compared to *N. gonorrhoeae*. This inference is supported by the observation that human-associated *Neisseria* induce lower Toll-like receptor (TLR4) response compared to *N. gonorrhoeae* and *N. meningitidis* (51). TLR4 is an innate immune receptor that responds to lipopolysaccharide and lipooligosaccharide (LOS) (52). LOS from *N. cinerea, N. flavescens, N. perflava* and *N. subflava* lack lipid A modification that is seen with LOS from *N. gonorrhoeae* and *N. meningitidis* (51).

As we continued to investigate PG fragment release by more strains of human- and animal-associated *Neisseria*, it became clear that the situation is not as simple as we initially hypothesized. There are both interspecies and intraspecies variations in PG fragment release patterns. *N. mucosa* and macaque isolate AP312 did not release PG dimers, while *N. lactamica, N. musculi* and macaque isolate AP678 released PG dimers (Figure 1). The two strains of *N. lactamica* evaluated showed differences in the relative amounts of PG monomer and PG peptides released (Figure 1). Labeling of *N. mucosa* with [^{3}H] DAP revealed that although *N. mucosa* released less PG monomer compared to *N. gonorrhoeae, N. mucosa* released more PG-derived peptides that might also activate hNOD1 (Figure 2). *N. meningitidis*, which despite its ability to

cause invasive disease is more commonly found as a colonizer of the nasopharyngeal space, also releases less PG monomer and more PG peptides compared to *N. gonorrhoeae*(53). While *N. musculi* did not release (NOD2-stimulatory) PG dimers, it did release large amounts of PG monomers with a dipeptide chain. Dipeptide monomers directly stimulate NOD2.

The two strains of *N. lactamica* used in the study displayed differences in the relative amounts of PG monomer and PG peptides released in part due to differences in the efficiency of PG recycling. Such intraspecies variation in PG monomer release has been previously observed with two different isolates of *N. meningitidis* that have polymorphisms at AmpG residues 398 and 402 (33). We identified another AmpG residue (residue 272) capable of modulating PG recycling efficiency and indirectly controlling the amount of PG monomer released. AmpG residue 272 is a threonine in *N. lactamica* ATCC 49142 and an alanine in *N. lactamica* ATCC 23970, and WT *N. gonorrhoeae* and WT *N. meningitidis*. This finding suggests that different species of *Neisseria* independently evolved strategies to fine-tune the amount of PG fragments released. According to ATCC, *N. lactamica* ATCC 49142 is a clinical isolate, while *N. lactamica* ATCC 23970 is nasopharyngeal isolate. *N. lactamica* can infrequently cause invasive disease in immunocompromised individuals (17), and it was not reported if the clinical isolate *N. lactamica* ATCC 49142 was isolated from a sick or a healthy individual. It is possible that alterations to PG fragment release allowed the two strains to colonize different body sites more effectively.

Through hNOD1and hNOD2 activation we were able to determine that despite the nonpathogenic nature of human-associated *Neisseria,* NOD activation was not lower in these species compared with *N. gonorrhoeae.* In fact, hNOD2 activation was higher in *N. lactamica* ATCC 23970 than in the pathogenic *N. gonorrhoeae.* Therefore, PG fragment release in significant amounts does not make commensal *Neisseria* pathogens during nasopharyngeal

colonization. Further research into the differences between NOD responses in the different niches infected by *Neisseria* needs to be done.

The recent establishment of macaque and mice-associated *Neisseria* as model organisms provide avenues for studying aspects of *Neisseria* colonization and infection in their cognate host organisms (9, 10, 16). One caveat of using mice as a model organism for understanding PG fragment release by humans is that the murine NOD1 detects tetrapeptide monomers instead of tripeptide monomers like the human NOD1 (54). Still, our findings indicate that both macaque isolates and *N. musculi* release PG monomers, and that macaque isolate AP312 and *N. musculi* recycle PG fragments liberated during turnover (Figure 1, 3 Our ability to mutate genes in macaque isolate AP312 and *N. musculi* provide us with tools to vary the amounts and types of PG fragments that the host organism is exposed to, and to study the corresponding host response. We have already constructed *ampG* mutants that release approximately 20 times more PG monomers, and we also have other peptidoglycanase mutants in *N. musculi* that have been used in infection and colonization studies.

There are exciting new avenues for further research on the consequences of PG fragment release during infections. The ability to manipulate genes and alter PG fragment release in a commensal can give us the ability to discern factors that distinguish a pathogen from a nonpathogen. Our study has provided insight into PG fragment release and immune responses to PG fragments in nonpathogenic *Neisseria*, revealing that despite their mainly nonpathogenic lifestyle they release PG fragments and activate NOD1 and NOD2 similarly to pathogenic *Neisseria.* Demonstrating that the factors that distinguish pathogenic and nonpathogenic *Neisseria* are more complex and perhaps less defined, than originally thought.

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Table 1. Strains and plasmids used in this study.

Strain/ Plasmid	Description	Source/ Reference
Strains		
N. mucosa ATCC 25996	*N. mucosa* pharyngeal isolate	ATCC
N. lactamica ATCC 49142	*N. lactamica* clinical isolate	ATCC
N. lactamica ATCC 23970	*N. lactamica* nasopharyngeal isolate	ATCC
N. musculi AP2031	*N. musculi* oral isolate	(10)
Macaque isolate AP312	*N. macacae* -like bite wound isolate	(9)
Macaque isolate AP678	*N. macacae* -like nasopharyngeal isolate	(9)
N. gonorrhoeae MS11	*N. gonorrhoeae* clinical isolate	(55)
DG132	Δ*ampG*$_{GC}$	(28)
EC2000	*ampG*$_{AP312}$::cat	This study
EC2003	*ampG*$_{Nmu}$::kan	(33)
EC2005	*ampG*$_{Nmus}$::kan	This study
EC540	*ampG*$_{GC}$- *ampG*$_{NL23970}$+	This study
EC544	*ampG*$_{GC}$- *ampG*$_{NL49142}$+	This study
EC561	*ampG*$_{GC}$- *ampG*$_{NL49142}$T272A+	This study
EC564	*ampG*$_{GC}$- *ampG*$_{NL23970}$ A272T+	This study

Plasmids		
pIDN3	Cloning vector (ErmR)	(56)
pKH6	Cloning vector (CmR); source of cat	(57)
pHSS6	Cloning vector (KanR); source of kan	(58)
pEC012	*ampG*$_{AP312}$::cat in pIDN3	This study
pEC088	P*ampG*$_{GC}$-*ampG*$_{NL49142}$ in pIDN3	This study
pEC089	P*ampG*$_{GC}$-*ampG*$_{NL23970}$ in pIDN3	This study
pEC095	*ampG*$_{Nmus}$ in pIDN3	This study
pEC096	*ampG*$_{Nmus}$::kan in pIDN3	This study
pEC113	P*ampG*$_{GC}$-*ampG*$_{NL49142}$T272A in pIDN3	This study
pEC116	P*ampG*$_{GC}$ -*ampG*$_{NL23970}$A272T in pIDN3	This study
pEC125	*ldcA*$_{Nmus}$::kan in pIDN3	This study
pKHmus502	Gibson cloning *ldcA*$_{Nmus}$ complementation plasmid	This study

Table 2. Primers used in this study.

Primer name	Sequence
MC *ampG* SacI F3	ATTCAGAGCTCCATCGGCGGCATCATCAAAC
ampG 3’ flank R BamHI	CTCAGGATCCGTTCTTTATATGAGCGGCAGG
AP312 *ampG* SacI F	GTATTGAGCTCGTCTTGTTCGGATTTCGTCTG
AP312 *ampG* BamHI R	GACTAGGATCCGTCCATTACTTCGGCGTATATGC
SOE-NL *ampG* F	GCTGTACGAACGATGACTGCATCGAAATCAGG

SOE-NL *ampG* R	GTTTGACGCGTTCATCTGCCTGCATCCTGAG
NL SOE-MS11 *ampG* 5' flank R	CCTGATTTCGATGCAGTCATCGTTCGTACAGC
NL SOE- MS11 *ampG* 3' flank F	GCAGGCAGATGAACGCGTCAAACTGGAGCG
NL49142 AmpG T272A F	GATTGCGAAAAATGCAGGACTGTGGC
NL49142 AmpG T272A R	GCCACAGTCCTGCATTTTTCGCAATC
NL23970 *ampG* A272T F	GATTGCGAAAAATACAGGACTGTGGC
NL23970 *ampG* A272T R	GCCACAGTCCTGTATTTTTCGCAATC
AP2031 *ampG* SacI F	CGGCCGAGCTCCTTCGTTGATACAATAACC
AP2031 *ampG* BamHI R	CATTAGGATCCCGCAAACGGGTTTGCTGTGG

Figures

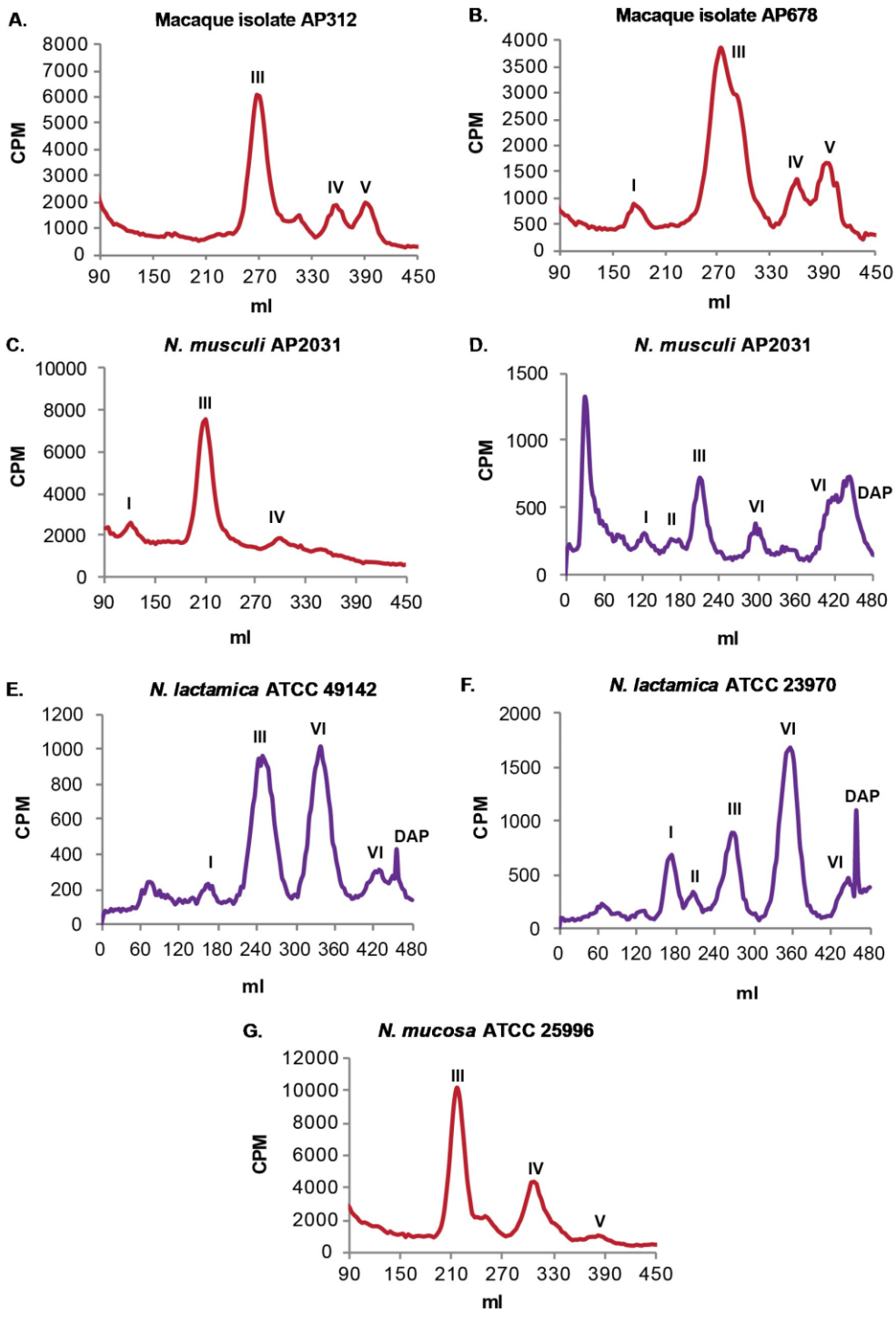
A.
Macaque isolate AP312
CPM
ml
III
IV
V
B.
Macaque isolate AP678
I
III
IV
V
C.
N. musculi AP2031
I
III
IV
D.
N. musculi AP2031
I
II
III
VI
DAP
E.
N. lactamica ATCC 49142
I
III
VI
DAP
F.
N. lactamica ATCC 23970
I
II
III
VI
DAP
G.
N. mucosa ATCC 25996
III
IV
V
H. Identity of PG fragments released
I - PG dimers
II - Tetrasaccharide-peptide
III - PG monomers
VI - PG-derived peptides
IV - anh-disaccharide
V - anhMurNAc
Key
anhMurNAc
MurNAc
GlcNAc
Alanine
Glutamate
meso-DAP

Figure 1. Nonpathogenic human-associated and animal-associated *Neisseria* release PG fragments. Metabolic labeling of peptidoglycan with [^{3}H] glucosamine (A-C,G; red) and [^{3}H] DAP (D-F; purple) was performed with macaque isolates AP312 (A) and AP678 (B), *N. musculi* AP2031 (C,D), *N. lactamica* ATCC 49142 (E), ATCC 23970 (F), and *N. mucosa* ATCC 25996 (G). (H) Cartoon depictions of the PG fragments found in each peak, using symbols from Jacobs et al. (35) All strains tested released PG monomers (III). *N. lactamica*, *N. musculi* and macaque symbiont AP678 released PG dimers (I). However, *N. mucosa* and macaque isolate AP312 released little to no PG dimers.

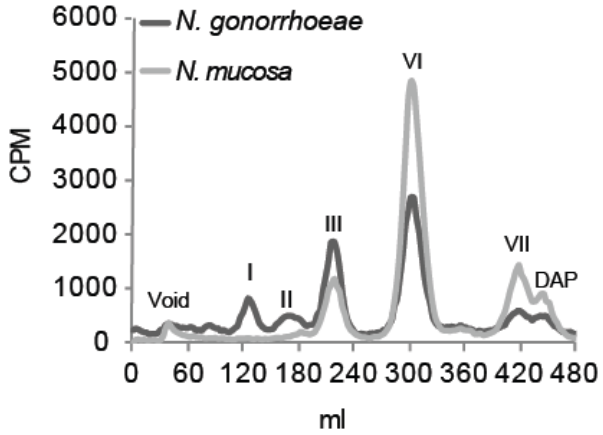

Figure 2. *Neisseria* mucosa released lower levels of PG monomer but higher levels of PG peptide compared to the pathogenic *Neisseria* gonorrhoeae. *N. mucosa* and *N. gonorrhoeae* were metabolically labeled with [3H] DAP and quantitative fragment release analysis was performed. The nonpathogenic *N. mucosa* released less pro-inflammatory PG monomer (III), but more PG-derived peptides (VI, VII) compared to *N. gonorrhoeae*.

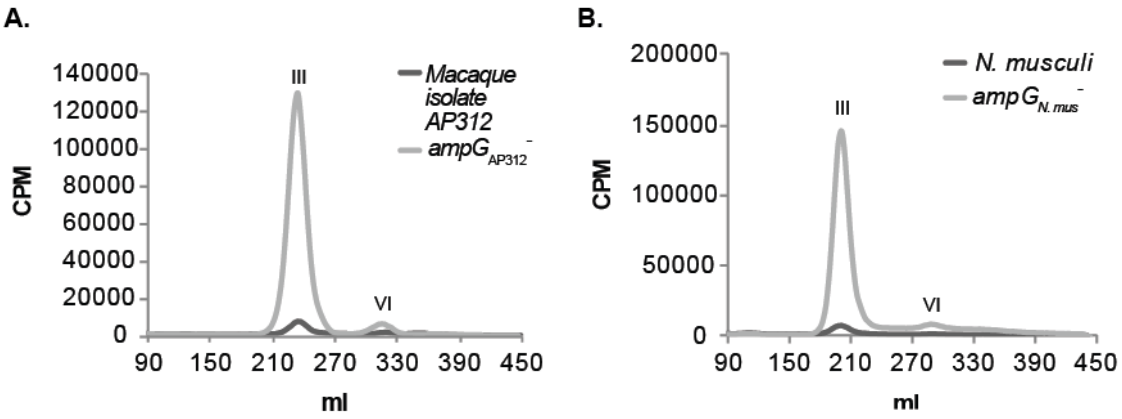

Figure 3. Mutation of *ampG* impaired PG fragment recycling in *N. musculi* and macaque symbiont AP312. Mutation of *ampG* in *N. musculi* (A) and the macaque isolate AP312 (B) resulted in a large increase in the amount of PG monomer (III) and disaccharide (IV) release, without affecting PG dimer release.

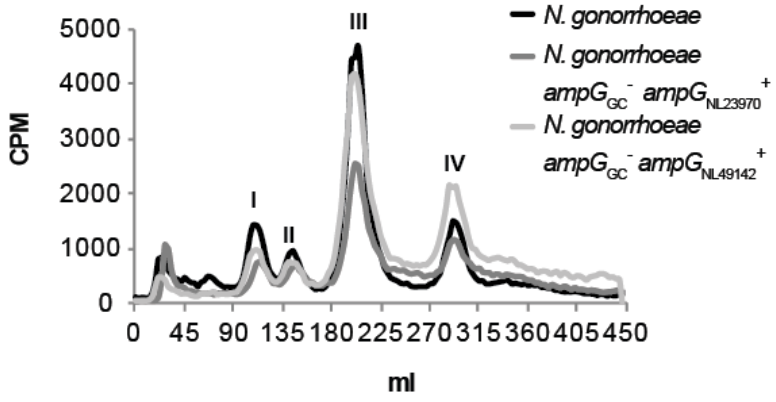

Figure 4. Gonococcal *ampG*- strains expressing *ampG* from two different strains of *N. lactamica* released different amounts of PG monomer. The *ampG* gene from *N. lactamica* ATCC 49142 or *N. lactamica* ATCC 23970 were expressed in *N. gonorrhoeae* in lieu of the native gonococcal *ampG* gene. Expression of $ampG_{NL23970}$ but not $ampG_{NL49142}$ in *N. gonorrhoeae* reduced the amount of PG monomer (III) released by the mutant strain.

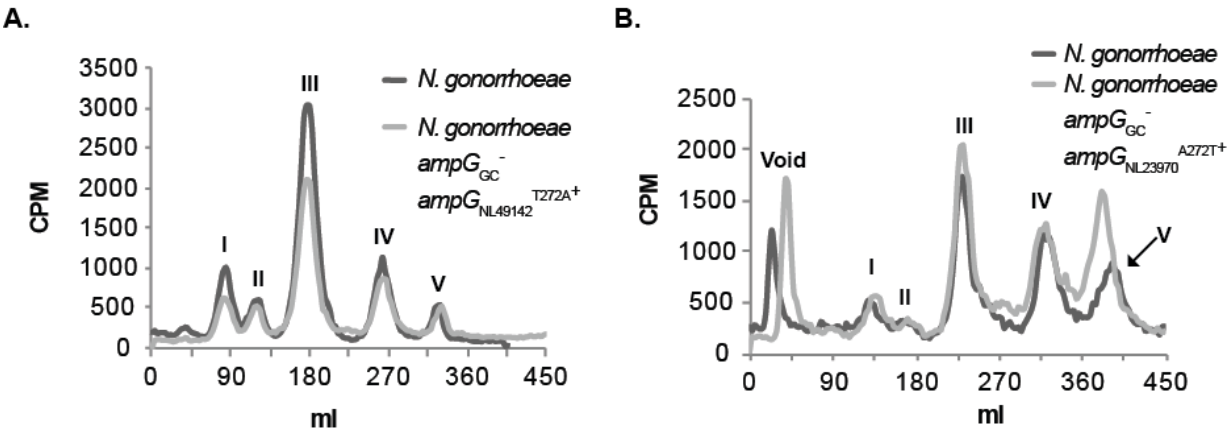

Figure 5. Polymorphism of *N. lactamica* AmpG contributes to the differences in the amounts of PG monomer released. A) *N. gonorrhoeae* expressing *ampG*$_{NL49142}$ with a single substitution at AmpG$_{NL49142}$ residue 272 from a threonine to an alanine show reduced PG monomer release by around half. B) *N. gonorrhoeae* expressing *ampG*$_{NL23970}$ with a single substitution at AmpG$_{NL23970}$ residue 272 from an alanine to a threonine released *N. gonorrhoeae* WT-like levels of PG monomer.

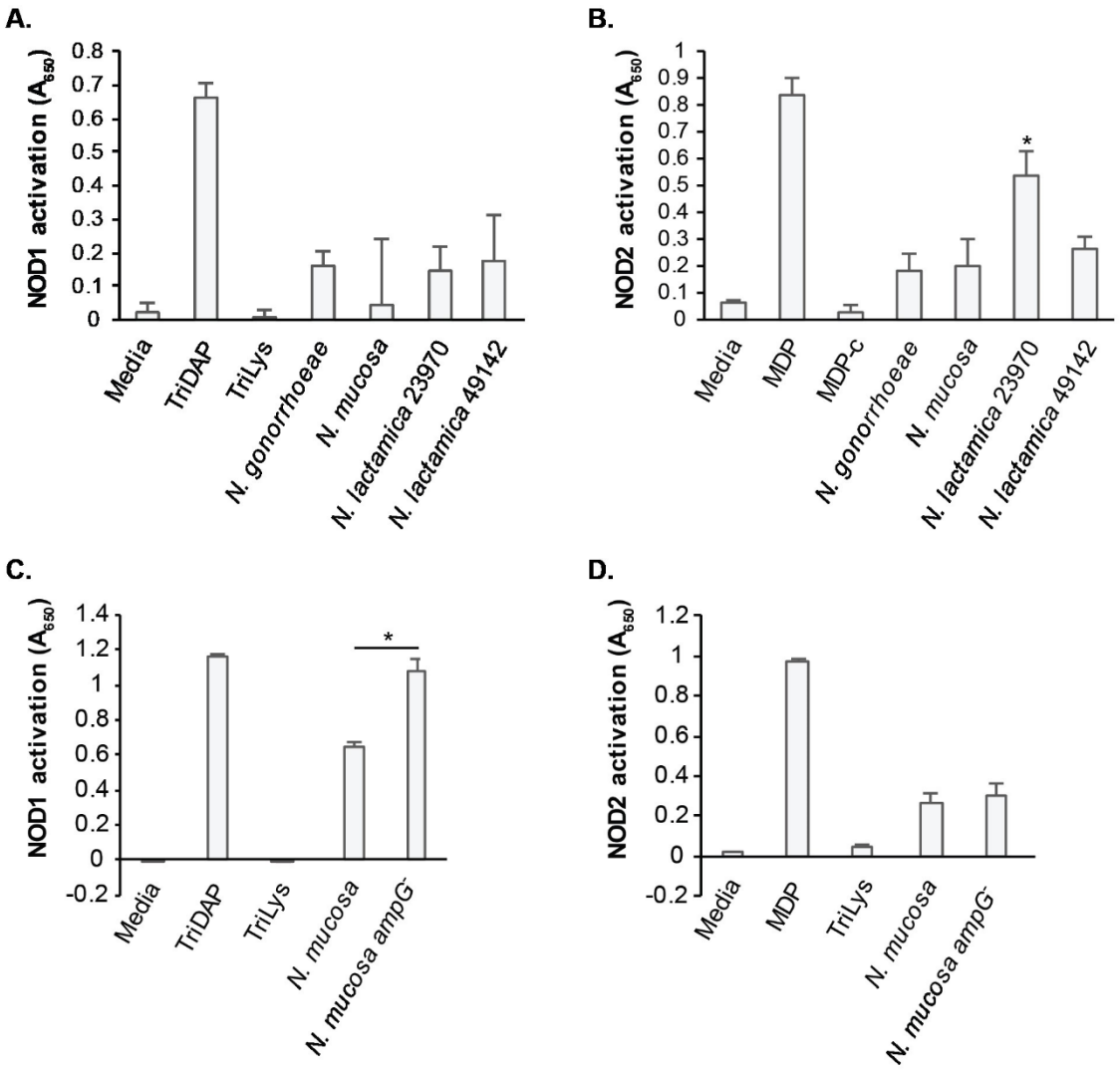

Figure 6. hNOD1 and hNOD2 activation by supernatant from different *Neisseria* species. Supernatant from *N. mucosa*, *N. lactamica* (A,B) and *ampG* mutants of and *N. mucosa* (C,D) induced hNOD1 (A,C) and hNOD2 (B,D) activation in HEK293 reporter cells overexpressing NOD1 or NOD2. Supernatant from *ampG* mutants of *N. mucosa* induced larger hNOD1 responses compared to wild-type. Supernatant from *N. lactamica* ATCC23970 induced the largest hNOD2 response of all treatment samples. Statistical significance was determined using Student's t-test. * indicates $p<0.05$.

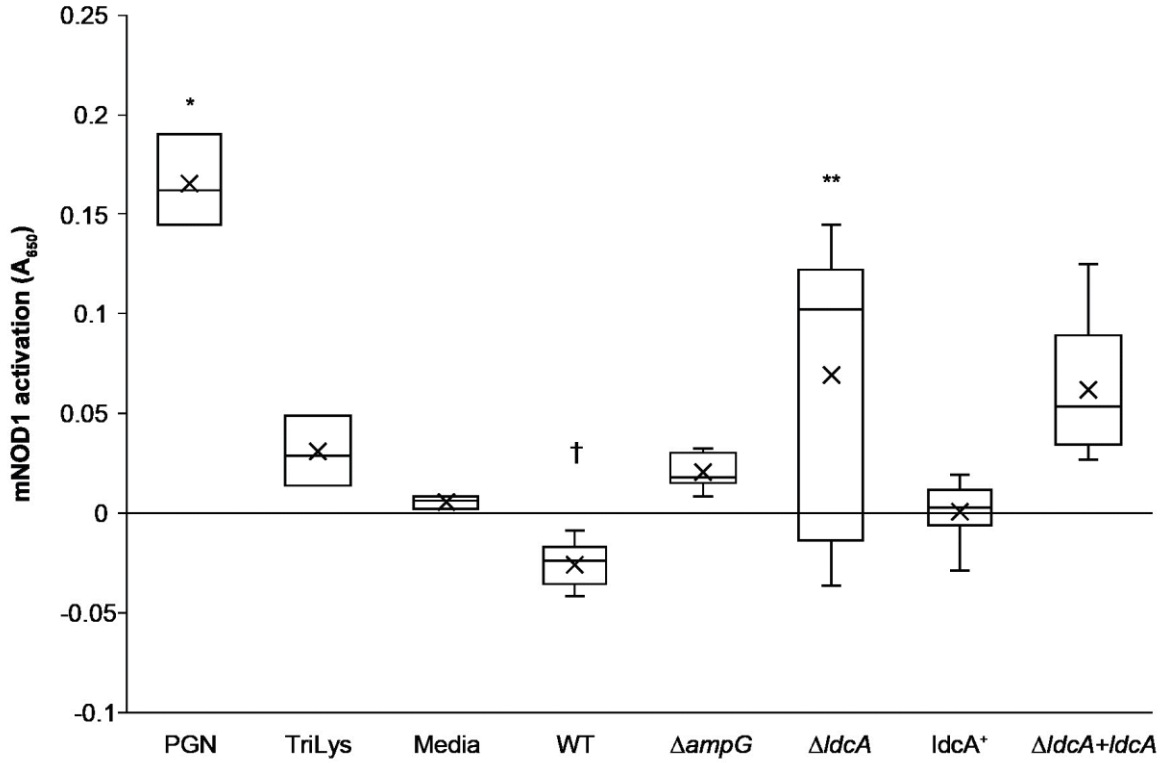

Figure 7. mNOD1 activation by supernatant from *Neisseria musculi*. Supernatant from *Neisseria musculi* induced low levels of mNOD1 activation in HEK293 reporter cells overexpressing NOD1. PGN was significantly increased from all samples. WT was significantly decreased from mutants of *ampG* (Δ*ampG*) and *ldcA* (Δ*ldcA*) as well as the *ldcA* overexpression mutant (*ldcA*$^{+}$) and ldcA complement (Δ*ldcA*+*ldcA*). The *ldcA* mutant (Δ*ldcA*) was significantly increased from the *ldcA* overexpression mutant (*ldcA*$^{+}$). *p<0.01, †p<0.01, **p<0.05

Chapter 5: Discussion

Tiffany N. Harris-Jones and Joseph P. Dillard

TN Harris-Jones and JP Dillard wrote the manuscript

For decades we have battled antibiotic resistance in *Neisseria gonorrhoeae* (Gc). The idea of a future of untreatable gonorrhea is no longer simply an idea but a fact. We see the spread of gonococcal strains that are completely resistant to all previously used antibiotics around the world, emphasizing the importance of developing new treatments for gonorrhea (1). Animal models, particularly mouse models, play a significant role in identifying and testing new treatments for gonorrhea. The chapters herein give an example of how an *in vivo* mouse model can be used to provide additional insight into the roles of proteins in bacterial survival and infection. Understanding how bacteria grow and thrive is critical to developing methods to combat infection.

Characterization of MltG

Through the characterization of the lytic transglycosylase, MltG, we found that antibiotic resistance was decreased for antibiotics that target the crosslinking of peptidoglycan strands in the cell wall. This identifies peptidoglycan (PG) synthesis as an important cellular process MltG is involved in. This result was no surprise as MltG was shown to be able to breakdown sacculi into PG monomers and dimers (Chapter 2, Fig. 1). Additionally, deletion of *mltG* altered the composition of cell sizes of the bacteria, where there were more cells larger than 0.7 μm^2 compared to the wild type, further indicating that PG is affected by the lack of MltG. Both PG turnover and cell lysis under non-growth conditions were significantly different between the wild type and the mutant. Particularly for cell lysis, there was not only a difference between the wild type and the deletion mutant but also the point mutant, indicating the presence of the protein (despite it being inactive) may affect the permeability of the outer membrane (OM) or structure of PG. Studies of gonococcal lytic transglycosylases LtgA and LtgD as well as studies of *Pseudomonas aeruginosa* lytic transglycosylases have shown that these membrane-bound

enzymes are important for envelope integrity(2, 3). For *N. gonorrhoeae*, loss of lytic transglycosylase function for LtgA and LtgD resulted in bacteria more sensitive to the enzymatic actions of lysozyme and neutrophil elastase but not cationic activities of these proteins or of antimicrobial peptides or polymyxin B(2). Thus, the bacteria may have gaps in the cell wall that allow increased access to immune system enzymes or antibiotics that target PG transpeptidation.

Most surprising was the increase in PG fragment release that occurred in the *mltG* mutants. As MltG is a lytic transglycosylase (LT), one might expect that PG fragment release would decrease. The opposite was observed, where PG monomer, dimer, and multimer fragment release were increased. To identify the LT that is responsible for producing the increased amounts of PG fragments released, double mutants of *ltgA* and *ltgD* with *mltG* were created and tested for which fragments and how much of each PG fragments were released. From this characterization, it is not clear if either one of the LTs are primarily responsible for the increase in PG fragment release. However, it is possible that a combination of both LTs is producing the PG fragments that are ultimately released. The characterization of PG fragments released for the double mutants were like the single *ltgA* and *ltgD* mutants for PG monomer release, while PG dimer release increased in the *mltG ltgA* and *mltG ltgD* double mutants compared to the *ltgA* and *ltgD* single mutants. These results are consistent with a model in which *mltG* mutants make overlong PG strands and LtgA and LtgD act to shorten the strands.

Using BACTH assays, we found positive interactions between MltG and PBP1, PBP2, or PBP4. Both PBP1 and PBP2 are involved in the synthesis of PG whilst PBP4 is an endopeptidase that cleaves bonds between amino acids within the peptide stem or the bonds crosslinking peptide stems(4–6). These interactions suggest, as PBP1 or PBP2 are acting in synthesis of the new PG strand, MltG will cleave the growing chain and stop elongation. The

interaction with PBP4 may facilitate opening of the PG mesh through PBP4 endopeptidase activity to allow space for the new PG strand and freeing up peptides in the cell wall to crosslink the new strand to.

Finally, we used our mouse model to determine the effects an *mltG* deletion would have on both oral colonization and blood infection. Contrary to our predictions, deletion of *mltG* had no effects on oral colonization nor on blood infection. Analysis of cytokine levels in the blood showed no significant differences between the mutant and the wild type. Yet, decreasing infection in the mutant correlated with decreasing concentrations of IL-6, IL-10, MCP-1, and TNF. This indicates that the amount of bacteria infecting affects the immune response to *Neisseria musculi* (Nmus). Given the large increase in PG fragment release from the *mltG* mutant, we had hypothesized that a large inflammatory response might occur that would result in clearing of the oropharyngeal infection. Since infection was not decreased in the mutant, it is possible that the oropharyngeal tissue does not respond strongly to NOD1 and NOD2 agonists or that the reponse was not effective in killing the bacteria. We demonstrated systemic infection with *N. musculi* for the first time. While we did not see a difference between *mltG* and wild type in this model either, this was a successful test of the model and it identified cytokines upregulated in response to *N. musculi* infection. The systemic infection model became useful in our study of *amiC* mutants.

Cell separation and AmiC

Investigations into AmiC in Nmus revealed that successful cell separation is important for successful infection. AmiC is an amidase that cleaves the bond between the *N*-acetylmuramic acid and the peptide stem in the cell wall. Previously, a cell separation defect was identified in *amiC* mutants of diplococcal *Neisseria* species *N. gonorrhoeae* and *N. meningitidis* (7). For the

diplococcobacillus species Nmus, we also observed a cell separation defect, which was seen as the bacteria growing in short chains. (7)Despite the similarity in the cell separation defect, the changes in PG fragments released were not comparable. In Nmus, all PG fragments released decreased in the mutant, whereas in *N. gonorrhoeae amiC* mutants, reductions in tetrasaccharide-peptide and free disaccharide are seen, but PG dimers increase and monomers are relatively unaffected(7). Consistent with the reduction in PG fragment release in Nmus, mNOD1 activation was also decreased in the mutant. The observation of decreased mNOD1 activation suggests that the immune response to the *amiC* mutant would be less than for the wild type, implying that the strain may be better suited to survive in an infection. However, infection of the mutant strain was decreased as compared to the wild type. This result suggests that the cell separation defect is a stronger determinant of infection than the effects on release of PG fragments. It is not known why *Neisseria* mutants with cell separation defects do not survive well in mice or blood. The bacteria grow at rates similar to wild type in vitro(7). *N. meningitidis nlpD* mutants show increased sensitivity to complement, and *N. gonorrhoeae amiC* mutants have increased sensitivity to vancomycin and deoxycholate(7, 8). So, increased permeability for the outer membrane in the mutants, where it has to surround multiple cells, may make the bacteria susceptible to multiple innate immune defenses. Further experiments using Nmus cell separation mutants may provide a better understanding of the infection defects of cell separation mutants.

Nonpathogenic *Neisseria*

Nonpathogenic *Neisseria* (*Neisseria* spp.) are found in many different animals including humans. These species are important reservoirs for antibiotic resistance genes for pathogenic *Neisseria* but also can prevent colonization by their pathogenic counterparts through competitive exclusion (9, 10). Despite their primarily nonpathogenic lifestyle, *Neisseria* spp. can also cause

opportunistic infections; however, this is typically in individuals with compromised immunity (source). The *Neisseria* spp. described here are all mucosal organisms of animals and humans.

Characterizations for released PG fragments revealed that nonpathogenic *Neisseria* spp. do release different PG fragments at varying amounts. Gc will release PG dimers, monomers, free disaccharide, free peptides, and anhydro-*N*-acetylmuramic acid (anhMurNAc) (11). From a characterization using [^{3}H]-glucosamine labeling, it was determined that *N. mucosa* and macaque isolate AP312 release PG monomers and free disaccharide. While *N. musculi* and macaque isolate AP678 release PG monomers, dimers, and disaccharide. Using [^{3}H]-DAP as a label, it was determined that two strains of *N. lactamica* released PG dimers, monomers, and peptides; however, the relative amounts of PG fragments released were different between strains. Strain ATCC 49142 released similar amounts of PG monomer and peptides while strain ATCC 23970 released lower levels of PG monomer. Contrastingly, Nmus released lower levels of peptides compared to PG monomers. Quantitative comparisons with Gc showed that *N. mucosa* releases more peptides, but lower levels of PG monomer compared with Gc.

Investigations into PG recycling were also conducted through analysis of the protein AmpG. AmpG is an inner membrane permease that allows the transport of PG monomer fragments and disaccharide fragments from the periplasm to the cytoplasm (12). This process allows the fragments to be recycled back into new PG synthesis. It was previously determined that deletion of *ampG* causes an increase in PG monomer and free disaccharide release in the two pathogenic *Neisseria* species *N. gonorrhoeae* and *N. meningitidis* (12). Mutation of *ampG* in *N. musculi* and macaque isolate AP312 resulted in an increase in PG monomer release with only modest changes in disaccharide release. Looking at *N. lactamica*, it was determined that a single nucleotide polymorphism in *ampG* was at least partially responsible for the difference in PG

monomer release between *N. lactamica* strains but could also confer changes in PG monomer release in Gc.

As these nonpathogenic *Neisseria* spp. release PG fragments, it follows that these same species should be able to activate NOD1 and NOD2; nevertheless, we predict that due to their nonpathogenic nature they will activate the receptors to a lesser degree than the pathogenic *Neisseria*. HEK293-reporter cells were used to measure activation of hNOD1 and hNOD2 for the human-associated nonpathogenic species and mNOD1 for Nmus. Human NOD1 and NOD2 activation analysis revealed that these human *Neisseria* spp. induced similar levels of hNOD1 and hNOD2 activation as Gc with *N. lactamica* ATCC 23970 actually inducing higher levels of hNOD2 activation than Gc. Mouse NOD1 activation analysis of Nmus was low with mutations of *ampG* and *ldcA* only giving slightly higher activation than wild-type.

Conclusions and future directions

Continuing to explore the uses and limitations of our mouse model will allow further investigations into aspects of gonococcal infection that were previously unable to be studied. The benefits of our model include using a *Neisseria* species in its native host that is genetically comparable to the pathogenic *Neisseria* and that it can be infected without the addition of antibiotics or hormones. While our investigations into different PG related proteins did not always show significant differences in infection, we can continue to look at unexplored proteins that have effects not only on PG fragment release but also in immune activation through NOD1 and NOD2 or in observations from cervical and fallopian tube explants. Additionally, our ability to mutate Nmus to make it release different amounts of fragments or different fragments can also be useful in making the bacteria release similar amounts and fragments as Gc for further study. Beyond PG, other immunologically relevant molecules such as lipooligosaccharide and outer

membrane vesicles can also be studied. The ability to look at specific interactions may be helpful in identifying ways to develop a successful vaccine or additional antimicrobials for gonorrhea. Despite the many possibilities for the model, it must be kept in mind that we are mimicking infection and not replicating it, and therefore, we can only infer so much. Nonetheless, our model can be an excellent step for further study into gonococcal infection with other established methods.

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Appendices

APPENDIX ONE: Oral colonization using a mouse model with *Neisseria musculi*

Abstract

Neisseria gonorrhoeae is a pathogen that causes infection in humans. The human specificity of this pathogen makes it difficult to model in mice. While currently there are several mouse models for gonorrhea, there are associated problems with these models including the necessity of adding hormones and antibiotics to achieve colonization as well as an inability to cause continuous infection. We propose using oral colonization with a mouse commensal in mice as an experimental model to understand gonococcal infection. *Neisseria musculi* is a mouse commensal that can colonize the oral cavity, nasopharynx, and the entire gastrointestinal tract of mice. Previous studies have shown mouse strain is important for both successful colonization as well as for how much colonization occurs. In this study we investigated the ability for colonization to occur in CAST and C57BL/6J mice and compared how much colonization occured in each strain. CAST mice had increased oral colonization compared with C57BL/6J mice. Additionally, we used a mutant with increased release of peptidoglycan fragments to determine if it would alter colonization levels in CAST mice. Despite the increased released of peptidoglycan fragments, this did not alter colonization levels.

Introduction

Neisseria gonorrhoeae (Gc) is a human specific pathogen that causes the sexually transmitted infection gonorrhea. Due to human specificity of many gonococcal pathogenesis factors, it is difficult to model this infection in mice. While there are previously developed mouse models for Gc, analyzing the specific *Neisseria*-host interactions is challenging because the bacteria do not form specific interactions with non-human hosts. Absence of binding receptors in mice for *N. gonorrhoeae* as well as the human specificity of gonococcal binding of iron scavenging proteins and complement regulators means that mice are missing many of the crucial components that determine gonococcal infection and survival. Additionally, infection is transient in mice and requires additional substrates, such as antibiotics for the commensal microbiota and estradiol, to foster long-term infection(1). We hypothesize that using *N. musculi* in mice can better mimic Gc infection in humans than using Gc in mice. A model that can mimic infection of Gc is will allow the study of specific *Neisseria*-host interactions and should work well for understanding the importance of immunostimulatory molecules such as peptidoglycan, that are highly similar in these two *Neisseria* species.

Neisseria musculi (Nmus) is a commensal of wild mice and has oropharyngeal colonization(2). There is 80% nucleotide identity between the mouse commensal and Gc(2). The use of animal colonizers to mimic infection of human pathogens has been previously used for Chlamydia and Tuberculosis, using *Chlamydia muridarum* and *Mycobacterium marinum*, respectively(3, 4). There are several benefits to using animal-specific bacteria over human pathogens, including the ease and low cost of animal use and the inability to study pathogens in humans. These *in vivo* systems using native hosts are preferred over other *in vivo* and *in vitro* methods because they allow the study of a bacterial species infecting a whole system that is its

native host over a reduced one or non-native host. Studies of complex interactions that would not occur in *in vitro* methods or in *in vivo* models using non-native hosts can be achieved. In this study, the mouse-specific species *N. musculi,* is used to analyze differences in oral colonization between two mouse strains. Additionally, analysis of the permease AmpG and the effect of a gene deletion on oral colonization was conducted using the mouse strain with the highest oral colonization.

Materials and Methods

Bacterial strains and growth

All *N. musculi* strains were derivatives of the smooth variant of strain AP2031. *N. musculi* strains were grown at 37 degrees Celsius and 5% CO_2 on GCB agar plates (Difco) with Kellogg's supplements. Strains were also grown in gonococcal base liquid medium (GCBL) containing 0.042% $NaHCO_3$ and Kellogg's supplements with aeration. Antibiotics were used at the following concentrations: kanamycin at 80 µg/mL and rifampicin at 40 µg/mL.

Plasmid and strain construction

The plasmids used in this study are listed in Table 1. Chromosomal DNA from *N. musculi* AP2031 was used as a PCR template unless otherwise noted.

Table 1.

Plasmid or Strain	Description	Reference
pIDN3/1	Insertion-duplication plasmid (Erm^R)	Hamilton et al. 2001
pEC096	*ampG* deletion/interruption constructed in pIDN3 (Kan^R)	This study
AP2031	Wildtype Nmus	Weyand et al. 2016
TNH2002	Wildtype Nmus with natural resistance to rifampicin (Rif^R)	This study
TNH2004	TNH2002 transformed with pEC096 (Rif^R, Kan^R)	This study

Infection of mice and cytokine analysis

All mice used were from the Jackson Laboratory. Animal protocols were approved by the University of Wisconsin-Madison Institutional Animal Care and Use Committee. All mice were

held in a room that was specific pathogen free 1 for 2 weeks prior to inoculation. Mice were 6-week-old female mice at the time of inoculation.

For the CAST and C57BL/6J experiment, on the day of infection, TNH2002, was swabbed from GCB agar, suspended in cGCBL at an OD540 of 0.2, centrifuged to pellet cells, and resuspended in Dulbecco's PBS. For the *ampG* experiment, on the day of infection, TNH2002 (WT) and TNH2004 (*ampG*) were swabbed from GCB agar, resuspended in cGCBL at an OD600 of 2.0, centrifuged to pellet cells, and resuspended in Dulbecco's PBS.

All mice were manually restrained and 50 µL of inoculum was pipetted into the oral cavity. For sample collection, the oral cavity was swabbed, and the swab was placed into warmed cGCBL. Fecal pellets were collected, weighed, and homogenized into warmed cGCBL. Samples were diluted, plated on GCB with rifampicin, and incubated for 48 hours before counting colony forming units (CFUs). Isolates obtained from GCB with rifampicin plates were verified by colony lysis PCR using ITS primers specific to *N. musculi*. Amplified sequences were screened by gel electrophoresis by comparison to the wild-type strain, TNH2002, and found to be identical (TNH2002 and AP2031 sequences were found to be identical in an unpublished observation).

Results

CAST mice are colonized at higher amounts than C57BL/6J mice

To analyze how mouse strain affects colonization, oral colonization of both CAST mice and C57BL/6J mice with *N. musculi* were measured. Mice were orally inoculated by pipetting 50 µL of inoculum into the oral cavity. Samples were taken 7-, 14-, and 21-days post inoculation by swabbing the oral cavity of the mice. Fecal pellets were also collected, and colonization measured for these samples. Both oral and fecal pellet colonization was decreased in C57BL/6J mice compared with CAST mice (Fig. 1). This result is consistent with previous observations(5).

Deletion of ampG *does not cause a change in oral colonization in CAST mice*

To analyze how deletion of the gene for the peptidoglycan fragment permease AmpG affects oral colonization, oral inoculation of CAST mice with *N. musculi* was completed. AmpG is found in the inner membrane and facilitates transfer of peptidoglycan fragments from the periplasm to the cytoplasm. When deleted, *ampG* mutants show increased peptidoglycan monomer release suggesting there will be increased immune activation through NOD1(6). This increased inflammatory response is expected due to the activation of NOD1 by peptidoglycan monomers(7). Mice were once again inoculated by pipetting 50 µL of inoculum into the oral cavity. Samples were taken 7-, 14-, and 21-days post inoculation by swabbing the oral cavity of the mice. There was not a significant difference in oral colonization observed between the mutant and wildtype infected mice (Fig. 2).

Discussion

Experiments using *N. musculi* in a mouse model were conducted to observe differences in oral colonization between mouse strains as well as between wildtype *N. musculi* and a mutant of *ampG.* CAST mice had higher oral colonization compared with C57BL/6J. This result was consistent with results observed previously in another study(5). Based upon the mouse strain results, experiments using CAST mice were then conducted. Using an *N. musculi* strain with a deletion of *ampG*, oral colonization was measured between a wildtype *N. musculi* strain and the mutant. Despite a deletion of *ampG*, which increases PG monomer release, there was not a difference in oral colonization between the two strains. It is possible that the amount of monomers released is not significant enough to impact oral colonization. There are millions of different microbes found in the oral cavity, and the increase in PG monomers may be diluted by other components released by the multitude of organisms, It is also possible that the PG fragments are being consumed or broken down further by other organisms. Finally, cells in the oropharyngeal space may not be very responsive to PG monomers.

Figures

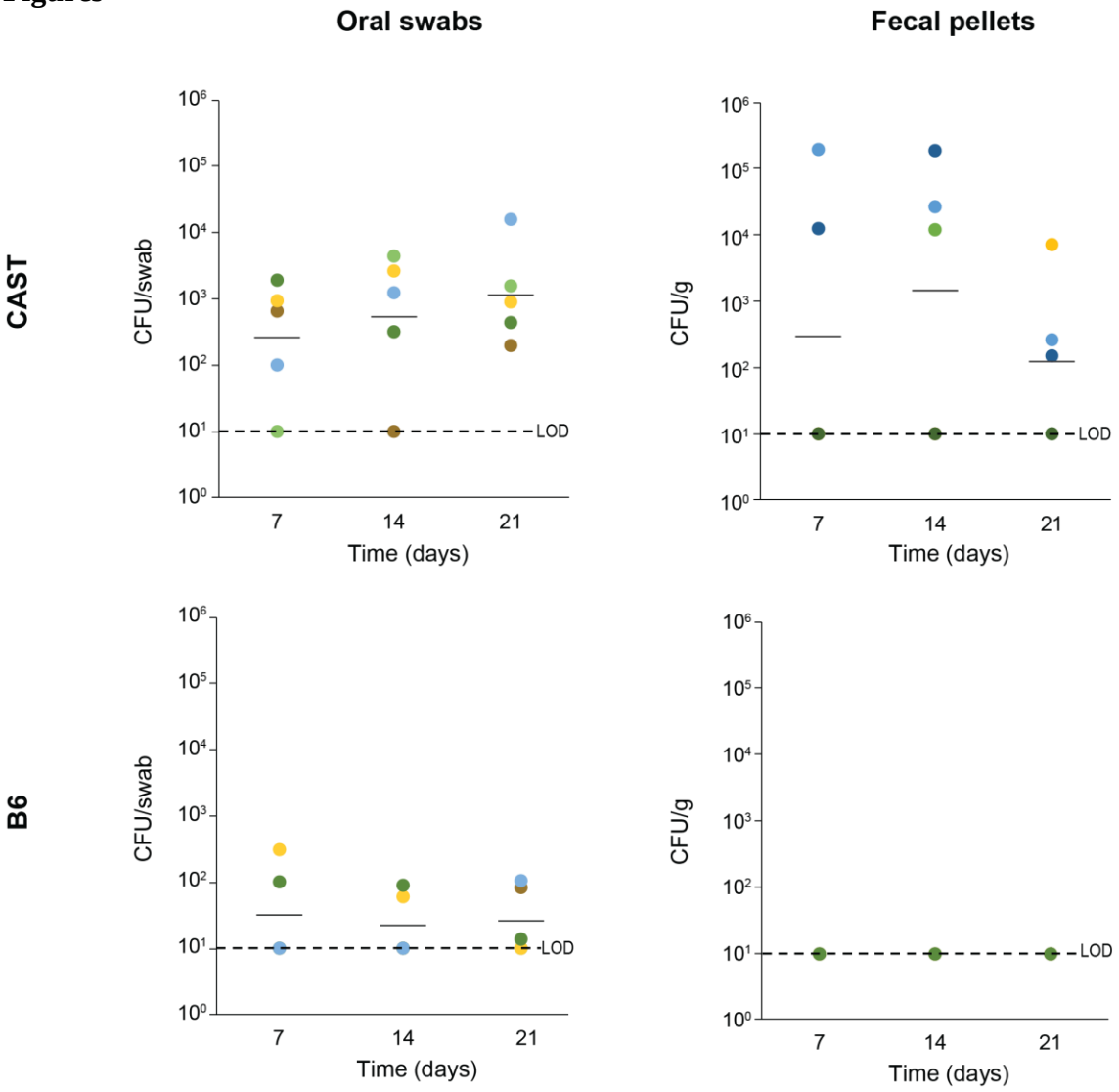

Figure 1. CAST mice are colonized at higher levels compared with C57BL/6J mice. CAST and C57BL/6J mice were orally inoculated with *N. musculi*. At 7-, 14-, and 21-days, mouth swabs were taken, and fecal pellets collected. Quantification of CFUs was determined for both the oral swabs and fecal pellets. Both the oral swabs and fecal pellets had higher CFUs for CAST mice as compared with C57BL/6J at all timepoints.

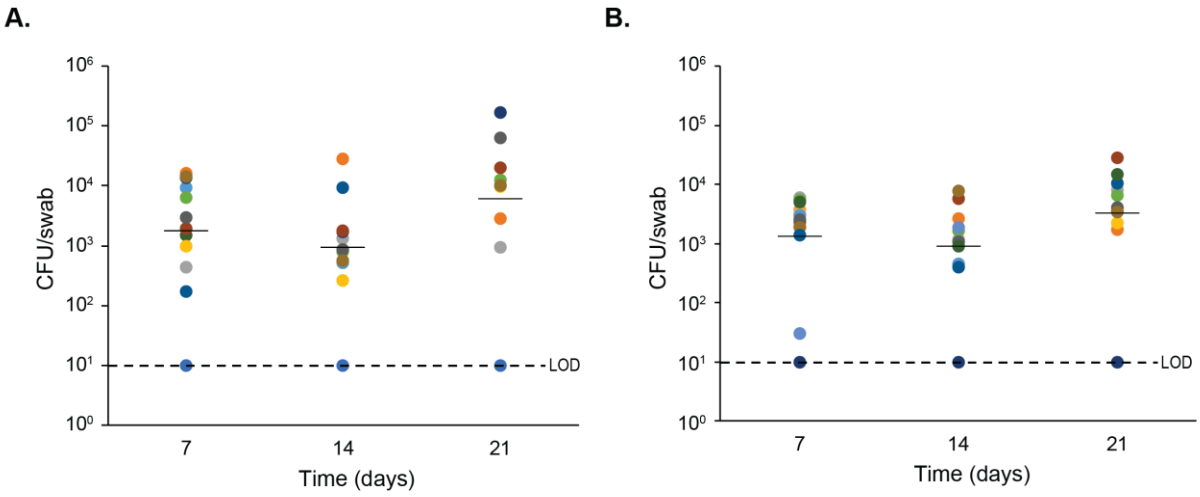

Figure 2. A mutation in *ampG* does not affect colonization of mice. CAST mice were orally inoculated with *N. musculi* and swabs were taken at 7-, 14-, and 21-days. Quantification of CFUs was determined. *a)* CFUs for mice inoculated with wildtype. *b)* CFUs for mice inoculated with the *ampG* mutant. For both strains of *N. musculi*, there were comparable levels of colonization, indicating *ampG* deletion has no effect on numbers of colonizing bacteria.

APPENDIX TWO: Analysis of transcript levels for peptidoglycanases in *Ngo1982* and *Ngo0393*

Abstract

Several proteins involved in the breakdown of peptidoglycan have been identified in *Neisseria gonorrhoeae.* These proteins are significant because they produce the peptidoglycan fragments that are released by the bacteria during growth that induce inflammatory responses in the host. What controls gene expression is currently unknown. In this study we analyze the transcript levels of different peptidoglycanases in two mutants of putative transcriptional regulators, *Ngo0393* and *Ngo1982.* We found changes in transcript levels of several different proteins in the *Ngo1982* strain but not the *Ngo0393 strain.* These data highlight the importance for this putative transcriptional regulator, *Ngo1982*.

Introduction

During normal growth of *Neisseria gonorrhoeae* (Gc), peptidoglycan fragments will be released(8). PG fragments are generated by a collection of peptidoglycanases including amidases, lytic transglycosylases, endopeptidases, and carboxypeptidases. The exact mechanism by which peptidoglycan fragments are released or how release is controlled is currently unknown. Transcript levels of peptidoglycanases in two mutants of putative transcriptional regulators in Gc were analyzed to determine if either of these regulators are involved in the regulation of any of the studied peptidoglycanases.

Materials and Methods

Bacterial strains and growth

All *N. gonorrhoeae* strains used in this study are derivatives of strain MS11(9). Piliated strains of MS11 were used for all transformations, whereas non-piliated strains were used for all other experiments. *N. gonorrhoeae* strains were grown at 37˚C and 5% CO_2 on GCB agar plates (Difco) with Kellogg's supplements(10). Strains were also grown in gonococcal base liquid medium (GCBL) containing 0.042% $NaHCO_3$ and Kellogg's supplements with aeration(10–12).

Plasmid and strain construction

The plasmids used in this study are listed in Table 1. Chromosomal DNA from *Neisseria gonorrhoeae* MS11 was used as a PCR template for Gc unless otherwise noted.

Table 1.

Plasmid or Strain	Description	Reference
pIDN3	Insertion-duplication plasmid (Erm^R)	Hamilton et al. (2001) (13)
pKH37	Complementation vector (Cm^R)	Kohler et al (2007) (14)
pEC189	*Ngo1982* deletion plasmid	Chan JM 2018 PhD thesis
EC574	*Ngo1982* deletion strain	This study
EC575	*Ngo1982* complement strain	This study
AKK689	*Ngo0393* deletion strain	This study

Reverse transcriptase PCR

Total RNA was extracted from gonococcal cultures grown for 3 hours at 37℃ from an OD_{540} of ~0.25. RNA isolation was done as previously described in Ramsey et al. (Targeted mutagenesis of intergenic regions in the *Neisseria gonorrhoeae* gonococcal genetic island reveals multiple regulatory mechanisms controlling type IV secretion, Mol Microbiol 2015). cDNA was made using iScript cDNA Synthesis Kit (BioRad), and qRT- PCR was done using SYBR green reagents (BioRad).

Results

Changes in transcript levels of peptidoglycanases occur in Ngo1982 *but not* Ngo0393

To determine possible regulators for LtgA, LtgB, LtgC, LtgE, MltG (YceG), and NagZ, analysis of transcript levels for these proteins in two different mutants of putative transcriptional regulators was conducted. LtgA, LtgB, LtgC, LtgE, and MltG total RNA was isolated, reverse transcribed to cDNA using primers specific for the genes of the proteins, which was then subjected to qRT-PCR. Comparisons were made between wildtype and the mutants. It was found that there were changes in transcript levels for certain peptidoglycanase genes in *Ngo1982* but no changes in the *Ngo0393* mutant. There was a decrease in transcript levels of *mltG*, while there were increases in transcript levels of *ltgA* and *nagZ.* These results indicate a role for Ngo1982 in direct or indirect regulation of these three genes.

Discussion

Although several different types of peptidoglycanases have been identified, how these proteins are regulated is unknown. To identify a possible transcriptional regulator for six peptidoglycanases, transcripts of these proteins were analyzed by qRT-PCR in two mutant strains lacking putative transcriptional regulators, Ngo0393 and Ngo1982. Three proteins had altered transcript levels in *Ngo1982*: *ltgA, nagZ,* and *mltG*. There were increased transcripts for *ltgA* and *nagZ,* and decreased transcripts for *mltG*. All other proteins did not show changes in transcript levels in either mutant. This indicates that *Ngo1982* could possibly be a positive transcriptional regulator for *mltG* and a negative transcriptional regulator for *ltgA* and *nagZ.*

Both LtgA and NagZ are involved in cell wall breakdown and recycling of PG fragments(15). LtgA is the major lytic transglycosylase in N. *gonorrhoeae*, cleaving PG strands into monomeric fragments, most of which are destined for uptake into the cytoplasm for PG recycling(16). The 1,6-anhydro bond that LtgA creates on these fragments is necessary for their recognition by the PG fragment permease AmpG(6). NagZ acts on the PG fragments after they are transported into the cytoplasm. It removes the N-acetylglucosamine residue from the monomers and free disaccharides that have been removed from the cell wall(17). By contrast, MltG cleaves the growing PG strand from the PG synthesis complex thereby terminates PG strand growth. This process is necessary for producing PG strands that are of the correct length for use in the cell wall. So overall, *Ngo1982* appears to repress PG breakdown and recycling genes and activate a PG synthesis gene.

Figures

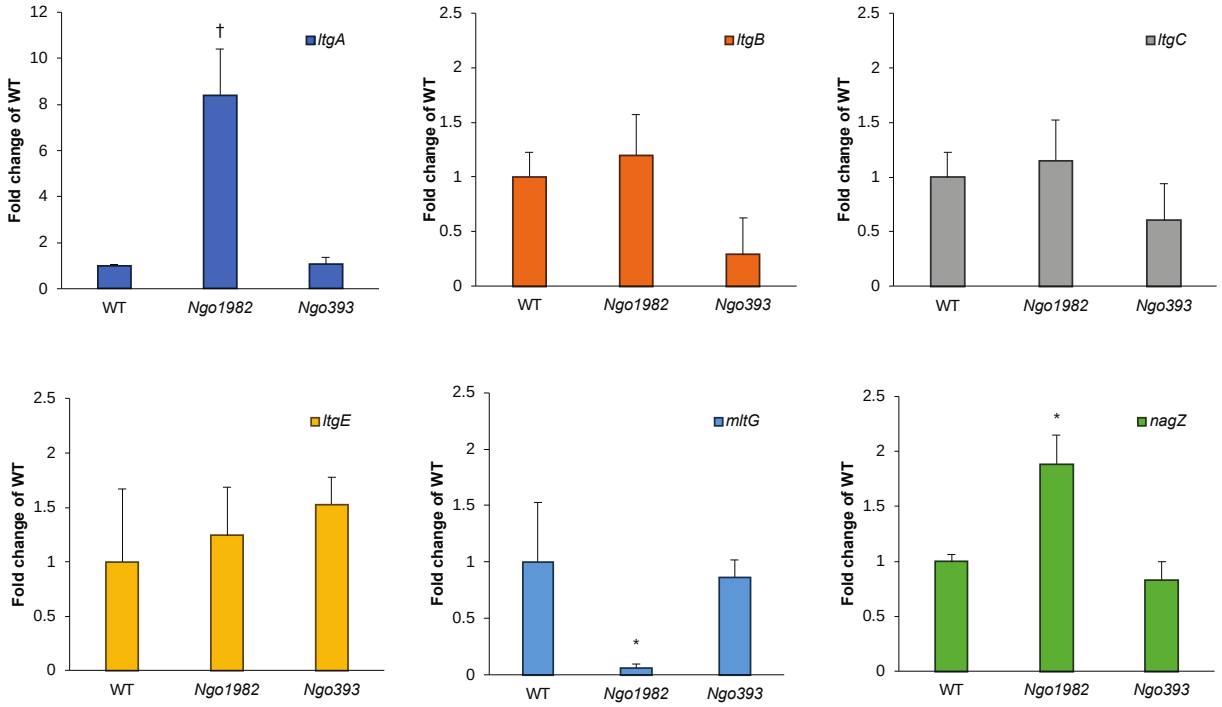

Figure 1. Transcript levels of peptidoglycanases. Analysis of transcript levels in mutants of putative transcriptional regulators *Ngo1982* and *Ngo393* was conducted. There were increased levels of transcripts of *ltgA* and *nagZ* in *Ngo1982* and a decreased level of *mltG* transcripts also in *Ngo1982*. No changes in levels of gene transcripts of the peptidoglycanases studied were identified in *Ngo393*. *p<0.01, †p<0.05

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