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Title: Characterization and Validation of *Neisseria gonorrhoeae* Proteins $GmhA_{GC}$ and NGO1985 as Molecular Targets for Development of Novel Anti-gonorrhea Therapeutic Interventions.

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Aleksandra E. Sikora

The sexually transmitted disease gonorrhea, caused by the Gram-negative bacterium and obligate human pathogen *Neisseria gonorrhoeae*, remains a significant health and economic burden worldwide. In the absence of a protective vaccine, antimicrobial agents are the only pharmacological intervention for patients with gonorrhea. However, due to the remarkable ability of gonococcus to develop antibiotic resistance, infections caused by *N. gonorrhoeae* are believed to become untreatable in the near future. Identification and elucidation of the physiological function of novel *N. gonorrhoeae* proteins is critical for the formulation of new therapeutic interventions. This work focuses on characterization and validation of two gonococcal proteins, $GmhA_{GC}$ and NGO1985, as targets for development of new antibiotics and a vaccine antigen, respectively. The sedoheptulose-7-phosphate isomerase, $GmhA_{GC}$, is the first enzyme in the biosynthesis of nucleotide-activated-*glycero*-*manno*-heptoses. We demonstrate that *N. gonorrhoeae* $GmhA_{GC}$ is essential for lipooligosaccharide (LOS) synthesis and pivotal for bacterial viability. Our crystallization studies have shown that $GmhA_{GC}$ forms a homo-tetramer in the closed conformation with four zinc ions in the active site. Site directed mutagenesis studies showed that active site residues E65 and H183 are important for LOS synthesis but not bacterial viability, suggesting that abolition of LOS synthesis is disconnected from the $GmhA_{GC}$

involvement in *N. gonorrhoeae* viability. NGO1985 was initially described as a hypothetical lipoprotein containing two BON (Bacterial OsmY and Nodulation) domains, hypothesized to be involved in maintaining bacterial cell envelope integrity. In our studies we demonstrate that NGO1985 is a surface exposed lipoprotein, conserved among diverse gonococcal isolates. Deletion of *ngo1985* results in bacterial cell envelope defects leading to a pleiotropic phenotype including increased susceptibility to antimicrobial agents, decreased survival during *in vitro* growth conditions mimicking the human host environment, and high attenuation in the murine model of infection. NGO1985 interactome studies indicated a broad network of interactions including potential association with β-Barrel Assembly Machinery (Bam) complex, antibiotic efflux pump(s), and several lipoproteins. Furthermore, we demonstrate that NGO1985 does not undergo lipoprotein sorting according to the +2 residue of the lipobox motif as characterized for *Escherichia coli*. We determined that both BON domains, in their native orientation, are essential for NGO1985 functionality and stability. Finally, for the first time we have investigated the importance of the BON domains' conserved glycine residues and showed that these amino acids play a critical role in protein stability. Based on the importance of both $GmhA_{GC}$ and NGO1985 on gonococcal physiology, we conclude that these proteins are promising molecular targets for development of new anti-gonorrhea interventions.

Characterization and Validation of *Neisseria gonorrhoeae* Proteins $GmhA_{GC}$ and NGO1985 as Molecular Targets for Development of Novel Anti-gonorrhea Therapeutic Interventions

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Igor H. Wierzbicki

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Major Professor, representing Pharmaceutical Sciences

Dean of the College of Pharmacy

Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Igor H. Wierzbicki, Author

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Chapter 1

General Introduction

Igor H. Wierzbicki

***Neisseria* genus**

Neisseria are Gram-negative, β-Proteobacteria bacteria belonging to the Neisseriaceae family (reviewed by Ryan and Ray, 2004; Bennett *et al*. 2014; Liu *et al*., 2015). This abundant and wide spread genus consists of free-living and host associated organisms. The animal reservoir spans from insects, lizards, waterfowl and ground-dwelling birds, to marine- and land-mammals. *Neisseria* contains many human-related commensals and only two pathogenic species. The human commensal species inhabit the mucosal surfaces of the upper respiratory and alimentary tracts, and are conserved across geographic regions, ethnic groups, and lifestyles. The pathogenic species, although closely related, reside in completely different environments and cause distinct diseases. *N. meningitidis*, the meningococcus, colonizes the nasopharynx and is the major cause of meningitidis and bacteremia, while *N. gonorrhoeae*, the gonococcus, primarily infects the urogenital tract and is the cause of gonorrhea.

Species belonging to this genus are non-motile, non-spore forming, and oxidase-positive microorganisms. The majority of representatives are cocci, with the exceptions of *N. elongata*, *N. weaveri*, *N. bacilliformis*, and *N. shayeganii* which are bacilli; usually appearing in pairs with flattened sides of the opposing cells which results in a coffee bean-shaped appearance. The cell envelope organization is typical for Gram-negative bacteria, with inner and outer membranes separated by a thin layer of peptidoglycan and aqueous periplasmic space (Fig. 1.1). *N. meningitidis*, is unique for this genus, as it also synthesizes group II type polysaccharide capsule. The outer membrane contains a variant of lipopolysaccharide (LPS) that is lacking the O-specific antigen, and is composed only of Lipid A and core oligosaccharide. This shorter version of LPS is called lipooligosaccharide (LOS).

The average *Neisseria* genome size is about 2.2 Mbp, with approximately 2000 open reading frames and a guanine-cytosine content of 51.5%. *N. gonorrhoeae* and *N. meningitidis* are polyploids, containing 2-5 copies of the genome per growing monococcal bacterial cell, with approximately two chromosomes per one diplococci unit (Tobiason and Seifert, 2010). Since non-pathogenic *Neisseria* were shown to be monoploid, it is hypothesized that the polyploidy is a virulence trait involved in genome plasticity. Non-chromosomal genetic elements such as plasmids, prophages, and

genetic islands are common among the members of the genus. A large number of restriction-modification systems have been noted (reviewed by Rotman and Seifert, 2014). All known species of this genus are naturally competent during all phases of growth for DNA transformation, a process of up-taking the genetic material from the environment (Sparling, 1966). Benefits of natural transformation include spread of antibiotic resistance determinants and beneficial alleles, providing a template for use in DNA repair, and as a source of nutrients. The DNA incorporation is facilitated by the presence of a nonpalindromic 10- or 12-base pair DNA uptake sequences (DUS), GCCGTCTGAA and ATGCCGTCTGAA, respectively. *Neisseria* contain over 2000 DUS within the genome, on average one per 1000 base pairs of DNA (Marri et al., 2010).

Many *Neisseria* genes were identified to be phase and/or antigen variable. Phase variation is defined as a reversible change in gene expression: either change between an expressed and unexpressed state or change between two distinct forms of a gene (Moxon *et al*., 2006). Most of the phase variation is a result of slipped-strand mispairing, a process of mispairing between mother and daughter DNA strands during replication, caused by the presence of either homopolymeric or polynucleotide repeats (Levinson, and Gutman, 1987). Slipped-strand mispairing either results in frame shift within the coding region leading to premature termination of translation or alteration in the promoter region which affects the transcription levels. Antigenic variation is the ability to alter the peptide sequence that results in production of variable forms of the antigens. This variation is a consequence of a low frequency recombination between similar coding regions (*opa* genes) or coding region and silent loci (*pilE* and *pilS*) (Aho *et al*., 1991; revieved by Obergfell and Seifert, 2015).

Neisseria gonorrhoeae and gonorrhea

In 1879 Albert Neisser identified a small diplococci microorganism, later named *Neisseria gonorrhoeae*, the gonococcus, as the causative agent of the sexually transmitted disease (STD) gonorrhea (Neisser, 1879). *N. gonorrhoeae* is an obligate human pathogen with no other natural reservoir. Although direct sexual contact is the main mode of gonococcal transmission, indirect ways of spreading were reported

(Kleist and Moi, 1993). Mucosal surfaces of the urogenital epithelia are the main colonization sites of this bacterium. However, the pharynx, rectal, and conjunctiva mucosa infections are also commonly reported (Ryan and Ray, 2004). The prolonged infection may progress from the surface of the tissues to the adjacent mucosal and glandular epithelial cells (McCormack, 1994).

Gonorrhea has been recorded throughout human history, specifically in ancient literature from China, Egypt, Rome, Greece, and the Old Testament of the Bible (Leviticus 15:1–3), which makes it one of the oldest recorded diseases in the history of the mankind. Gonorrhea was described in fifth century B.C. by Hippocrates as "strangury" acquired from the "pleasures of Venus" (Holmes *et al*., 1999). In 130 A.D., Galen confused the purulent discharge caused by the gonococcal urethritis with the semen, and introduced the term "flow of seed" for gonorrhea (Mandell *et al*., 1990; Holmes *et al*., 1999). The common name for this disease, "clap" has been in use for almost 700 years and finds it origin in French language from the archaic word clapoir which stands for "a rabbit warren"; later "the brothel" (Ryan and Ray, 2004). In present times, gonorrhea still remains a major global health concern. In 2012 the World Health Organization (WHO) estimated 78.3 million gonorrhea cases worldwide (Newman *et al*., 2015). In the United States of America, gonorrhea holds the status of the second most common sexually transmitted disease, with 333,004 reported cases and frequency of 106.1 per 100,000 population in 2013 (http://www.cdc.gov/std/stats13/gonorrhea.htm). The total amount of the globally reported cases is believed to be much lower than the actual number of infections due to inappropriate diagnostics, absence of laboratory testing, and incomplete reporting of the identified cases.

The disease is transmitted more frequently from men to women than from women to men, with 50-73% and 20-35% probability, respectively (Mandell *et al*., 1990; Holmes *et al*., 1999; Brooks and Donegan, 1985). In men, the urethra is the primary site of the gonococcal infection. Symptoms are usually experienced 2 to 7 days following contact with the pathogen and consist predominantly of purulent urethral discharge and dysuria (Ryan and Ray, 2004). In rare situations, infection may progress and cause epididymitis, as well as prostatitis. In women, the endocervix is the main infection site, and disease symptoms include vaginal discharge, increased urinary

frequency, dysuria, abdominal pain, and dysregulation in the menstrual cycle. The symptoms of gonorrhea are usually mild and can be absent among women (≥50%) as well as men (≤10%) (Bignell *et al*., 2013). Although the urethra and uterine cervix are the main infective sites among men and women respectively, infections of the rectum, pharynx, and the conjunctiva are also commonly reported (Ryan and Ray, 2004). Rectal infection is usually caused by rectal intercourse or, for women, as a consequence of contamination from the infected vaginal fluids. Rectal conditions are mostly asymptomatic however tenesmus, discharge, and rectal bleeding may occur. Pharyngeal gonorrhea is a consequence of the genital-oral sex practices and symptoms as in the case of the rectum infections are predominantly absent. If manifested, this condition usually causes sore throat and cervical adenitis. Presence of gonococcus in conjunctiva can occur at any age and leads to a severe, acute, purulent conjunctivitis. Newborns may acquire a gonococcal opthalmia neonatorum from infected mothers during delivery, which commonly leads to blindness.

Prolonged, untreated gonorrhea can be a cause of Pelvic Inflammatory Disease (PID) among 10 to 20% of women (Holmes *et al*., 1999). This condition is a consequence of gonococcal spread along the fallopian tube into the pelvic cavity causing salpingitis, pelvic peritonitis, and abscesses. Symptoms of PID include fever, lower abdominal pain, adnexal tenderness, and leukocytosis (Holmes *et al*., 1999). The complications of PID include infertility and ectopic pregnancy. Local *N. gonorrhoeae* infections as well as PID can lead to Disseminated Gonococcal Infection (DGI), a spread of the bacteria into the bloodstream. Symptoms of bacteremic spread include fever, polyarthritis, pustules, petechial, and maculopapular rash (Ryan and Ray, 2004). Proper diagnosis of DGI is very often difficult because the blood culture are often negative for *N. gonorrhoeae*. This can result in an inappropriate treatment increasing both treatment coast and chance of health complications. PDI most commonly sequel in purulent arthritis, however it may additionally lead to metastatic infections including endocarditis and meningitis, and in rare situations death.

Gonococcal infections participate in enhancement of acquisition as well as transmission of human immunodeficiency virus (HIV) type 1 (Workowski and Levine, 2002). The acute inflammatory response to *N. gonorrhoeae* infection and the following

disruption in mucosal integrity is the underlying cause of this increased susceptibility (Chen *et al*., 2003). Moreover, symptomatic gonorrhea infections are associated with increased expression of HIV-1 RNA in the genital tract which results in a two- to five-fold increased rate of virus transmission from men to women (Cohen *et al*., 1997; Ghys *et al*., 1997; Moss *et al*., 1995; Fleming and Wasserheit, 1999).

Treatment and prevention of gonorrhea

In the absence of a protective vaccine, the spread of gonorrhea is contained by blockage of direct mucosal contact with condoms, proper diagnosis, epidemiological surveillance, and mainly by the use of the antimicrobial agents (Ryan and Ray, 2004; Unemo *et al*., 2016). Initially *N. gonorrhoeae* infections were easily treated with sulfonamides and penicillin (Kampmeier, 1983; Mahoney *et al*., 1943; Sternberg and Turner, 1944). However, because of the natural competence for DNA transformation, this pathogen throughout 70-80 years was able to rapidly evolve resistance to virtually all clinically used compounds. Gonococcus either acquired or developed the majority of known antimicrobial resistance mechanisms, which includes enzymatic destruction/modification of the drug, target modification/protection, and decreased influx or increased efflux of the antimicrobials (Unemo and Shafer, 2014).

The problem of constant emerging antimicrobial resistance, in particular to extended-spectrum cephalosporins (ESC), among *N. gonorrhoeae* isolates; has been recognized as a great concern in public health communities, scientific societies, and media internationally (Unemo and Shafer, 2014). In 2012 the Centers for Disease Control and Prevention (CDC) certified *N. gonorrhoeae* with the “Superbug” status (Groopman, 2012). The rapid acquisition of antibiotic resistance by gonococcus has caused monotherapies to be excluded from clinical use. Currently, the only treatment for gonorrhea recommended by the CDC in 2015 is dual therapy with azithromycin (1000 mg) taken orally and either intramuscular injection of ceftriaxone (250 mg) or oral cefixime (400 mg) (World Health Organization, 2016). However, this dual antimicrobial regiment is believed not to be an effective long-term solution (Unemo *et al*., 2016). Decreased susceptibility to ceftriaxone has been increasingly reported worldwide and high-level azithromycin resistant strains have already been isolated

from various parts of the globe (reviewed by Unemo *et al*., 2016). Moreover, the first failure of the recommended dual antibiotic treatment has been reported for a male patient with pharyngeal gonorrhea in the UK, who was infected in Japan (Golparian *et al*., 2016). The recommended dual treatment is not affordable in many low-resource settings. Because of the dire possibility of untreatable gonorrhea, the WHO and CDC emphasize the urgent need for the development of new cost-effective antimicrobial agents with novel modes of action or other therapeutic compounds for monotherapy, as well as formulation of new dual therapy strategies (World Health Organization, 2012; Centers for Disease Control and Prevention, 2012).

Two new dual antibiotic regiments have been evaluated for treatment of gonorrhea. Gentamicin (240 mg×1 intramuscularly) combined with azithromycin (2 g×1 orally), and gemifloxacin with (320 mg×1 orally) plus azithromycin (2 g×1 orally) have been shown to have a cure rate of 100% and 99.5%, respectively (Kirkcaldy et al., 2014). However, nausea and vomiting are the common side effects, causing a decrease in total absorbed antimicrobials (3.3% and 7.7% of patients, respectively). These two options are being considered as alternatives for failures of the currently recommended treatment and for people with allergies to extended-spectrum cephalosporins. Derivatives and analogues of known antibiotics are being actively pursued for their activity against *N. gonorrhoeae* (reviewed by Unemo *et al*., 2016). Solithromycin, a macrolide derivative, is currently in a multi-center, open-label, randomized Phase 3 clinical trial (www.clinicaltrials.gov). However, gonococcal isolates with high levels of resistance to azithromycin also have decreased susceptibility to solithromycin (Golparian *et al*., 2012). Several compounds with novel mechanisms of action have been described to possess *in vitro* activity against *N. gonorrhoeae* (reviewed by Unemo *et al*., 2016). Most advanced in development is a novel bacterial topoisomerase inhibitor, (different target than fluoroquinolones) spiropyrimidinetrione ETX0914 (AZD0914) which currently is the subject of a multi-center, open-label, randomized Phase 2 clinical trial (Jacobsson *et al*., 2014; Huband *et al*., 2015; Unemo *et al*., 2015; Alm *et al*., 2015).

The need for new antimicrobial agents for treatment of *N. gonorrhoeae* infections is undeniable. However, the speed of acquiring resistance suggests that

antibiotics will never be a single permanent pharmacological solution for gonorrhea. According to the simulation models, development of a protective vaccine may be the best intervention for limiting the spread of gonorrhea (Craig *et al*., 2015). It has been estimated that the burden of gonococcal infections would be reduced by 90% after either 20 years or 7.5 years of administration, with a vaccine possessing 50% or 100% efficacy and providing 20 years of protection, respectively. A vaccine of short duration (7.5 years) and 100% efficacy would also have a similar impact of a 90% decrease in gonorrhea prevalence during 20 years of usage. Moreover, a vaccine with a protection rate of only 20% is predicted to have a significant impact of a 40% reduction in gonorrhea prevalence. Furthermore, this simulation suggests that vaccination of only 50% of 13-year-olds (the sex of the individuals was found not to be an important factor) would reduce gonococcal infection frequency by 90% after approximately 17 years (only ~five years longer in comparison to a vaccination campaign including 100% of 13-year-olds). Targeting 75% of high-risk "core group" members (assumed to be 5% of the overall population) would achieve a comparable population-level output as vaccination of 50% of all 13-year-olds. Mathematical simulations performed by Craig *et al*., 2015 assumed heterosexuality of the entire population. Because men who have sex with men (MSM) tend to display an increased prevalence of gonorrhea (Mayer, 2011; Roberts-Witteveen *et al*., 2014), the authors speculated that the vaccine would have an even greater impact on these communities.

Research attempts for developing a gonorrhea vaccine have been ongoing for decades (reviewed by Jerse *et al*., 2014). One of the major recognized obstacles for progress in this field is the high phase and antigenic variation of the targeted gonococcal surface antigens. So far only two potential vaccines have entered clinical trials. The first vaccine utilized killed whole cells of *N. gonorrhoeae* and was carried in a population of Inuit in northern Canada (Greenberg *et al*., 1974; Greenberg, 1975). The vaccination induced an antibody response in 90% of the test subjects; however, it did not provide protection from the infection. The second potential vaccine was composed of purified pilin protein that elicited a broad antibody response in the serum and provided protection from urethral infection with the parental strain (Brinton *et al*., 1982). However, follow up studies using infection with a heterologous strain showed

no protective properties of this single-antigen vaccine (Tramont and Boslego, 1985). The pilin vaccine was further tested in a large-scale trial utilizing US military personnel stationed in Korea, unfortunately resulting in the same lack of protection (Boslego *et al*., 1991). Since that time, research on gonococcal vaccines has been limited. This discontinuance is mainly a consequence of the mentioned gonococcal antigen variability and heterogeneity, incomplete knowledge regarding factors that influence protective immune response against *N. gonorrhoeae*, and lack of an adequate animal model for studying this bacterium (Levine *et al*., 2004; Genco and Wetzler, 2010; Liu *et al*., 2011). Recent advancements in the development of a small model for *N. gonorrhoeae* infection have opened new possibilities for testing potential vaccine antigens. The current model for female infection uses female mice treated with 17-β-estradiol during the diestrus stage of the estrus cycle and an antibiotic cocktail (streptomycin sulfate, vancomycin HCl, trimethoprim sulfate) for prevention of commensal vaginal bacteria overgrowth (Jerse, 1999). Bacteria are introduced intra-vaginally and can be recovered 12.2 days post-inoculation. Furthermore, new transgenic mice expressing human CEACAM proteins have been developed for better mimicking of the human host environment (Gu *et al*., 2010). Although availability of the animal models can greatly facilitate the vaccine development, the process still remains challenging due to numerous host restrictions (reviewed by Jerse *et al*., 2014) and identification of suitable, conserved, and stable antigens is needed.

Development of a successful vaccine against *N. meningitidis* serogroup B has been a challenge for a long time. In contrast to capsules of serogroup A and C, which are formulated into protective vaccines, the capsule of meningococcal serogroup B is identical to the human central nervous system (Maiden *et al*., 2008; Butler, 2010). Because of the cross-reactive anti-CNS immune response, the development of MenB vaccine was focused on non-capsular targets. Similar to *N. gonorrhoeae*, many *N. meningitidis* proteins are known to undergo phase and/or antigenic variation, making them difficult targets. Initially, almost 600 candidates were selected through a reverse-vaccinology approach, a method pioneered by Rino Rappuoli that identifies plausible vaccine antigens using different genomic and proteomic methodologies (reviewed by Serruto *et al*., 2012). Out of these candidates, 350 recombinant proteins were

successfully expressed in *Escherichia coli*, validated for their surface availability, and antibodies against 28 of them showed bactericidal properties against *N. meningitidis* serogroup B *in vitro*. The identified Neisserial Heparin-Binding Antigen (NHBA), factor H-binding protein (fHbp), and Neisserial adhesion protein (NadA) have been included in the successful 4CMenB vaccine (Seib *et al*., 2012; Delany *et al*., 2013; Jerse *et al*., 2014). Currently, only about 12 gonococcal antigens are being pursued as vaccine candidates (reviewed by Jerse *et al*., 2014; Edwards *et al*., 2016) (Table 1.1). The history behind the success of the 4CMenB vaccine development clearly suggests that a much larger pool of antigens should be taken under investigation in order to generate a vaccine against gonorrhoeae.

Proteomic mining of cell envelopes and naturally released membrane vesicles for identification of novel targets for development of anti-gonorrhea interventions

In response to the threat of potentially untreatable *N. gonorrhoeae* infections, studies involving high-throughput quantitative proteomic mining of gonococcal cell envelopes and naturally released membrane vesicles were recently employed by our laboratory for the purpose of identifying novel targets for development of anti-gonorrhea compounds and vaccines (Zielke *et al*., 2014; Zielke *et al*., 2016).

Composition and properties of bacterial cell envelopes are crucial factors affecting the physiology, morphogenesis, susceptibility to chemicals, uptake of substances, and interaction with the environment. The cell envelopes of pathogenic bacteria are the primary site of interaction with the host, and thus play a fundamental role for pathogen survival throughout infection. In *N. gonorrhoeae*, a relatively small portion of cell envelope components, with emphasis on pili, opacity (opa) proteins, porins, components of iron uptake, and LOS have been studied with respect to their importance for pathogenesis. Pili are critical for initial attachment to host cells, ascending mucosal surfaces (by twitching motility), defense against neutrophil killing, and natural transformation (Swanson, 1973; Craig *et al*., 2004; Merz *et al*., 2000; Stohl *et al*., 2013). Opa proteins (11 isoforms) mediate adherence to and invasion of human epithelial cells and neutrophils through binding either heparin sulfate proteoglycans (HSPG) (class Opa50) or to the carcinoembryonic antigen-related family of cell

adhesion molecules (CEACAM or CD66) (class Opa52) (Bhat *et al*., 1991; Bos *et al*., 2002; Hauck and Meyer, 2003; Sadarangani *et al*., 2010). Major outer membrane porin protein (PorB) is essential for *N. gonorrhea* viability and forms a voltage-gated pore allowing for nutrient flux, participates in inhibition or induction of apoptotic signaling in eukaryotic cells, confers serum resistance through modulation of classical and alternative pathways, and affects reactive oxygen species formation by innate immune cells (Haines *et al*., 1988; Muller *et al*., 1999; Binnicker *et al*., 2004; Ram *et al*., 1998; Ngampasutadol *et al*., 2008; Lorenzen *et al*., 2000). The TbpAB and LbpAB protein systems are involved in acquisition of iron from human transferrin and lactoferrin, respectively (Anderson *et al*., 1994; Biswas and Sparling, 1995).

LOS is an essential non-protein component of the outer membrane involved in maintaining its integrity as well as adhesion and invasion of host epithelial cells, host immune evasion through variation of sugar moieties' structures, and molecular mimicry, which provides protection from killing by classical and alternative complement pathways (Ghuysen and Hakenbeck, 1994, Griffiss *et al*., 1987; Song *et al*., 2000; Elkins *et al*., 1992; Ram *et al*., 1998).

N. gonorrhoeae, like other Gram-negative bacteria, release outpouchings of the membranes called membrane vesicles (Ellis and Kuehn, 2010). These nano-sized bilayered proteolipids were shown in gonococcus to be involved in biofilm formation, which may be crucial for asymptomatic infection in women, resistance to antimicrobial agents, and suppression of the host immune system (Greiner *et al*., 2005; Steichen *et al*., 2008; Falsetta *et al*., 2011).

Phase and/or antigenic variation of many described virulence determinants makes them difficult therapeutic targets. However, a large body of membrane proteins of *N. gonorrhoeae* have not been characterized with respect to their function and orientation in the cell envelope. Potential importance in physiology and surface topology of these antigens makes them interesting and promising novel molecular targets for developing pharmacological interventions against gonorrhea. Based on findings from the two proteomic studies (Zielke et al., 2014; Zielke et al., 2016) our laboratory is currently pursuing 20 novel cell envelope proteins for development of a

gonococcal vaccine. Among these potential antigens is hypothetical lipoprotein NGO1985.

NGO1985

NGO1985 was found to be ubiquitously expressed in cell envelopes and membrane vesicles of four analyzed temporally and geographically diverse *N. gonorrhoeae* isolates, as well as during exposure to environmental cues relevant to infection sites (Zielke *et al*., 2014; Zielke *et al*., 2016). Initial studies showed that deletion of the *ngo1985* gene resulted in dramatic increase in bacterial susceptibility to membrane perturbing probes and chloramphenicol antibiotic (Zielke *et al*., 2014). Thus, NGO1985 was hypothesized to be involved in maintenance of bacterial cell envelope integrity.

Bioinformatic predictions of NGO1985 domain architecture, using the UniProt search engine (http://www.uniprot.org/), SignalP 4.1 Server (http://www.cbs.dtu.dk /services/SignalP/), and LIPO algorithm (http://services.cbu.uib.no/tools/lipo) revealed the presence of a signal peptide containing putative lipobox motif LALGGCF with a conserved cysteine residue (C23) and two putative membrane-binding BON (Bacterial OsmY and Nodulation) domains. Lipobox motifs are a C-terminal hallmark of signal peptides found in lipoproteins (von Heijne, 1989). The invariant cysteine within this sequence is the site of protein maturation and lipid modification that allows for anchoring to the cell envelope (reviewed by Konovalova and Silhavy, 2015). Following removal of the signal peptide, cysteine becomes the new N-terminal amino acid of the protein, thus it is referred to as the +1 residue. Localization of lipoproteins either to the inner or outer membrane depends on residues following cysteine. Among enterobacteria, most of lipoproteins destined to the inner membrane have aspartic acid in position +2 (Lewenza *et al*., 2006). The +2 rule however, was shown not always to be applicable to bacteria from other families (Schulze and Zuckert, 2006; Narita and Tokuda, 2007). BON domains are usually about 60 amino acids in length, and based on the alignment predictions, contain a conserved glycine with an unknown role and several hydrophobic regions (Yeats and Bateman, 2003). These domains are present in the osmotic-shock-resistance protein OsmY, nodulation proteins, hemolysins,

secretory channels, and several hypothetical proteins. Amino acid organization suggests a binding and structural function, rather than a catalytic role, and it is currently hypothesized that these domains bind phospholipids. Most Proteobacteria encode two BON-containing proteins, although the Burkholderia genus tends to have an extended repertoire.

Function of the *E. coli* homologue of NGO1985, YraP protein, was also assigned to maintenance of cell envelope integrity (Onufryk *et al*., 2005). YraP mutants were selectively sensitive to sodium dodecyl sulfate (SDS), but not to rifampin. Authors suggested a potential role of this protein in OMP folding. Although initial studies of β-barrel assembly machinery (BAM) complex did not detect YraP to be associated with BamA-BamB-BamC-BamD, it was speculated that it could be a loosely associated component of this complex (Wu *et al*., 2005; Onufryk *et al*., 2005). Another homologue of NGO1985 from *N. meningitidis*, GNA2091 - a component of the 4CMenB vaccine - was characterized recently (Bos *et al*., 2014). GNA2091 was proposed to reside on the periplasmic site of the outer membrane. Separate studies provide evidence that this protein can be recognized by the component of the humoral arm of innate immunity, long pentraxin 3 (PTX3), suggesting that some part of the protein could be accessible on the surface of the bacterium (Bottazzi *et al*., 2015). GNA2091 was linked to cell envelope biogenesis, as mutations in the gene encoding this protein led to accumulation of misassembled outer membrane proteins. Similar to YraP, the meningococcal protein was proposed to interact BAM complex, yet this interaction could not be shown and transient interactions have been suggested.

β-barrel Assembly Machinery (BAM) complex

Known constituents of *N. gonorrhoeae* cell envelopes are indispensable for establishing infection and evasion of the human immune system. Proper biogenesis of these components is crucial for their physiological function and maintaining cell envelope integrity. Bacterial outer membrane proteins (OMPs) begin their life in the cytosol as precursors with a leader sequence which is guided by cytoplasmic chaperones and the SecYEG complex through the inner membrane (Driessen and Nouwen, 2008). Upon entering the periplasmic compartment, the leader peptide is

cleaved by a signal peptidase, and OMPs are transported towards the outer membrane with the help of periplasmic chaperones (Sklar *et al*., 2007). The nascent OMPs are being recognized at the inner leaflet of the outer membrane by the β-barrel assembly machinery (BAM) complex which folds them and inserts into the outer membrane (reviewed by Ricci and Silhavy, 2012). In *Escherichia coli*, BAM is composed of five components: an integral β-barrel membrane protein BamA (previously YeaT, Omp85), and four accessory outer membrane lipoproteins: BamB (YfgL), BamC (NlpB), BamD (YfiO), and BamE (SmpA). BamA, initially characterized in *N. meningitidis* and *N. gonorrhoeae* as Omp85 (Manning *et al*., 1998), is an essential protein for bacteria viability and the major component of BAM. It is composed of a C-terminal membrane-bound β-barrel domain and an N-terminal periplasmic domain containing five Polypeptide Translocation Associated (POTRA) motifs (Kim *et al*., 2007). POTRA domains are believed to mediate OMP binding and are the site of interactions with other BAM components in the periplasmic space (Gatzeva-Topalova *et al*., 2008). BamB is a non-essential protein required for the folding of a subset of OMPs including porins OmpF, LamB, OmpT (Malinverni *et al*., 2006). Together, BamB and BamD were shown to be crucial for BamA biogenesis (Misra *et al*., 2015). BamC is another non-essential component of BAM, important for membrane integrity; yet its function in protein folding still remains unclear (Wu *et al*., 2005). Studies have shown that two helix-grip domains of BamC are surface exposed, and it was suggested that they can provide, in addition to POTRA domains, interaction with the BamA surface (Webb *et al*., 2012). BamD is the last part of the BAM complex essential for bacterial viability. It is important for BamA stability as well as mediating and facilitating interaction of BamC and BamE with BamA (Malinverni *et al*., 2006, Kim *et al*., 2007). Additionally, BamD has been shown recently to interact directly with substrate OMPs through recognition of consensus sequences (Hagan *et al*., 2015). The function of BamE is not exactly clear; however, mutations in this protein result in moderate OMP folding defects and increased susceptibility to membrane perturbing agents (Sklar *et al*., 2007). BamE has been shown to modulate conformational changes of BamA and is required for surface exposed lipoprotein RcsF/OMP complex formation (Rigel *et al*., 2012; Konovalova *et al*., 2016).

Although the BAM complex is conserved among proteobacteria, organizational differences have been documented among species. Bioinformatic analysis indicate that BamC is absent in α-proteobacteria, while δ-, and ε-proteobacteria contain only homologues of BamA and BamD (Anwari *et al*., 2012). Additionally, the α-proteobacterial lineage has uniquely evolved an additional component of BAM, BamF (Anwari *et al*., 2012). This suggests that the presence of BamB, BamC, and BamE is unique to β- and γ-proteobacteria. Differences in BAM among these bacteria have also been noted. For example, the *Burkholderia* genus encodes two copies of BamC while *Neisseria* genomes do not encode a BamB homologue (Anwari *et al*., 2012; Volokhina *et al*., 2009). Moreover, the BAM complex of *N. meningitidis* was shown to include the RmpM protein and authors of this work speculated the possible existence of other potential components (Volokhina *et al*., 2009).

Nucleotide-activated-glycero-manno-heptoses and GmhA

Sedoheptulose-7-phosphate isomerases are important for bacterial physiology, and therefore it has been suggested that these enzymes are potential targets for the development of new classes of antimicrobial agents (Taylor *et al*., 2008; Umamaheswari *et al*., 2010; Harmer, 2010).

Synthesis of LPS/LOS is a complex, multifactorial, and discontinuous process involving synthesis and assembly of multiple distant components. The core oligosaccharide can be structurally divided into the inner and outercore. The outer core is typically composed of hexoses and hexosamines, while the inner core consists of two residues of 3-deoxy-D-*manno*-octulosonic acid (KDO) and two or three molecules of L-*glycero*-D-*manno*-heptoses (L,D-heptose) (Raetz and Whitfield, 2002). Heptose residues have been shown to be crucial for LPS/LOS physiological functions. Phosphate groups of heptoses are involved in ionic interaction with the outer membrane and divalent cations, which provide a barrier for hydrophobic compounds like dyes, antibiotics, and detergents (Nikaido, 1994; Nikaido & Vaara, 1985). In *E. coli*, mutations leading to formation of heptose-less inner core oligosaccharides results in hypersensitivity to novobiocin, detergents, and bile salts; as well as defects in plasmid F conjugation and bacteriophage P1 transduction (Tamaki *et al*., 1971; Havekes *et al*.,

1976; Sherburne and Taylor, 1997). In some bacteria L,D-heptose were found in the outer core of LPS (Melaugh *et al*., 1992; Holst *et al*., 1991). Moreover, L-D-heptoses are used for glycosylation of capsular polysaccharides and serine modification of certain autotransporters, which play an important role in pathogenesis (Valvano *et al*., 2002; Lu *et al*., 2015). Recently it was shown that in *N. gonorrhoeae*, free L-D-heptoses released to the extracellular milieu elicit an innate immune response and stimulate expression of Human Immunodeficiency Virus (Malott *et al*., 2013). Another form of heptoses, the D-glycero-D-manno-heptoses (D,D-heptose), is required for decoration of flagella and capsular polysaccharides, and these are part of S-layer glycan in some Gram-positive bacteria (Kosma *et al*., 1995; Wugenditsch *et al*., 1999; Eidels and Osborn, 1974).

The first step in the biosynthesis of ADP- and GDP-activated L,D- and D,D-heptoses, respectively, is catalyzed by the sedoheptulose-7-phosphate isomerase GmhA (previously annotated as TrfA and LpcA) (Havekes *et al*., 1986; Brooke and Valvano, 1996). This enzyme is conserved among Gram-negative and some Gram-positive bacteria, and is involved in isomerization of D-sedoheptulose-7-phosphate into D-*glycero*-α,β-D-*manno*-heptose 7-phosphate. Mutations of genes encoding GmhA among different bacteria have resulted in production of LPS/LOS molecules composed of only lipid A and KDO residues, which leads to increased susceptibility to membrane perturbing agents, defects in biofilm formation, and decreased virulence (Bauer *et al*., 1998; Brooke and Valvano, 1996; Darby *et al*., 2005; Aballay *et al*., 2003).

Scope of Dissertation

Identification, characterization, and elucidation of the physiological function of new *N. gonorrhoeae* proteins is crucial for facilitating the development of new therapeutic interventions. Cell envelopes are the site of interaction between the pathogen and its host, and therefore play a crucial role in bacterial fitness. Many described gonococcal cell envelope components are indispensable for establishing infection and persistence in the human body. The objectives of this dissertation work is to, 1) gain insight into the implications of the GmhA homologue of *N. gonorrhoeae* in LOS synthesis and other physiological processes, 2) determine NGO1985

orientation within the cell envelope and its role in maintaining its integrity, 3) identify amino acid and protein motifs that are important for the proper function of NGO1985. These are some of the important aspects that the work presented in the following chapters aims to address. Chapter 2 focuses on characterization of the GmhA homologue as a potential drug target, and provides understanding of its impact on the physiology of *N. gonorrhoeae*. In chapter 3, we study NGO1985 for the purpose of validating it as potential vaccine development candidate and investigate its function in cell envelope homeostasis. Finally, chapter 4 is an analysis of amino acid components of NGO1985 and their implied effect on the protein function.

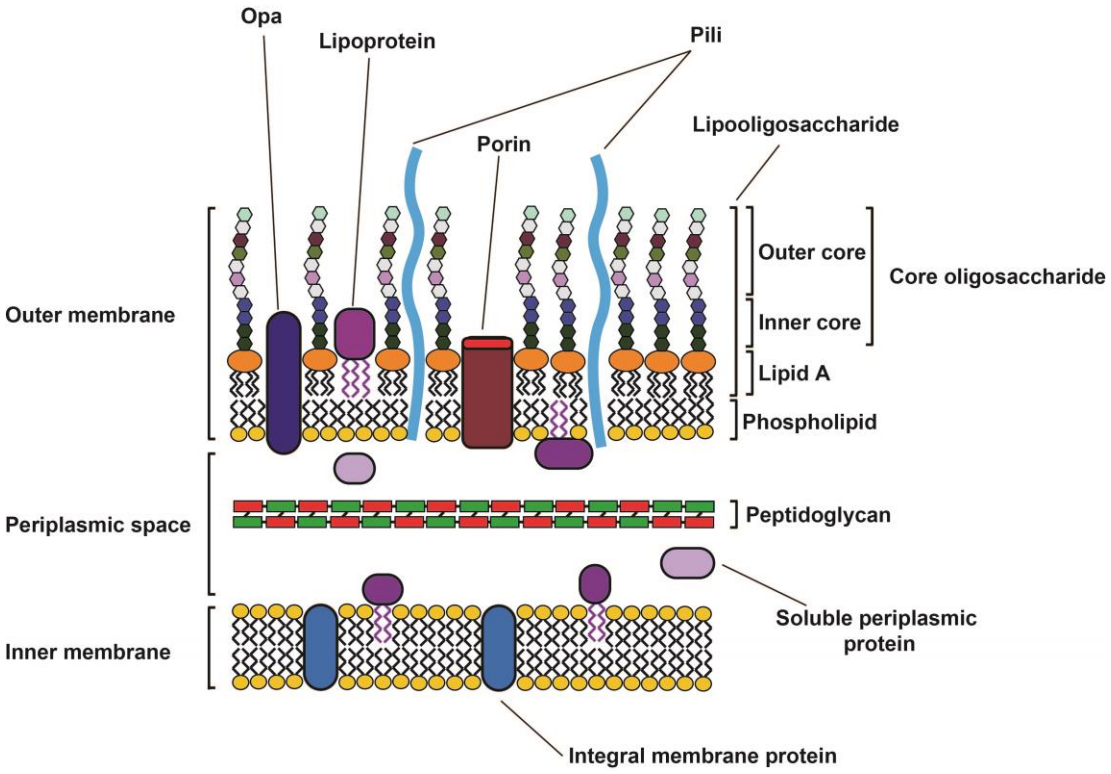

Figure 1.1. Cell envelope of *N. gonorrhoeae*.

Table 1.1. Potential gonococcal vaccine antigens

Antigen	Function	Expression	Variability	Immunogenicity	Reference
OpcA	Adherence, invasion	Stable	Conserved	Bactericidal antibodies	Zhu *et al*., 2003
OmpA	Adherence, invasion	Transcriptio-nal regulation	N/A	Bactericidal antibodies	Serino *et al*., 2007
PilC	Pilus-associated adhesion	Phase variable	Conserved and variable regions	N/A	Rahman *et al*., 1997; Rudel *et al*., 1995
PilQ	Channel formation for pilus extrusion	Stable	Conserved C-terminus	Surface-binding and bactericidal antibodies	Haghi *et al*., 2012
Opa	Adherence, invasion	Phase variable	Variable	Bactericidal antibodies	Callaghan *et al*., 2011; de Jonge *et al*., 2004; Cole and Jerse, 2009
AniA	Nitrate reductase	Induced by nitrate and anoxia	Conserved, variable glycosylati on	Antibodies block function of AniA	Shewell *et al*., 2013
TdfJ	Zinc transporter	Regulated	Conserved	Bactericidal antibodies (when raised against meningococcal homologue)	Cornelissen and Hollander, 2007; Stork *et al*., 2010
PorB	Nutrient acquisition, antibiotic and serum resistance, invasion	Stable	Variable	Bactericidal antibodies; antibodies block use of transferrin iron source	Garvin *et al*., 2010 IPNC Abstract #P235
Lst	Serum resistance	Constitutive	Conserved	Antisera reduces sialylation of LOS	Shell *et al*., 2002
TbpB, TbpA	Transferrin receptor	Induced during iron depletion	TbpA, conserved; TbpB variable	Bactericidal antibodies when transferrin is a sole iron source	Hobbs *et al*., 2011; Price *et al*., 2007
2C7 epitope	Monoclonal antibody to (LOS)	Phase variable	Common epitope of LOS	Antisera to peptide mimic are bactericidal	Gulati *et al*., 1966; Ngampasutadol *et al*., 2006

Chapter 2

Functional and structural studies on the *Neisseria gonorrhoeae* GmhA, the first enzyme in the *glycero-manno*-heptose biosynthesis pathways, demonstrate a critical role in lipooligosaccharide synthesis and gonococcal viability

Igor H. Wierzbicki, Ryszard A. Zielke, Konstantin V. Korotkov, and Aleksandra E. Sikora

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Abstract

Sedoheptulose-7-phosphate isomerase, GmhA, is the first enzyme in the biosynthesis of nucleotide-activated-*glycero*-*manno*-heptoses and an attractive, yet underexploited, target for development of broad-spectrum antibiotics. We demonstrated that GmhA homologues in *Neisseria gonorrhoeae* and *N. meningitidis* (hereafter called $GmhA_{GC}$ and $GmhA_{NM}$, respectively) are interchangeable proteins essential for lipooligosaccharide (LOS) synthesis, and their depletion had adverse effects on neisserial viability. In contrast, the *Escherichia coli* orthologue failed to complement $GmhA_{GC}$ depletion. Further, we showed that $GmhA_{GC}$ is a cytoplasmic enzyme with induced expression at mid-logarithmic phase, upon iron deprivation and anaerobiosis, and conserved in contemporary gonococcal clinical isolates including the 2016 WHO reference strains. The untagged $GmhA_{GC}$ crystalized as a tetramer in the closed conformation with four zinc ions in the active site, supporting that this is most likely the catalytically active conformation of the enzyme. Finally, site directed mutagenesis studies showed that the active site residues E65 and H183 were important for LOS synthesis but not for $GmhA_{GC}$ function in bacterial viability. Our studies bring insights into the importance and mechanism of action of GmhA and may ultimately facilitate targeting the enzyme with small molecule inhibitors.

Introduction

The World Health Organization (WHO) and the Centers for Disease Control and Prevention (CDC) emphasized an urgent need for the development of antimicrobials with novel modes of action against antibiotic resistant threats with severe consequences for public health, including infections caused by drug-resistant *Neisseria gonorrhoeae* (World Health Organization, 2012; World Health Organization, 2015; Centers for Disease Control and Prevention, 2013b, Centers for Disease Control and Prevention, 2013a). Gonorrhea is highly prevalent throughout the world, and if untreated or inadequately treated, often leads to serious repercussions on reproductive health including ectopic pregnancy, pelvic inflammatory disease, and infertility (Centers for Disease Control and Prevention, 2013b; World Health Organization, 2011; Low *et al*., 2014). In the absence of a protective gonorrhea vaccine, antibiotics remain the sole therapeutic intervention. However, the well-documented ability of gonococci to acquire antibiotic resistance continues to threaten available treatment options (Unemo and Shafer, 2014; Unemo, 2015). To meet the needs raised by WHO and CDC our laboratory focuses on identification and validation of new molecular targets for the development of gonorrhea treatments (Zielke *et al*., 2014; Zielke *et al*., 2015; Bonventre *et al*., 2016; Zielke *et al*., 2016).

Targeting the first enzymes in the nucleotide-activated-*glycero*-*manno*-heptose pathways is a relatively unexplored field, although it appears to be an alternative approach to the discovery of broad-spectrum antibacterial drugs (Tamaki *et al*., 1971; Bauer *et al*., 1998; Darby *et al*., 2005; Valvano *et al*., 2002). Nucleotide-activated-*glycero*-*manno*-heptoses, while absent in eukaryotic cells, are widely present in bacteria and are crucial components of the lipopolysaccharides (LPS), lipooligosaccharides (LOS), capsules, O antigens, and glycan moieties of bacterial cell surface (S-layer) glycoproteins (Valvano *et al*., 2002). In particular, one such potential drug target of significant interest is sedoheptulose-7-phosphate isomerase, GmhA, annotated previously as TfrA (Havekes *et al*., 1976) and LpcA (Brooke and Valvano, 1996). GmhA is conserved in many Gram-negative and some Gram-positive bacteria and is responsible for catalyzing isomerization of the D-sedoheptulose 7-phosphate into D-glycero-α,β-D-manno-heptose-7-phosphate (Eidels and Osborn, 1974), which is the

first and common step for parallel biosynthetic pathways leading to generation of GDP-D-glycero-α-D-manno-heptose (D,D-heptose) and ADP-L-glycero-β-D-manno-heptose [L,D-heptose; reviewed in Ref: (Valvano *et al*., 2002)]. D,D-heptose is required for glycosylation of flagella and capsular polysaccharides, and for the assembly of disaccharide repeating units (D,D-heptose linked to L-rhamnose) composing the S-layer glycan in the Gram-positive *Eubacterium, Aneurinibacillus thermoaerophilus* DSM 10155 (Kosma *et al*., 1995; Wugeditsch *et al*., 1999; Eidels and Osborn, 1974). L,D-heptose is used for glycosylation of capsular polysaccharides (Valvano *et al*., 2002) and as a primary building block of LPS/LOS core oligosaccharide (Eidels and Osborn, 1971; Brooke and Valvano, 1996). In addition, a large family of bacterial autotransporter heptosyltransferases (BAHTs) utilizes L,D-heptose as a sugar donor to modify serine residues on their substrate autotransporters, which has a significant impact on the virulence of Gram-negative pathogens (Lu *et al*., 2015). The L,D-heptose is synthesized in sequential reactions catalyzed in order by GmhA-HldE (HldA)-GmhB-HldE (HldC)-HldD [reviewed in Refs: (Valvano *et al*., 2002; Raetz and Whitfield, 2002)]. Usually one or more L,D-heptose molecules and two 2-keto-3-deoxy-D-manno-oct-2-ulosonic acid (KDO) residues form the inner portion (lipid A proximal) of the LPS/LOS core oligosaccharide, which is typically more conserved than the structurally diverse outer core (Raetz and Whitfield, 2002). Similarly, gonococcal LOS contain two basal heptose molecules, designated Hep I and Hep II, forming elongation centers α, β, and χ (Gibson *et al*., 1989; John *et al*., 1991; Yamasaki *et al*., 1991). The individual chains can be decorated with structures that mimic human carbohydrate epitopes [α chain linked to Hep I; (Apicella and Mandrell, 1989)]; a single glucose, lactose, or glucose with additional sugars [β chain extending from Hep II; (Gibson *et al*., 1989; Yamasaki *et al*., 1994)]; and GlcNac, GlcNac acetate, or occasionally galactose [χ chain; (McLeod Griffiss *et al*., 2000)]. In addition, phosphate or phosphoethanolamine groups may be attached to the heptose residues (Raetz and Whitfield, 2002; Preston *et al*., 1996). The phosphoric residues participate in the ionic interactions between LPS/LOS and outer membrane proteins, as well as divalent cations, providing a barrier against entry of detergents, antibiotics, and hydrophobic compounds (Raetz and Whitfield, 2002; Preston *et al*., 1996). The *N.*

gonorrhoeae heptose-monophosphate was recently linked with the clinical and epidemiological synergy of gonorrhea and HIV (Malott *et al*., 2013). At the molecular level, this interplay involves the unique ability of gonococci to efficiently liberate phosphorylated L,D-heptose into the extracellular milieu, which elicits an immune response and induces HIV-1 expression and viral production in cluster of differentiation 4-positive ($CD4^+$) T-cells (Malott *et al*., 2013).

Mutations in genes encoding GmhA in different bacterial species examined to date resulted in pleiotropic effects including production of truncated LPS/LOS composed of lipid A and KDO residues, increased susceptibility to antibiotics and detergents, impaired biofilm formation, and attenuated virulence (Bauer *et al*., 1998; Brooke and Valvano, 1996; Darby *et al.,* 2005; Aballay *et al*., 2003). In addition, lack of HldA, which acts immediately downstream from GmhA in the L,D-heptose biosynthetic pathway, rendered gonococci unable to induce HIV-1 expression (Malott *et al*., 2013). Therefore, we propose GmhA in *N. gonorrhoeae*, $GmhA_{GC}$, as a molecular target for the development of new antigonococcal drugs. Herein we performed characterization of $GmhA_{GC}$ at the molecular, functional, and structural levels to facilitate the future targeting of this enzyme with small molecule inhibitors.

Materials and methods

Bacterial strains and growth conditions. Strains of bacteria used in this study are listed in Table 2.1. *Neisseria gonorrhoeae* and *N. meningitidis* were cultured either on gonococcal base solid medium (GCB, Difco), or in gonococcal base liquid (GCBL) medium supplemented with Kellogg's supplement I and II in ratios 1:100 and 1:1000, respectively (Spence *et al*., 2008). GCBL was additionally supplemented with sodium bicarbonate at a final concentration of 0.042%. *In vitro* host relevant growth conditions (iron deprivation, presence of normal human serum, anoxia) were procured as described previously (Zielke *et al*., 2016). *Neisseria* were cultured on solid media for 18-22 h at 37 °C in the presence of 5% atmospheric CO_2. For *N. gonorrhoeae*, piliated or non-piliated variants were passaged onto GCB and incubated for an additional 18-22 h. Colonies with piliated morphology were used for DNA transformation, while non-piliated variants were used in all other experiments. *Escherichia coli* strains were grown either on Luria-Bertani agar (LBA, Difco) or cultured in Luria-Bertani broth (LB, Difco) at 37 °C.

Antibiotics were used on selected bacteria in the following concentrations: for *N. gonorrhoeae*: kanamycin 40 µg/mL, erythromycin 0.5 µg/mL; for *N. meningitidis*: kanamycin 80 µg/mL, erythromycin 2 µg/mL; for *E. coli*: kanamycin 50 µg/mL, erythromycin 250 µg/mL.

Genetic manipulations, site-directed mutagenesis and transcomplementation. Plasmids and primers used in this study are listed in Supplemental Table S2.1. Oligonucleotides were designed based on the genomic sequence of *N. gonorrhoeae* FA1090 (NC_002946), *N. meningitidis* MC58 (NC_003112), and *E. coli* BL21(DE3) (NC_012892) using SnapGene software version 2.8 (GSL Biotech LLC) and synthesized by Integrated DNA Technologies. Genomic DNA of gonococcal isolates, *N. meningitidis* MC58, and *E. coli* BL21(DE3) was isolated with the Wizard Genomic DNA Purification Kit (Promega). PCR products and plasmid DNA were purified using QIAprep Spin Miniprep Kit (QIAGEN). PCR reactions were performed using chromosomal or plasmid DNA as template, appropriate oligonucleotides, and Q5® High-Fidelity DNA Polymerase (NEB). *E. coli* MC1061 was used as the host during the molecular cloning and site-directed mutagenesis

procedures. All obtained constructs were verified by Sanger Sequencing at the Center for Genomic Research and Biocomputing at Oregon State University. Transformation of *N. gonorrhoeae* and *N. meningitidis* was performed as described previously (Zielke *et al*., 2014, Alexander *et al*., 2004).

The recombinant GmhAGC (rGmhAGC) containing N-terminal-6×His-tag followed by the Tobacco Etch Virus (TEV) protease recognition site was obtained by amplifying the DNA region of the *ngo1986* gene lacking a stop codon with primers rNGO1986-F and rNGO1986-R and cloning the obtained PCR product (615 bp) into NcoI/HindIII sites of pRSF-NT (Supplemental Table S2.1).

The conditional GmhAGC mutant, FA1090 Δ$gmhA_{GC}$/$Plac::gmhA_{GC}$, was constructed using the strategy described by Zielke *et al*., 2016. An additional copy of *ngo1986* was placed under the control of the isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible promoter, P_{lac}, within an intergenic region located between *lctP* and *aspC* in the FA1090 chromosome (Mehr *et al*., 2000), followed by an in-frame replacement of *ngo1986* in its native chromosomal locus with the nonpolar kanamycin resistance cassette. Specifically, *ngo1986* containing its native ribosomal binding site (RBS) was amplified with primers NGO1986-RBS-F and NGO1986-RBS-R. The resulting 628 bp PCR product was digested with FseI and inserted into ScaI/FseI treated pGCC4, yielding pGCC4-GmhAGC. After transformation of FA1090 with pGCC4-$GmhA_{GC}$, gonococci were selected on GCB with 0.5 µg/mL erythromycin and verified by PCR with primers pGCC4-Ver-F and pGCC4-Ver-R.

The constructs for deletion of $gmhA_{GC}$ were obtained by amplification of the 552 bp upstream DNA region and 571 bp downstream from *ngo1986* with primers NGO1986-UP-F/NGO1986-UP-R and NGO1986-Down-F/NGO1986-Down-R, respectively. The upstream fragment was digested with EcoRI/KpnI and cloned into similarly cleaved pUC18K, yielding pUC18K-GmhAGC-Up. Next, the downstream fragment was inserted into the BamHI/HindIII-cleaved pUC18K-GmhAGC-Up. The resulting pUC18K-GmhAGC was linearized with HindIII and used to transform FA1090 carrying the second copy of *gmhA* on the chromosome, FA1090 $P_{lac}::gmhA_{GC}$. Clones were selected on solid medium supplemented with kanamycin and 0.1 mM IPTG, and verified by PCR with NGO1986-Ver-F and NGO1986-Ver-R primers.

Subsequently, selected gonococci were further verified by immunoblotting analyses using anti-GmhAGC antisera.

To generate $GmhA_{GC}$E65A and $GmhA_{GC}$H183A, the template for site-directed mutagenesis (pUC18-GmhAGC) was constructed by amplifying *ngo1986* with its native RBS using primers NGO1986-RBS-F and NGO1986-RBS-R and cloning into SmaI-cleaved pUC18. Site-directed mutagenesis was performed using the template; primer pairs E65A-F/E65A-R or H183A-F/H183A-R, respectively, and Q5 Site-Directed Mutagenesis Kit (NEB), according to the manufacturer's manual. Subsequently, the mutated variants of $GmhA_{GC}$ were amplified with NGO1986-RBS-F and NGO1986-RBS-R, cleaved with FseI, cloned into ScaI/FseI digested pGCC4, introduced into the FA1090 chromosome, the native *ngo1986* was deleted as described above.

For transcomplementation studies, GmhA homologues from *N. meningitidis* MC58 (nmb2090) and *E. coli* BL21(DE3) (ECBD_3400) were amplified with primer pairs NGO1986-RBS-F/NGO1986-RBS-R and ECBD3400-RBS-F, respectively. The resulting PCR products were digested with FseI and introduced into ScaI/FseI-treated pGCC4. Introduction into the FA1090 chromosome and deletion of GmhA were performed as outlined above.

The cytoplasmic marker control for subfractionation experiments, the *zwf* gene (*ngo0715*) encoding glucose-6-phosphate-1-dehydrogenase was amplified using primers rZwf-F and rZwf-R. The PCR product was cleaved with NcoI and HindIII and cloned into similarly digested pRSF-NT to obtain plasmid pRSF-NT-Zwf.

Proteome subfractionation procedures. Colonies of N. gonorrhoeae FA1090 were collected from GCB, suspended in 500 mL of GCBL to OD_{600} of 0.1 and cultured with aeration at 37 °C until OD_{600} of ~0.8. Bacterial cells were separated from the suspension by centrifugation (10 min, 6,000 × g, 4 °C). The crude cell envelopes were purified using a sodium carbonate extraction procedure while naturally released membrane vesicles (MVs) and soluble proteins (SS) were fractionated from culture supernatants by ultracentrifugation as described previously (Zielke *et al*., 2016).

$GmhA_{GC}$ depletion studies. Colonies of FA1090 $\Delta gmhA_{GC}/P_{lac}::gmhA_{GC}$ were collected with a cotton swab from GCB supplemented with 10 µM IPTG and

suspended in GCBL to OD_{600} of 0.1. After two washes in pre-warmed GCBL, bacterial suspension was split and incubated with shaking (220 rpm) with or without IPTG for 3 h at 37 °C. Cultures were back-diluted to OD_{600} of 0.1 in fresh GCBL, as described above, and cultured for additional 6 h. At specific time points indicated in the text, OD_{600} measurements were taken, samples for western blotting and LOS isolation were withdrawn, and cultures were serially diluted followed by plating on GCB for enumeration of colony forming units (CFUs). Experiments were performed on three separate occasions and mean values and SEM are presented.

Isolation of LOS and silver staining. LOS was isolated from *N. gonorrhoeae* and *N. meningitidis* based on the method described previously (Hitchcock and Brown, 1983). Bacteria were either collected from GCB or GCBL, as specified in the text, suspended in 1.5 mL of GCBL to OD_{600} of 0.2 and spun down for 1.5 min at 15,000 × *g*. Pelleted cells were lysed by addition of 50 µL of lysis buffer (2% SDS, 4% β-mercaptoethanol, 10% glycerol, 1M Tris-HCl pH 6.8, and 0.01% bromophenol blue) and incubation at 100 °C for 10 min. Samples were allowed to cool down to room temperature and proteins were digested by addition of 25 µg proteinase K in 10 µL of lysis buffer and incubated for 1 h at 60 °C. Isolated LOS was resolved on 16.5% Mini-PROTEAN® Tris-Tricine Gel (BioRad) and visualized by a silver staining procedure (Tsai and Frasch, 1982).

Fitness assessment. Colonies of different neisserial strains, as indicated in the text, were collected from GCB and reconstituted in GCBL to OD_{600} of 0.1. Bacterial cultures were incubated in the absence of IPTG for 3 h at 37 °C with aeration and subsequently back-diluted to OD_{600} of 0.2, serially diluted, and plated on GCB with or without IPTG for CFUs scoring. Experiments were repeated in three biological replicates and averaged values with corresponding SEM are presented.

Purification of the rGmhAGC and rZwf and production of polyclonal rabbit antibodies. *E. coli* BL21(DE3) strain carrying either pRSF-NT-GmhAGC or pRSF-NT-Zwf was used as heterologous host for overproduction and purification of $rGmhA_{GC}$ and recombinant Zwf (rZwf), respectively. Overnight cultures were back-diluted in 2.0 L of LB supplemented with kanamycin and incubated with aeration at 37 °C until the optical density (OD_{600}) reached ~0.5. Overproduction of $rGmhA_{GC}$ and

rZwf was induced with 0.1 and 1 mM IPTG, respectively, and cultures were incubated for additional 3 h at 37 °C. Cells were harvested by centrifugation (6,000 × *g*, 10 min, 4 °C). Pelleted bacteria carrying pRSF-NT-GmhAGC were suspended in lysis buffer (20 mM Tris-HCl pH 7.0, 1 M NaCl, 10 mM imidazole, 5% glycerol) supplemented with a Pierce Protease Inhibitor Mini Tablet (Thermo Scientific) and lysed by passaging six times through a French pressure cell at 12,000 psi. Cell debris and unbroken cells were separated from soluble protein fraction by centrifugation at 16,000 × g for 30 min at 4 °C. The obtained supernatant was passed through 0.22 µm membrane filter (VWR International) and applied onto Bio-Scale MiniProfinity IMAC cartridges (Bio-Rad) on the NGC Scout Chromatography system (Bio-Rad). Loosely bound proteins were removed with 10 column volumes of wash buffer (20 mM Tris pH 8.0, 500 mM NaCl, 40 mM imidazole) and elutions were conducted with a 40-250 mM imidazole gradient. Fractions containing rGmhA$_{GC}$ were combined and a PD-10 column (GE Healthcare) was used to exchange the buffer to 20 mM HEPES pH 7.5, 100 mM NaCl, 5% glycerol. Subsequently, the N-terminal-6×His-tag of the rGmhA$_{GC}$ was removed by TEV protease. Cleavage reaction was prepared by mixing rGmhA$_{GC}$ with TEV in 20:1 (w:w ratio) in 500 µL of cleavage buffer (0.5 M Tris-HCl pH 8.0, 5 mM EDTA), 10 µL of 0.5 M DTT, and 500 µL of Ni-NTA agarose (Qiagen) equilibrated with cleavage buffer. Following overnight incubation at room temperature, cleavage mixture was loaded onto a 5 mL polypropylene column (Thermo Scientific) and supernatant containing 6×His-tag-free rGmhA$_{GC}$ was collected. To remove residual TEV protease that co-eluted with rGmhAGC, the mixture was incubated again with 500 µL of Ni-NTA agarose (Qiagen) equilibrated with cleavage buffer for 1 h with rotation at 4 °C, and rGmhA$_{GC}$ was eluted. The purified rGmhA$_{GC}$ was applied to a PD-10 column (GE Healthcare) for a buffer exchange into 20 mM HEPES pH 7.5, 100 mM NaCl, and 5% glycerol.

Purification of rZwf was accomplished using the same procedures as described above with the following modifications. *E. coli* cells were resuspended in lysis buffer (20 mM Tris-HCl pH 8.0, 10 mM imidazole, 450 mM NaCl). Cells were lysed using French Press and rZwf was purified using 5 mL Bio-Scale Mini Nuvia IMAC Ni-Charged column (Bio-Rad) connected to a NGC Chromatography System (Bio-Rad).

Bound peptides were eluted using elution buffer (20 mM Tris-HCl pH 8.0, 250 mM imidazole, 450 mM NaCl). Fractions containing proteins were pooled, EDTA and DTT were added to final concentrations of 0.5 mM and 1 mM, respectively, and the His-tag was removed by overnight incubation with TEV protease (ratio 1:100 w/w). After cleavage the proteins were separated using Hi Load 16/600 Superdex 75 pg column (GE Healthcare) and buffer containing 20 mM Tris pH8, 150 mM NaCl connected to the NGC Chromatography System. Fractions containing Zwf were pooled together and concentrated using 10 kDa Vivaspin 20 concentrators (GE Healthcare). Glycerol was added to a final concentration of 10% and the protein was aliquoted and stored at -80 °C.

The polyclonal rabbit anti-$GmhA_{GC}$ and anti-Zwf antibodies were generated using 6×His-tag-free r$GmhA_{GC}$ and rZwf, respectively. Standard 13-week antibody production protocols were applied, utilizing four New Zealand White rabbits, animal handling was performed according to the Animal Protocol #1 approved by IACUC, in a certified animal facility (USDA 93-R-283) and the NIH Animal Welfare Assurance Program (#A4182-01) at the Pacific Immunology Corporation. Anti-GmhAGC and anti-Zwf antisera were used at 1:10,000.

Size exclusion chromatography. The NGC Scout Chromatography system (Bio-Rad) employing a HiLoad 16/600 Superdex 75 pg column (GE Healthcare Life Sciences) was used to separate purified r$GmhA_{GC}$ based on the molecular size. Buffer for the chromatography (20 mM Tris-HCl pH 8.0, 500 mM NaCl) was applied at 1 mL/min flow rate. Gel Filtration Standard (BioRad) was used to determine the size of the separated proteins.

Crystallization and structure determination of *N. gonorrhoeae* $GmhA_{GC}$. The screening for initial crystallization conditions was performed using JCSG Core Suites I-IV (Qiagen) (Newman *et al*., 2005). The optimized crystals were grown using 0.1 M Tris-HCl pH 8.5, 0.2 M magnesium chloride, 30% PEG4000. Crystals were transferred to a cryo-protectant solution supplemented with 20% glycerol and flash-frozen in liquid nitrogen. The diffraction data were collected from a single crystal at the beamline 22-ID, Southeast Regional Collaborative Access Team (SER-CAT) at the Advanced Photon Source, Argonne National Laboratory. Data were integrated and

scaled using XDS and XSCALE (Kabsch, 2010). The structure was solved by molecular replacement using Phaser (McCoy *et al*., 2007) and structure of *Pseudomonas aeruginosa* GmhA (PDB 3BJZ) as a search model (Taylor *et al*., 2008). The electron density modification was performed using Parrot (Cowtan, 2010) followed by automated model rebuilding using Buccaneer (Cowtan, 2006). The model was completed by manual rebuilding in Coot (Emsley *et al*., 2010) and was refined using REFMAC5 (Murshudov *et al*., 2011). The structure was validated using Coot and the MolProbity server (Chen *et al*., 2010). The structural figures were generated using PyMol (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC.).

SDS-PAGE and immunoblotting. Whole cell lysates of *Neisseria* strains indicated in the text were either collected from solid media after 22 h of aerobic or 48 h of anaerobic growth, or harvested from liquid media following procedures described (Zielke *et al*., 2014, Zielke *et al*., 2016). *E. coli* cells were collected from LB agar plates after overnight incubation. Samples were normalized based on OD_{600} values or based on total protein concentration, and SDS-PAGE, staining with Coomassie Brilliant Blue G-250, and immunoblotting analyses were performed exactly as described previously (Zielke *et al*., 2014, Zielke *et al*., 2016).

Statistical analysis. GraphPad Prism's build-in *t*-test was used for determination of statistically significant differences between obtained experimental results. A confidence level of 95% was used for all analyses.

Accession numbers. The coordinates and structure factors were deposited to the Protein Data Bank with accession code 5I01.

Results

Chromosomal location and purification of GmhA$_{GC}$. Genes encoding the enzymes of the L,D-heptose biosynthesis pathway are scattered throughout the chromosome in the majority of bacteria, including *N. gonorrhoeae* (Valvano *et al*., 2002). The GmhA homologue in *N. gonorrhoeae* strain FA1090 is encoded by *ngo1986*, which is located between *ngo1985*, coding for an outer membrane lipoprotein (Zielke *et al*., 2014) and *ngo1987* encoding a putative endonuclease (YraN). Comparison of the *gmhA* location using BioCyc Pathway/Genome Database Collection (http://biocyc.org/) showed that this genetic arrangement is conserved among the various deposited *Neisseria* species and isolates (n=70) with only a few exceptions, including *N. bacilliformis* ATCC BAA-1200, *N. sp.* oral taxon 020 F0370, *N. shayeganii* 871, and *N. subflava* NJ9703.

GmhA$_{GC}$ consists of 197 amino acids with residues 37–197 comprising a sugar isomerase domain (SIS) (Bateman, 1999) that is shared between all ketose/aldose isomerases (Golinelli-Pimpaneau, 1989). At the amino acid level, GmhA$_{GC}$ shows 43-57% identity with crystallized orthologous proteins (Table 3) and contains the key conserved residues observed in all GmhA homologues, including three serine residues and a threonine that presumably interacts with the phosphate group of sedoheptulose 7-phosphate or D-glycero-D-manno-heptose 7-phosphate (Harmer, 2010, Valvano *et al*., 2002).

To characterize GmhA$_{GC}$, we first purified recombinant protein with a N-terminal 6×His-tag followed by the Tobacco Etch Virus (TEV) protease cleavage site and prepared untagged protein. Size exclusion chromatography indicated that the native GmhA$_{GC}$ forms tetrameric structures in the solution (Supplemental Fig. S2.1 A). The denatured protein migrated on SDS-PAGE according to the predicted molecular mass of 21.093 kDa (Supplemental Fig. S2.1 B). Untagged GmhA$_{GC}$ was subsequently used in crystallization and to obtain polyclonal rabbit antisera.

***N. gonorrhoeae* deprived of GmhA$_{GC}$ displays defect in LOS synthesis and growth cessation.** The genetic inactivation of *gmhA* in *E. coli*, *Haemophilus influenzae*, *H. ducreyi*, *Salmonella enterica*, and *Yersinia pestis* resulted in altered outer membrane permeability and decreased virulence in animal infection models, however

the mutants did not display drastic growth defects *in vitro* (Bauer *et al*., 1998; Brooke and Valvano, 1996; Darby *et al*., 2005; Aballay *et al*., 2003). Therefore, we were surprised by the failure of our numerous attempts to generate a clean deletion of *ngo1986* in *N. gonorrhoeae* FA1090. As an alternative strategy, an additional copy of $gmhA_{GC}$ was placed under the control of the IPTG inducible P_{lac} promoter and introduced into the chromosome of wild type (wt) FA1090, followed by an allelic replacement of *ngo1986* with the kanamycin resistance cassette. The obtained $\Delta gmhA_{GC}/P_{lac}::gmhA_{GC}$ mutant grew scarcely after passage on solid media without IPTG, whereas abundant colonies were observed in the presence of the inducer (Fig. 2.1 A). The depletion of $GmhA_{GC}$ was corroborated by probing whole cell lysates of these gonococci with anti-$GmhA_{GC}$ antisera (Fig. 2.1 B). Visualization of LOS species by Tricine-SDS-PAGE coupled with silver staining showed, as expected, that bacteria deprived of $GmhA_{GC}$ carried a truncated version of LOS that migrated faster in comparison to the LOS species isolated from wt and $\Delta gmhA_{GC}/P_{lac}::gmhA_{GC}$ grown in the presence of IPTG (Fig. 2.1 B).

Subsequently, time course experiments during standard laboratory growth conditions were conducted to examine the correlation between depletion of $GmhA_{GC}$, LOS formation, and *N. gonorrhoeae* fitness. An initial suspension of non-piliated colonies of $\Delta gmhA_{GC}/P_{lac}::gmhA_{GC}$ collected from solid media supplemented with IPTG was divided and cultured in liquid media in the presence or absence of IPTG [(+) or (-); respectively]. After 3 h, to ensure $GmhA_{GC}$ depletion, the corresponding cultures were diluted to OD_{600} of 0.1 in media with or without IPTG. From this point onward (shown as 0 h in Fig. 2.1 C-E), samples were withdrawn every hour to assess $GmhA_{GC}$ levels and LOS formation (Fig. 2.1 C), culture density (Fig. 2.1 D), and Colony Forming Units (CFUs; Fig. 2.1 E). Immunoblotting with anti-$GmhA_{GC}$ antisera showed that in the absence of IPTG, $GmhA_{GC}$ levels were slightly decreased at 0 h and complete $GmhA_{GC}$ depletion was achieved three hours later (Fig. 2.1 C). Also, it was noted that despite the presence of inducer, $GmhA_{GC}$ had a peculiar expression pattern; decreased amounts of protein in comparison to these observed at 0 h were detected at 1 h and in early log phase (corresponding to 2-3 h data points). Further, increased $GmhA_{GC}$ levels were found in late-log phase (4, 5 h) followed by a decline in stationary phase (6 h; Fig.

2.1 C-E). In contrast, no major differences were noticed in the overall protein profiles and the level of Ng-MIP (Fig. 2.1 C), which is ubiquitously expressed throughout phases of gonococci growth (Zielke *et al*., 2016). As expected based on data presented in Fig. 2.1 B, the wt LOS species diminished over time and were barely detectable upon $GmhA_{GC}$ diminution (Fig. 2.1 C). Finally, analysis of growth rates showed that in media lacking IPTG, bacterial growth terminated concomitant to depletion of $GmhA_{GC}$ (Fig. 2.1 C-E), whereas in the presence of IPTG, the generation time of the $\Delta gmhA_{GC}/P_{lac}::gmhA_{GC}$ mutant was 61.21 min (Fig. 2.1 D-E), a value similar to the parental wt strain (56.5 min; Fig. 2.2 B).

Together, these findings demonstrated that GmhAGC is pivotal for LOS synthesis and optimal growth of N. gonorrhoeae.

GmhAGC subcellular localization and expression patterns. Preliminary results and absence of signal peptide suggested that GmhA of *E. coli* is a cytoplasmic enzyme (Brooke and Valvano, 1996). However, subcellular localization of GmhA and expression patterns were never assessed. To examine the localization of $GmhA_{GC}$, *N. gonorrhoeae* FA1090 was harvested at mid-logarithmic phase of growth and subjected to subfractionation procedures. Equal amounts of extracted proteome fractions including cytoplasm, cell envelopes, naturally released membrane vesicles, and soluble proteins in culture supernatants were separated by SDS-PAGE and probed with polyclonal antibodies (Fig. 2.2 A). $GmhA_{GC}$ was found solely in the cytoplasmic protein fraction, similarly to the cytoplasmic enzyme glucose-6-phosphate 1-dehydrogenase, Zwf. As expected, the GTPase Obg (Obg_{GC}), which primarily associates with 50S ribosomal subunits and partly with the peripheral inner membrane proteome (Zielke *et al*., 2015; Papanastasiou *et al*., 2013), was detected mainly in the cytoplasm and minute amounts were also found in the cell envelope fraction; whereas antisera against the outer membrane protein marker BamA (Zielke *et al*., 2016) cross-reacted with fractions containing cell envelopes and membrane vesicles.

The expression of $GmhA_{GC}$ was subsequently examined in wt FA1090 during routine aerobic growth in liquid media (Fig. 2.2 B-C) and on solid media under conditions that more closely mimic clinical infection, such as iron deprivation, exposure to human serum, and anoxia (Fig. 2.2 D). Antibodies against Ng-MIP, TbpB,

and AniA were used as markers for ubiquitous expression, iron-limiting conditions and anaerobiosis, respectively (Cornelissen, 2008; Zielke *et al*., 2014; Zielke *et al*., 2016). Immunoblotting analyses of whole cell lysates showed that expression of $GmhA_{GC}$ peaked during mid-exponential phase (3 and 4h, Fig. 2.2 B-C). Increased $GmhA_{GC}$ levels were also detected during iron deprivation and anaerobic growth in comparison to standard laboratory conditions (Fig. 2.2 D). As expected, expression of Ng-MIP remained constant throughout different phases of gonococcal growth and was unchanged under all tested conditions (Zielke *et al*., 2015; Zielke *et al*., 2016), while TbpB and AniA were the most highly upregulated during iron deprivation and anaerobic growth, respectively (Fig. 2.2 D).

The increase in $GmhA_{GC}$ expression did not influence LOS migration patterns in wt gonococci, albeit increased amounts of total LOS were observed in bacteria cultured in the presence of normal human serum and anaerobically (Fig. 2.2 D). To further assess the possible correlation between expression of $GmhA_{GC}$ and LOS levels, $\Delta gmhA_{GC}/P_{lac}::gmhA_{GC}$ was cultured in increasing concentrations of IPTG. The vast overexpression of $GmhA_{GC}$ achieved with 1000 µM IPTG did not have adverse effect on bacterial growth (data not shown) and had no effect on the LOS quantities (Fig. 2.2 E).

Cumulatively, these experiments demonstrated that $GmhA_{GC}$ is a cytoplasmic enzyme with augmented expression during mid-logarithmic phase, iron depletion and anaerobiosis, and that overproduction of GmhAGC alone does not alter gonococcal LOS abundance.

Conservation of gmhA among *Neisseria*. Analysis of *gmhA* conservation showed that the gene (locus NGO1986, NMB2090, NMC2070) is present in all of the 39,182 *Neisseria spp.* genomes deposited into the PubMLST database (http://pubmlst.org/neisseria/ as of July, 20, 2016) and that there are 340 alleles and 323 single nucleotide polymorphic sites (Supplemental Fig. S2.3).

Expression of $GmhA_{GC}$ among 36 different *N. gonorrhoeae* strains isolated from patients at different times and geographic locations, including the 2016 WHO reference strains (Unemo *et al*., 2016), was also assessed by immunoblotting. Whole cell lysates were resolved by SDS-PAGE and probed with either polyclonal anti-

$GmhA_{GC}$ antisera or Zwf antibodies, or stained with Coomassie Brilliant Blue G-250 as a loading control. Antisera against $GmhA_{GC}$ cross-reacted with all clinical isolates of *N. gonorrhoeae* but no cross-reactivity was detected for the *E. coli* GmhA homologue (Fig. 2.3). In addition, there were noticeable differences in $GmhA_{GC}$ protein abundance between the strains, while Zwf was uniformly expressed and there were no discrepancies in regards to samples normalization and loading (Fig. 2.3 and Supplemental Fig. S2.2).

Hindering GmhAGC isomerase activity does not influence *N. gonorrhoeae* growth. The crucial side chains for GmhA enzymic activity appeared to be E65 and H180 in the *E. coli* orthologue as analyzed by *in vitro* kinetic assays, LOS synthesis, and novobiocin sensitivity studies. It has been proposed that these two residues act as the base and the acid, respectively, to promote the isomerization reaction of D-sedoheptulose 7-phosphate into D-glycero-α,β-D-manno-heptose-7-phosphate (Taylor *et al*., 2008). Therefore, to determine whether the observed decrease in *N. gonorrhoeae* survival upon $GmhA_{GC}$ depletion is a consequence of abolished LOS synthesis, site-directed mutagenesis of corresponding residues (E65 and H183 in $GmhA_{GC}$) was employed. The obtained $GmhA_{GC}$ E65A and H183A variants were placed under the IPTG inducible promoter and introduced into the chromosome of wt FA1090, followed by allelic exchange of *ngo1986* with the kanamycin resistance cassette. Both mutated proteins were stably produced in *N. gonorrhoeae*; however, the H183A variant was present at higher levels than the wt $GmhA_{GC}$ (Fig. 2.4 A). Silver staining analysis of LOS revealed that bacteria expressing either E65A or H183A constructs produced truncated LOS, regardless of the presence of IPTG (Fig. 2.4 A). Further, under non-permissive conditions (without IPTG), the $\Delta gmhA_{GC}/P_{lac}::gmhA_{GC}$E65A and $\Delta gmhA_{GC}/P_{lac}::gmA_{GC}$H183A, similarly to the $\Delta gmhA_{GC}/P_{lac}::gmhA_{GC}$, had decreased viability, which was demonstrated by a 3214-, and 8653-fold decline, respectively, in CFUs in comparison to wt gonococci (Fig. 2.4 B). In contrast, the expression of either mutated version of $GmhA_{GC}$ rescued bacterial viability to the wt level.

These *in vivo* studies demonstrated the importance of residues E65 and H183 in $GmhA_{GC}$ activity in the production of full length LOS and suggested that abolition

of LOS synthesis is disconnected from the $GmhA_{GC}$-dependent effect on N. *gonorrhoeae* viability.

Trans-complementation studies of GmhA. GmhA homologues of *H. influenzae* and *H. ducreyi* restored the synthesis of full length LPS in the *E. coli* Δ*gmhA* mutant (Brooke and Valvano, 1996; Bauer *et al*., 1998). This suggested that GmhA proteins can function interchangeably. However, failure of multiple attempts to remove *ngo1986* in FA1090 carrying the *E. coli gmhA* gene cloned under the P_{lac} promoter and integrated into the chromosome ruled out the possibility of a functional inter-species complementation. The *E. coli* GmhA ($GmhA_{EC}$) shares 74% and 75% amino acid identity with *H. influenzae* and *H. ducreyi* proteins, respectively, while only 50% identity exists between $GmhA_{EC}$ and $GmhA_{GC}$ (Table 2.3). In contrast, *N. meningitidis* GmhA ($GmhA_{NM}$) shows 98% identity to $GmhA_{GC}$, and anti-$GmhA_{GC}$ antisera readily recognized $GmhA_{NM}$ (Fig. 2.5 A). Therefore, we decided to use an analogous strategy to create *N. gonorrhoeae* and *N. meningitidis* Δ*gmhA* strains expressing either endogenous or non-endogenous GmhA. Immunoblotting experiments demonstrated that under permissive conditions, both proteins were stably expressed in each host, while without IPTG, neither $GmhA_{GC}$ nor $GmhA_{NM}$ were detected (Fig. 2.5 A). Functional interchangeability of GmhA between *N. gonorrhoeae* and *N. meningitidis* was then evaluated by analysis of LOS patterns. In FA1090 $\Delta gmhA_{GC}/P_{lac}::gmhA_{NM}$ expression of $GmhA_{NM}$ resulted in LOS migrating exactly as LOS species extracted from the wt gonococci (Fig. 2.5 A). Likewise, expression of $GmhA_{GC}$ restored LOS synthesis in the *N. meningitidis* MC58 $\Delta gmhA_{NM}/P_{lac}::gmhA_{GC}$. Further, as expected from our studies in *N. gonorrhoeae* (Fig. 2.1), depletion of $GmhA_{NM}$ in *N. meningitidis* MC58 $\Delta gmhA_{NM}/P_{lac}::gmhA_{NM}$ had adverse effects on bacterial viability and resulted in a 300- and 235.7-fold decrease in CFUs in comparison to wt and $\Delta gmhA_{NM}/P_{lac}::gmhA_{NM}$ cultured in the presence of IPTG, respectively (Fig. 2.5 B). There was no statistically significant difference in the number of CFUs between the wt *N. gonorrhoeae* and its isogenic $\Delta gmhA_{GC}$ mutant expressing either $GmhA_{GC}$ or $GmhA_{NM}$ (Fig. 2.5 B). Similarly, both $GmhA_{NM}$ and $GmhA_{GC}$ fully complemented the lack of $GmhA_{NM}$ in *N. meningitidis*.

Together, the trans-complementation studies showed that the *N. gonorrhoeae* and *N. meningitidis* GmhA can function interchangeably. Expression of either of the homologues restored both viability and LOS synthesis. Additionally, we concluded that *E. coli* $GmhA_{EC}$ is not able to complement $GmhA_{GC}$ functions, as we were not able to generate a viable *N. gonorrhoeae* $\Delta gmhA_{GC}/P_{lac}::gmhA_{EC}$ mutant.

The structure of *N. gonorrhoeae* GmhA. To gain insights into the function of $GmhA_{GC}$ and to facilitate the future targeting of this molecule with small molecule inhibitors, we obtained recombinant protein for structural studies (Supplemental Fig. S2.1). The structure of *N. gonorrhoeae* GmhA was determined by molecular replacement and was refined to 2.37 Å resolution with Rwork 0.207, R_{free} 0.267, and excellent stereochemical parameters (Table 2.2). Four monomers of $GmhA_{GC}$ were present in the asymmetric unit (Fig. 2.6 A). The tetrameric architecture is consistent with the results of size-exclusion chromatography (Supplemental Fig. S2.1 A), and with the previously determined structures of GmhA homologs from other bacteria (Table 2.3). The interface area of the $GmhA_{GC}$ tetramer is extensive and buries 14,160 Å2 of surface area as calculated by the PISA server (Krissinel and Henrick, 2007). Four subunits of $GmhA_{GC}$ adopt highly similar structures with root mean square deviation (r.m.s.d.) 0.1–0.2 Å between subunits. Residues 69–74 (chains A and B) and 69–75 (chains C and D) are disordered in the structure. These residues form a loop in the vicinity of the active site and could become ordered upon substrate binding. A similar disorder was observed in the homologous region of *E. coli* GmhA (Taylor *et al*., 2008).

Each monomer of $GmhA_{GC}$ is composed of a central five-stranded parallel α–sheet flanked by four α–helices from each side (Fig. 2.6 B). Four zinc ions are present in the $GmhA_{GC}$ tetramer. The zinc ions are coordinated by the side chains of residues H61, E65 and H183 of one monomer, and the side chain of Q175 of the neighboring monomer (Fig. 2.6 C). Therefore, the zinc-binding sites of $GmhA_{GC}$ are identical to the zinc-binding sites of *B. pseudomallei* GmhA (Harmer, 2010).

Overall, the structure of $GmhA_{GC}$ is similar to the previously determined structures of GmhA from other bacteria, as well as *E. coli* DiaA (Table 2.3). The monomer structure of $GmhA_{GC}$ could be superimposed to the homologous structures with r.m.s.d. of 0.5–1.4 Å. The tetramer structure of $GmhA_{GC}$ forms a ‘closed’

conformation, similar to the GmhA structures of *P. aeruginosa*, *V. cholerae*, *B. pseudomallei* and *C. psychrerythraea* (Harmer, 2010; Do *et al*., 2015; Seetharaman e*t al*., 2006; Taylor *et al*., 2008).

Discussion

GmhA is a conserved sedoheptulose-7-phosphate isomerase involved in the first biosynthesis step of the L,D-heptose component of the LPS/LOS. Deprivation of heptoses results in pleiotropic phenotypes including synthesis of LPS/LOS molecules composed only of lipid A and KDO residues, increased susceptibility to antimicrobial agents, defects in plasmid F conjugations and P1 bacteriophage transduction, and decreased virulence and biofilm formation (Tamaki *et al*., 1971; Havekes *et al*., 1976; Brooke and Valvano, 1996; Malott *et al*., 2013; Bauer *et al*., 1998; Earl *et al*., 2015). In *N. gonorrhoeae*, heptoses are also crucial for LOS function in the evasion of the host immune system and induction of HIV expression (Malott *et al*., 2013; Preston *et al*., 1996). Variability of LOS molecules on the gonococcal surface is driven by phase variable enzymes responsible for decorating the sugar moiety of the outer core oligosaccharide (Danaher *et al*., 1995; Apicella *et al*., 1987). *N. gonorrhoeae* scavenges sialic acid from the human body and modifies the lacto-N-neotetraose attached to the HepI of the LOS core oligosaccharide, which provides protection from killing by classical and alternative complement pathways (Elkins *et al*., 1992; Ram *et al*., 1998). Thus, inhibition of GmhA could aid in treatment of infections caused by different pathogenic bacteria including *N. gonorrhoeae*.

Accordingly, in this work we presented initial characterization of $GmhA_{GC}$ at the functional and structural levels. Our experiments demonstrated that gonococci and meningococci depleted in GmhA produced overall significantly less LOS molecules that migrated faster in comparison to the LOS-derived from wt bacteria (Figs. 2.1 B and 2.5 A). In contrast to other orthologues, however, diminution of the GmhA cellular pool had severe consequences on neisserial growth (Figs. 2.1 A, D-E and 2.5 B). This protein is likely not essential for bacterial viability, as GmhA-depleted neisserial cells still arose on solid media under nonpermissive conditions (Fig. 2.1 A, D-E). Corroborating this observation, $gmhA_{GC}$ was not found among 827 gonococcal core essential genes identified using a high density Tn5 transposon library (Remmele *et al*., 2014). In addition, expression of $GmhA_{GC}$ variants carrying substitutions in the catalytic residues coordinating zinc ions, E65 and H183 (Fig. 2.6 C), led to synthesis of truncated LOS while retaining ample *N. gonorrhoeae* viability (Fig. 2.4). These

findings suggested that $GmhA_{GC}$ may be involved in additional physiological function(s) in *Neisseria*. Not surprisingly, a complete functional trans-complementation was achieved between $GmhA_{GC}$ and $GmhA_{NM}$ (Fig. 2.5 A-B), whereas multiple attempts to generate a viable strain of *N. gonorrhoeae* expressing only $GmhA_{EC}$ were unsuccessful (data not shown). Based on these findings, we concluded that GmhA plays a critical role in LOS synthesis and is a fundamental growth factor for both *N. gonorrhoeae* and *N. meningitidis*.

Profiling of $GmhA_{GC}$ expression showed that amounts of $GmhA_{GC}$ increased during exponential growth of wild type *N. gonorrhoeae* (Fig. 2.2 C). Likewise, the isogenic conditional $\Delta gmhA_{GC}/P_{lac}::gmhA_{GC}$ mutant cultured in the presence of IPTG had elevated levels of $GmhA_{GC}$ during the logarithmic phase (Fig. 2.1 C-E), suggesting that expression of $GmhA_{GC}$ is regulated at the post-transcriptional level. Higher amounts of $GmhA_{GC}$ were also noted upon exposure of *N. gonorrhoeae* to environmental cues relevant to infection; iron deprivation and anaerobiosis (Fig. 2.2 D). Transcriptomic studies, however, did not identify $GmhA_{GC}$ as an iron-regulated gene (Ducey *et al*., 2005; Jackson *et al*., 2010). The observed upregulation of $GmhA_{GC}$ under anaerobic conditions was in agreement with deep sequencing analysis of the *N. gonorrhoeae* anaerobic stimulon, where $gmhA_{GC}$ was found to be 4.2- and 6.1-fold upregulated in two biological experiments (Isabella and Clark, 2011). Higher levels of enzymes acting downstream from GmhA including HldA (NGO0402) and GmhB (NGO2070) were identified in anaerobically versus aerobically cultured gonococci in our recent high-throughput proteomic studies (Zielke *et al*., 2016), suggesting that the L,D-heptose biosynthesis pathway is upregulated during anoxia. Accordingly, increased amounts of LOS were detected in anaerobically grown gonococci (Fig. 2.2 D), while vast overexpression of $GhmA_{GC}$ alone had no effect on LOS levels (Fig. 2.2 E). $GmhA_{GC}$ expression was also studied in a diverse collection of gonococcal isolates, including the 2016 WHO reference strains (Unemo *et al*., 2016) containing multidrug resistant *N. gonorrhoeae* (Fig. 3). Potential involvement of GmhA in antibiotic resistance via increased synthesis of LPS was suggested recently in *Salmonella typhimurium* DT104B multiresistant strain with additional fluoroquinolone resistance (Correia *et al*., 2016). Notwithstanding this suggestion, the amounts of $GmhA_{GC}$ varied

widely between the WHO strains K, L, V, W, X, Y, and Z, which display overall high levels of ciprofloxacin resistance (MIC>32 mg/L) and decreased susceptibility to gemifloxacin and moxifloxacin (Unemo *et al*., 2016). Additionally, strain L, which has high MICs for all quinolones (MICs in mg/L for ciprofloxacin, gemifloxacin, and moxifloxacin of >32, 8, and 16, respectively), showed significantly lower $GmhA_{GC}$ levels in comparison to WHO isolates F, O, P, and U, which are all ciprofloxacin sensitive (MIC of 0.004 mg/L) and are significantly more sensitive to gemifloxacin and moxifloxacin (MICs ranging from 0.004 to 0.016 mg/L).

Finally, to better understand the pivotal function of $GmhA_{GC}$, we have determined the three-dimensional structure of the untagged enzyme (Fig. 2.6). Comparison of our $GmhA_{GC}$ crystal structure with the structures of homologues from other bacteria did not reveal significant differences in the general organization of the structure or within the catalytic site of the enzyme. Zinc ions were not added to any of the protein purification steps or crystallization buffers, yet similarly to *Burkholderia pseudomallei* GmhA (Harmer, 2010), $GmhA_{GC}$ held zinc ions in the active site (Fig. 2.6 C), providing further support that GmhA is a metalloenzyme. Zinc is likely retained from the cell of the heterologous host during protein overproduction and natively bound to the active site of the enzyme. It likely drives the "closed" conformation of $GmhA_{GC}$, which was also observed in structures of *B. pseudomallei*, *P. aeruginosa* and *V. cholerae* GmhA (Table 2.3), thus providing further support that the "closed" conformation is most probable catalytically relevant. In addition, site-directed mutagenesis coupled with *in vivo* studies confirmed the pivotal function of $GmhA_{GC}$ E65 and H183 in the isomerization of the sedoheptulose-7-phosphate (Figs. 2.4 and 2.6).

In conclusion, understanding the function and structure of individual GmhA proteins will facilitate drug discovery approaches focused on targeting this protein with small molecule inhibitors. In particular, our work demonstrated the crucial function of GmhA in neisserial growth and LOS synthesis, positive regulation of expression by host-relevant environmental stimuli, and conservation among different isolates, as well as provided further support for the mode of action of GmhA. These findings underscore the significance of $GmhA_{GC}$ as a target for anti-gonorrhea therapeutics. Future work

involving determining the interacting partner(s) of $GmhA_{GC}$ and analysis of global changes at the proteome and metabolome levels are required to elucidate the whole scope of physiological function(s) of $GmhA_{GC}$ in *N. gonorrhoeae*.

Acknowledgements

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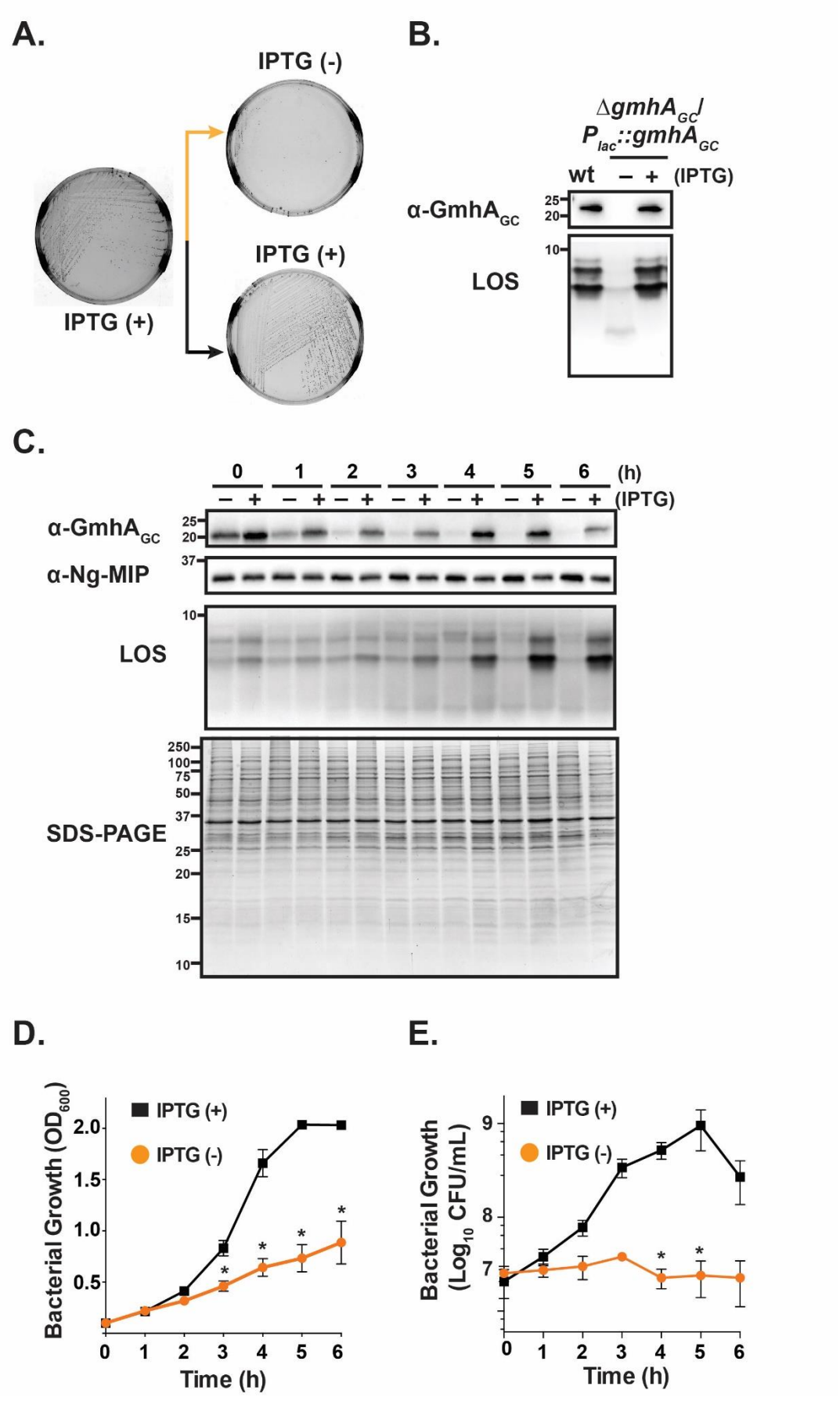

A.
IPTG (-)
IPTG (+)
IPTG (+)
B.
ΔgmhA_GC/
P_lac::gmhA_GC
wt – + (IPTG)
α-GmhA_GC
LOS
25
20
10
C.
0 1 2 3 4 5 6 (h)
– + – + – + – + – + – + – + (IPTG)
α-GmhA_GC
α-Ng-MIP
LOS
SDS-PAGE
250
100
75
50
37
25
20
15
10
D.
Bacterial Growth (OD600)
IPTG (+)
IPTG (-)
Time (h)
E.
Bacterial Growth (Log10 CFU/mL)
IPTG (+)
IPTG (-)
Time (h)

Figure 2.1. GmhA$_{GC}$ is pivotal for *N. gonorrhoeae* growth and LOS synthesis. (A) The *N. gonorrhoeae* FA1090 *gmhA$_{GC}$* conditional knockout strain, Δ*gmhA$_{GC}$*/*P$_{lac}$::gmhA$_{GC}$*, was streaked out from frozen glycerol stock on solid media supplemented with 20 μM IPTG. After 18 h incubation at 37 °C in the presence of 5% atmospheric CO_2, the colonies were passaged onto plates either with (+) or without IPTG (-). (B) Whole cell lysates of FA1090 wt and isogenic Δ*gmhA$_{GC}$*/*P$_{lac}$::gmhA$_{GC}$* harvested from plates with (+) or without 20 μM IPTG (-) were either probed with polyclonal rabbit antisera or subjected to LOS extraction using proteinase K followed by silver staining. (C-E) The FA1090 Δ*gmhA$_{GC}$*/*P$_{lac}$::gmhA$_{GC}$* cells were collected from solid media supplemented with 20 μM IPTG, suspended to OD_{600} of 0.1, washed twice, divided, and cultured either in the presence or absence of IPTG for 3 h. At time point designated as 0 h, corresponding cultures were back-diluted to the same density (OD_{600} of 0.1) into fresh media with or without IPTG and incubated for additional 6 h. Samples of bacterial cultures were collected every hour for GmhA$_{GC}$ and Ng-MIP immunoblotting analysis, LOS, and whole cell protein profiles (C), monitoring of bacterial proliferation by measurement of density of the cultures at OD_{600} (D) and spotting serially diluted bacteria on solid media with IPTG for CFU scoring (E). Whole cell lysates were matched by the same OD_{600} units. As loading control, samples separated by SDS-PAGE samples were either probed with anti-Ng-MIP antibodies or stained with Coomassie Brilliant Blue G-250. The migration of molecular mass marker (kDa) is indicated on the left. All experiments were performed in three biological replicates. Means and SEM are presented on graphs; *$p<0.05$.

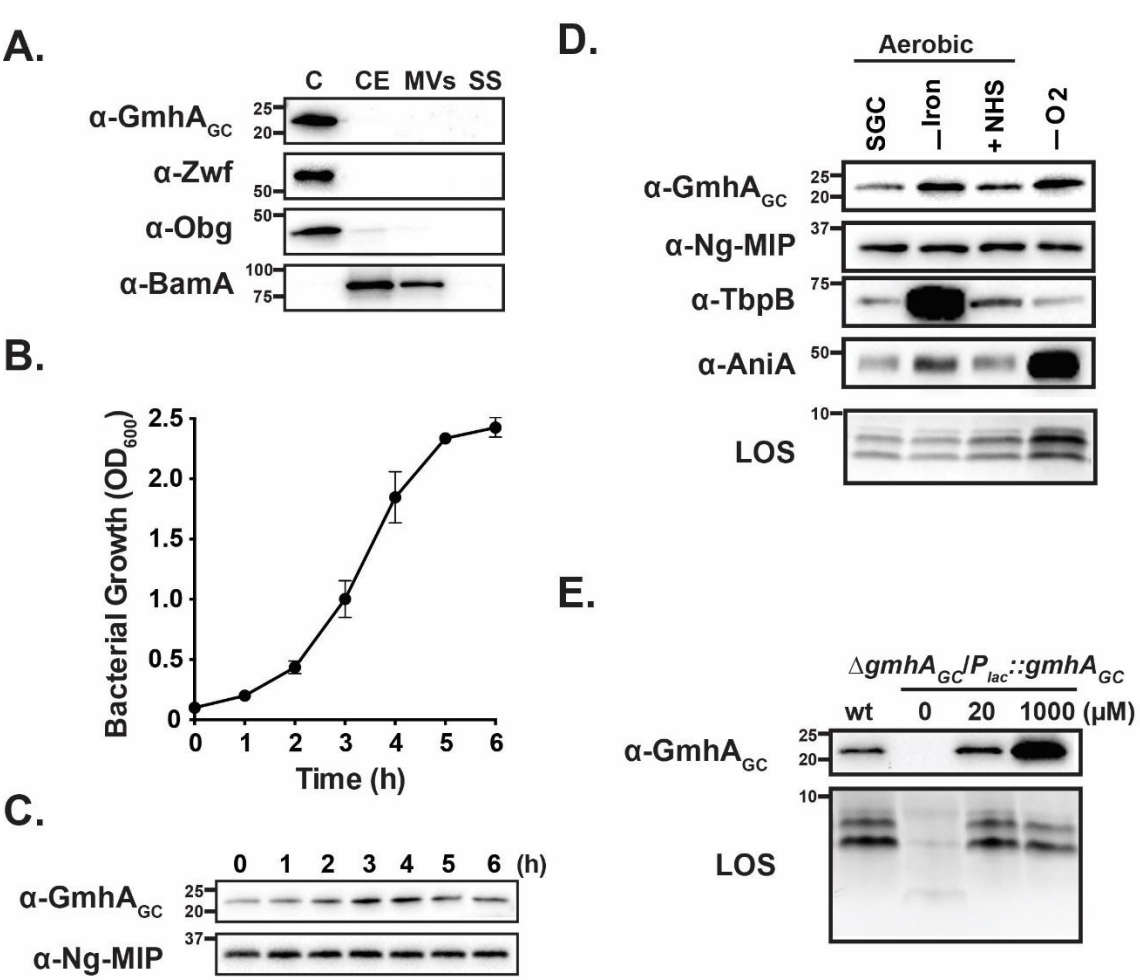


Figure 2.2. Assessment of GmhA$_{GC}$ subcellular localization and expression patterns. (A) *N. gonorrhoeae* wt FA1090 cells were harvested during mid-exponential phase and subjected to proteome extraction to separate cytoplasmic/periplasmic proteins [C], cell envelopes [CE], naturally released membrane vesicles [MVs], and soluble proteins in culture supernatants [SS]. Individual fractions were loaded based on the total amount of protein (μg) and probed with antisera as indicated on the left. (B-C) Wt *N. gonorrhoeae* FA1090 was cultured in liquid media and at indicated time points OD_{600} measurements were taken (B) and samples were withdrawn and processed for immunoblotting analysis of GmhA$_{GC}$ and Ng-MIP (C). D. Quantities of GmhA$_{GC}$ and LOS in wt FA1090 during *in vitro* conditions relevant to different infection sites [standard growth under aerobic conditions (SGC), iron deprivation (-Iron), presence of

normal human serum (+NHS), and anaerobiosis ($-O_2$)], were assessed by probing the whole cell lysates with anti-$GmhA_{GC}$ antibodies and silver staining of proteinase K-extracted LOS, respectively. Immunoblotting experiments with antisera against Ng-MIP, TbpB and AniA were used as markers for ubiquitous expression, iron-limiting and anaerobic conditions, respectively. (E) Effect of overexpression of $GmhA_{GC}$ on LOS amounts was examined by harvesting wt FA1090 and isogenic $\Delta gmhA_{GC}/P_{lac}::gmhA_{GC}$ grown on solid media with different IPTG concentrations (0, 20, or 1000 μM). Whole cell lysates were either probed with anti-$GmhA_{GC}$ antisera or treated with proteinase K and LOS was visualized by silver staining. Experiments were performed in biological triplicates and representative immunoblots and silver stained gels are shown. Mean values and corresponding SEM are presented on the graph. Migration of a molecular mass marker (kDa) is indicated on the left**.**

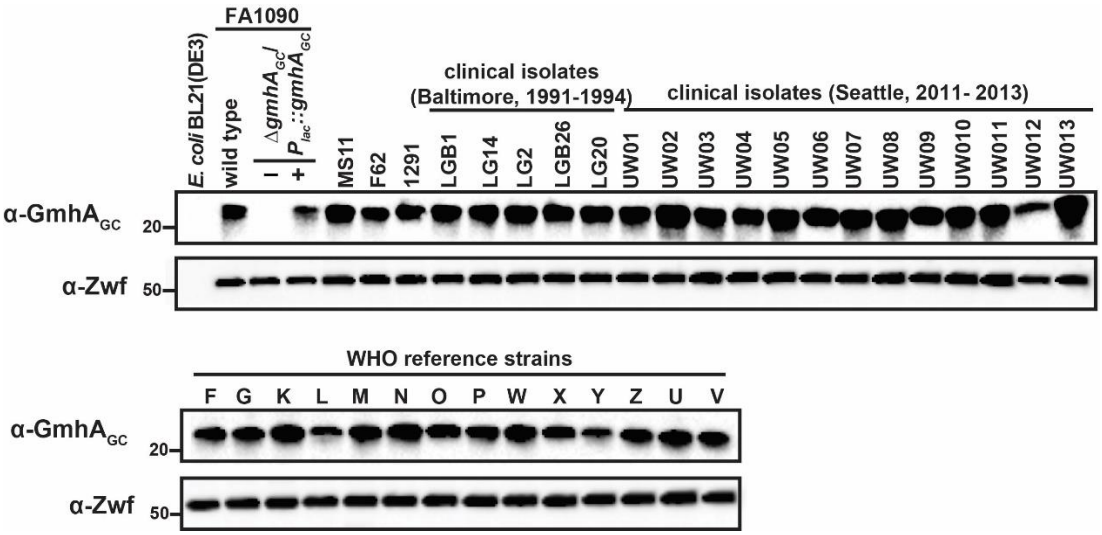


Figure 2.3. GmhA$_{GC}$ expression in a panel of *N. gonorrhoeae* isolates. Whole cell lysates of *E. coli*, wt FA1090, Δ*gmhA*$_{GC}$/*P*$_{lac}$*::gmhA*$_{GC}$ grown either with (+) or without (-) IPTG, and 36 additional strains of *N. gonorrhoeae* were resolved on a 10-20% Tris-Glycine gel and probed with anti-GmhAGC antibodies. Immunoblotting with anti-Zwf antisera was used as a loading control. Migration of a molecular mass marker (kDa) is indicated on the left.

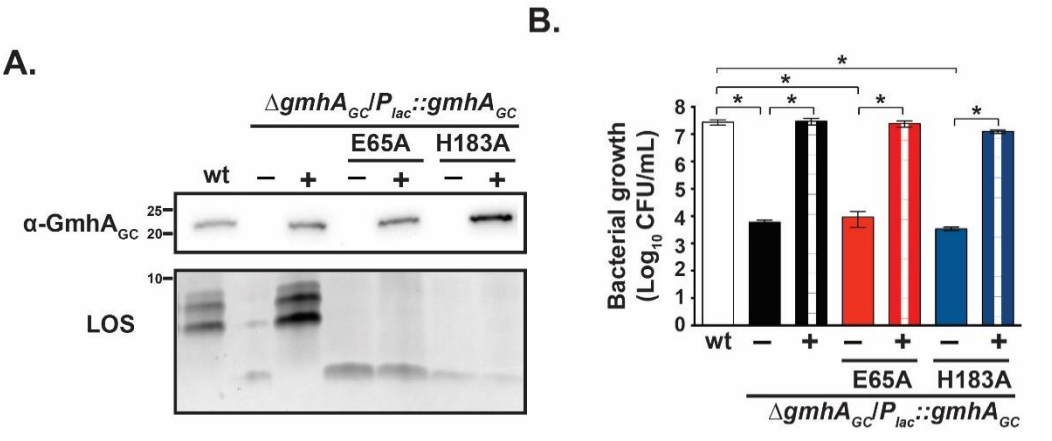


Figure 2.4. Hindering GmhA$_{GC}$ isomerase activity does not influence *N. gonorrhoeae* growth. (A) Wt FA1090 and isogenic conditional mutants carrying either native *gmhA$_{GC}$* (Δ*gmhA$_{GC}$*/P$_{lac}$::*gmhA$_{GC}$*) or mutated variants of GmhA$_{GC}$ (Δ*gmhA$_{GC}$*/*P$_{lac}$::gmhA$_{GC}$*E65A or Δ*gmhA$_{GC}$*/*P$_{lac}$::gmhA$_{GC}$*H183A) were collected from solid media with (+) and without (-) IPTG. Expression of individual GmhA*GC* variants and LOS patterns were examined in whole cell extracts by immunoblotting and silver staining, respectively. Samples were matched by equivalent OD_{600} units. Migration of a molecular mass marker (kDa) is indicated on the left. (B) Wt FA1090 and conditional mutants Δ*gmhA$_{GC}$*/*P$_{lac::}$gmhA$_{GC}$*, Δ*gmhA$_{GC}$*/*P$_{lac}$::gmhA$_{GC}$*E65A, and Δ*gmhA$_{GC}$*/*P$_{lac}$::gmhA$_{GC}$*H183A were collected from solid media supplemented with 20 µM IPTG, suspended in liquid media to OD_{600} of 0.1, cultured for 3 h, back-diluted to equal OD_{600} of 0.2, serially diluted, and spotted on solid media in the presence (+) and absence (-) of IPTG. CFUs were scored. The data show averages of CFUs with corresponding SEM of at least three separate experiments; *p<0.05.

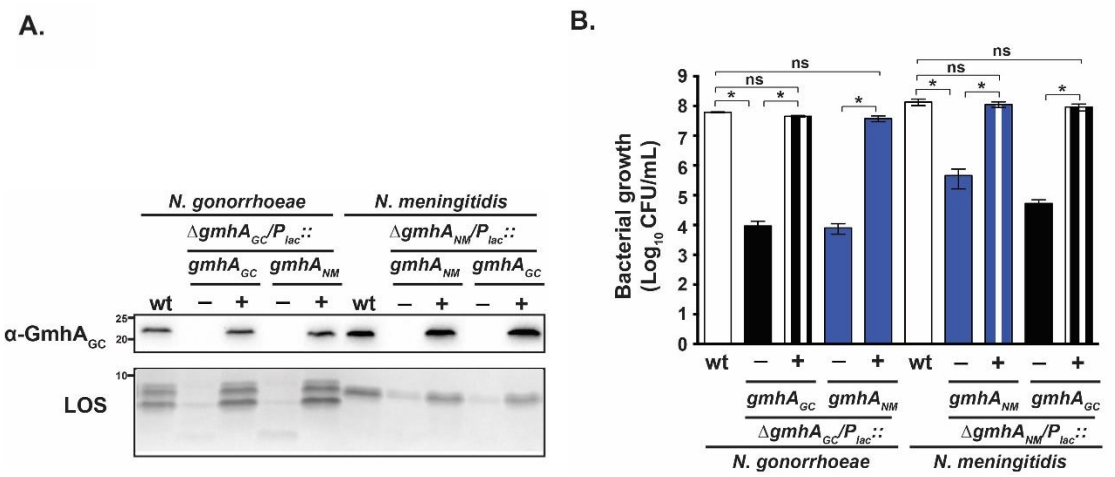


Figure 2.5. Homologues of GmhA from *N. gonorrhoeae* and *N. meningitidis* function interchangeably. (A) *N. gonorrhoeae* FA1090 wt and isogenic conditional *gmhA* mutants bearing either endogenous (Δ*gmhA*$_{GC}$/*P*$_{lac}$*::gmhA*$_{GC}$) or *N. meningitidis*-derived *gmhA*$_{NM}$ (Δ*gmhA*$_{GC}$/*P*$_{lac}$*::gmhA*$_{NM}$), as well as *N. meningitidis* MC58 wt and conditional *gmhA* mutants carrying *gmhA* alleles (Δ*gmhA*$_{NM}$/*P*$_{lac}$*::gmhA*$_{NM}$ or Δ*gmhA*$_{NM}$/*P*$_{lac}$*::gmhA*$_{GC}$) were harvested from GCB after 22 h of incubation either with (+) or without (-) IPTG. Whole cell lysates were probed with anti-GmhA$_{GC}$ antibodies or treated with proteinase K and LOS was visualized by silver staining. Migration of a molecular mass marker (kDa) is indicated on the left. (B) Wt and mutant strains of *N. gonorrhoeae* and *N. meningitidis*, as described above, were collected from GCB supplemented with 20 µM IPTG and 15 µM IPTG, respectively. Bacteria were suspended in GCBL to OD$_{600}$ of 0.1, cultured for 3 h, back-diluted to equal OD600 of 0.2, serially diluted and plated on GCB with (+) and without (-) IPTG. CFUs were counted following 22 h of incubation. Experiments were performed in three independent replicates. Mean values and SEM are presented; *p<0.05.

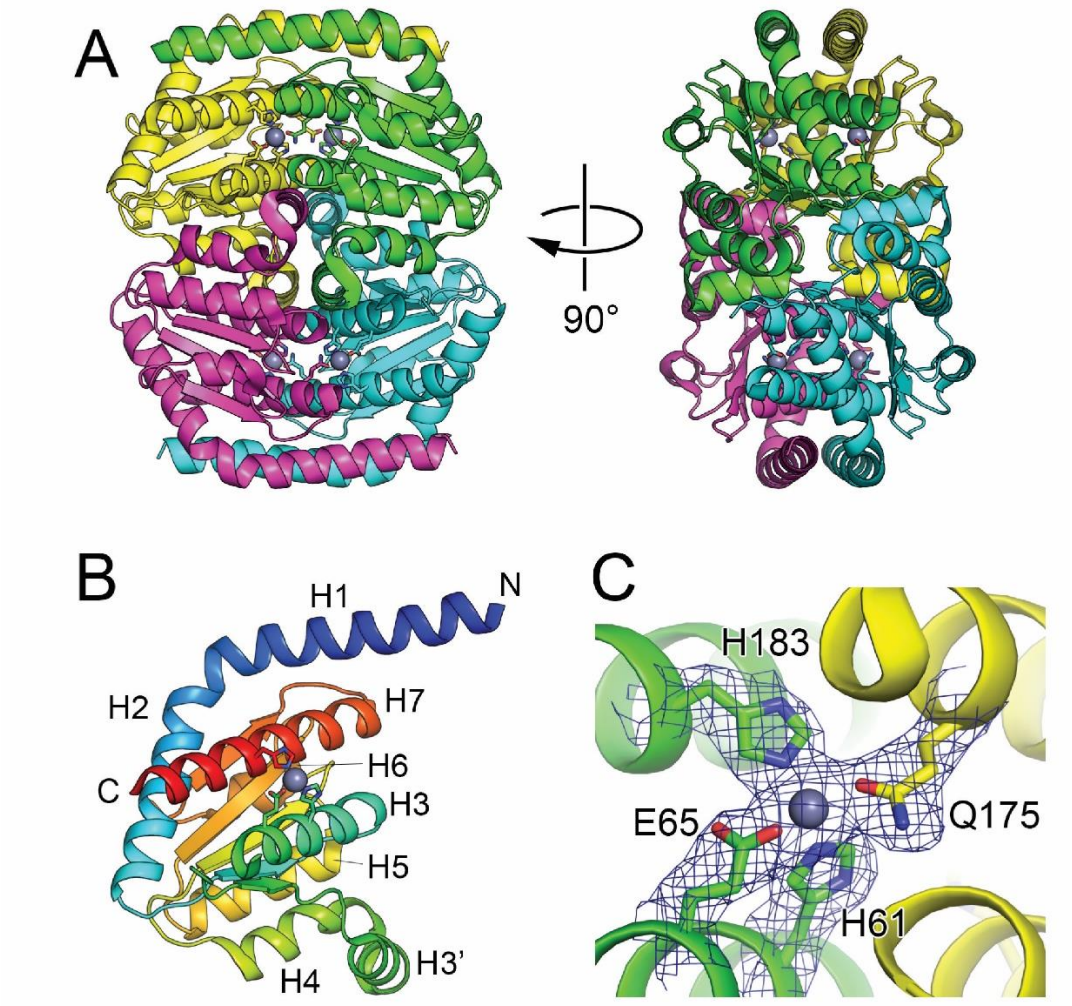


Figure 2.6. The structure of *N. gonorrhoeae* GmhA$_{GC}$. (A) The ribbon representation of GmhA$_{GC}$ tetramer with monomers colored in green, yellow, cyan and magenta. Zn^{2+} ions are shown as grey spheres. (B) The structure of GmhA$_{GC}$ monomer is colored in rainbow colors from blue (N-terminus) to red (C-terminus). The α–helices are labeled according to (Seetharaman *et al*., 2006). (C) A close-up view of the Zn^{2+}-binding site of GmhA$_{GC}$. The coordinating side-chains are shown in stick representation. The σ_A-weighted $2F_O–F_C$ electron density map countered at 1.0 σ is displayed as blue mesh.

Table 2.1. Bacterial strains used in this study.

Bacterial strain	Reference
N. gonorrhoeae	
FA1090	(Connell *et al.*, 1988)
MS11	(Meyer *et al.*, 1982)
1291	(Apicella *et al.*, 1978)
F62	(Sparling, 1966)
FA1090 Δ$gmhA_{GC}$/P_{lac}:: $gmhA_{GC}$	This study
FA1090 Δ$gmhA_{GC}$/P_{lac}:: $gmhA_{GC}$ E65A	This study
FA1090 Δ$gmhA_{GC}$/P_{lac}:: $gmhA_{GC}$ H183A	This study
FA1090 Δ$gmhA_{GC}$/P_{lac}:: $gmhA_{NM}$	This study
Baltimore collection 1991-1994: LGB1, LG14, LG2, LGB26, LG20	(Garvin *et al.*, 2008, Zielke *et al.*, 2014)
Clinical isolates from Public Health–Seattle & King County Sexually Transmitted Disease Clinic: UW01, UW02, UW03, UW04, UW05, UW06, UW07, UW08, UW09, UW10, UW11, UW12, UW13	(Zielke *et al.*, 2016)
2016 WHO reference strains: F, G, K, L, M, N, O, P, W, X, Y, Z, U, V	(Unemo *et al.*, 2016)
N. meningitidis	
MC58	(McGuinness *et al.*, 1991)
MC58 Δ$gmhA_{NM}$/P_{lac}:: $gmhA_{NM}$	This study
MC58 Δ$gmhA_{GC}$/P_{lac}:: $gmhA_{GC}$	This study
E. coli	
MC1061	(Casadaban & Cohen, 1980)
BL21(DE3)	(Studier & Moffatt, 1986)

Table 2.2. Data collection and refinement statistics.

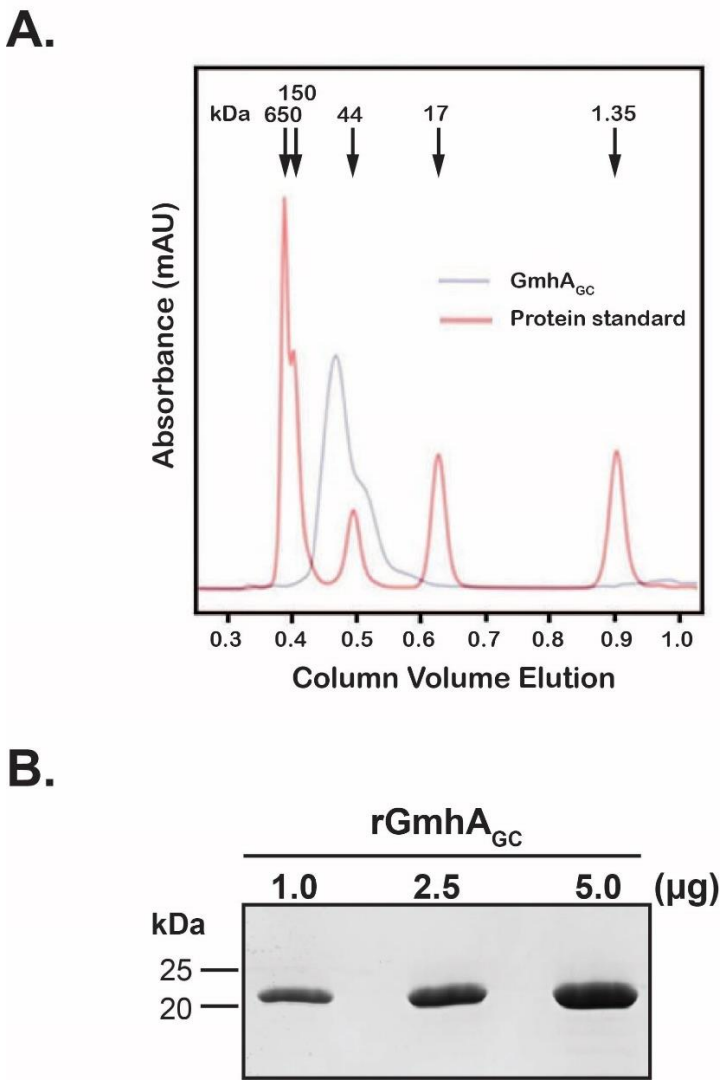
	***N. gonorrhoeae* $GmhA_{GC}$ (PDB 5I01)**
Data collection	
Wavelength (Å)	1.0000
Space group	$P2_12_12$
Cell dimensions:	
a, *b*, *c* (Å)	114.36, 130.15, 47.15
α, β, γ (°)	90, 90, 90
Resolution (Å)	85.91–2.37 (2.43–2.37)[a]
R_{sym}	0.134 (1.032)
$CC_{1/2}$[b]	99.6 (62.4)
$I / \sigma I$	9.3 (1.7)
Completeness (%)	95.8 (97.8)
Multiplicity	3.7 (3.6)
Refinement	
Resolution (Å)	85.91–2.37
No. reflections (total / free)	28191 / 1405
R_{work} / R_{free}	0.207 / 0.267
Number of atoms:	
Protein	5411
Ligand/ion	4
Water	101
B-factors:	
Protein	37.6
Ligand/ion	54.9
Water	32.7
All atoms	37.2
Wilson *B*	41.2
R.m.s. deviations:	
Bond lengths (Å)	0.009
Bond angles (°)	1.224
Ramachandran distribution[c] (%):	
Favored	97.8
Outliers	0

[a]Values in parentheses are for the highest-resolution shell.
[b]$CC_{1/2}$ correlation coefficient as defined in (Karplus & Diederichs, 2012) and calculated by *XSCALE* (Kabsch, 2010b).
[c]Calculated using the MolProbity server (http://molprobity.biochem.duke.edu) (Chen *et al.*, 2010).

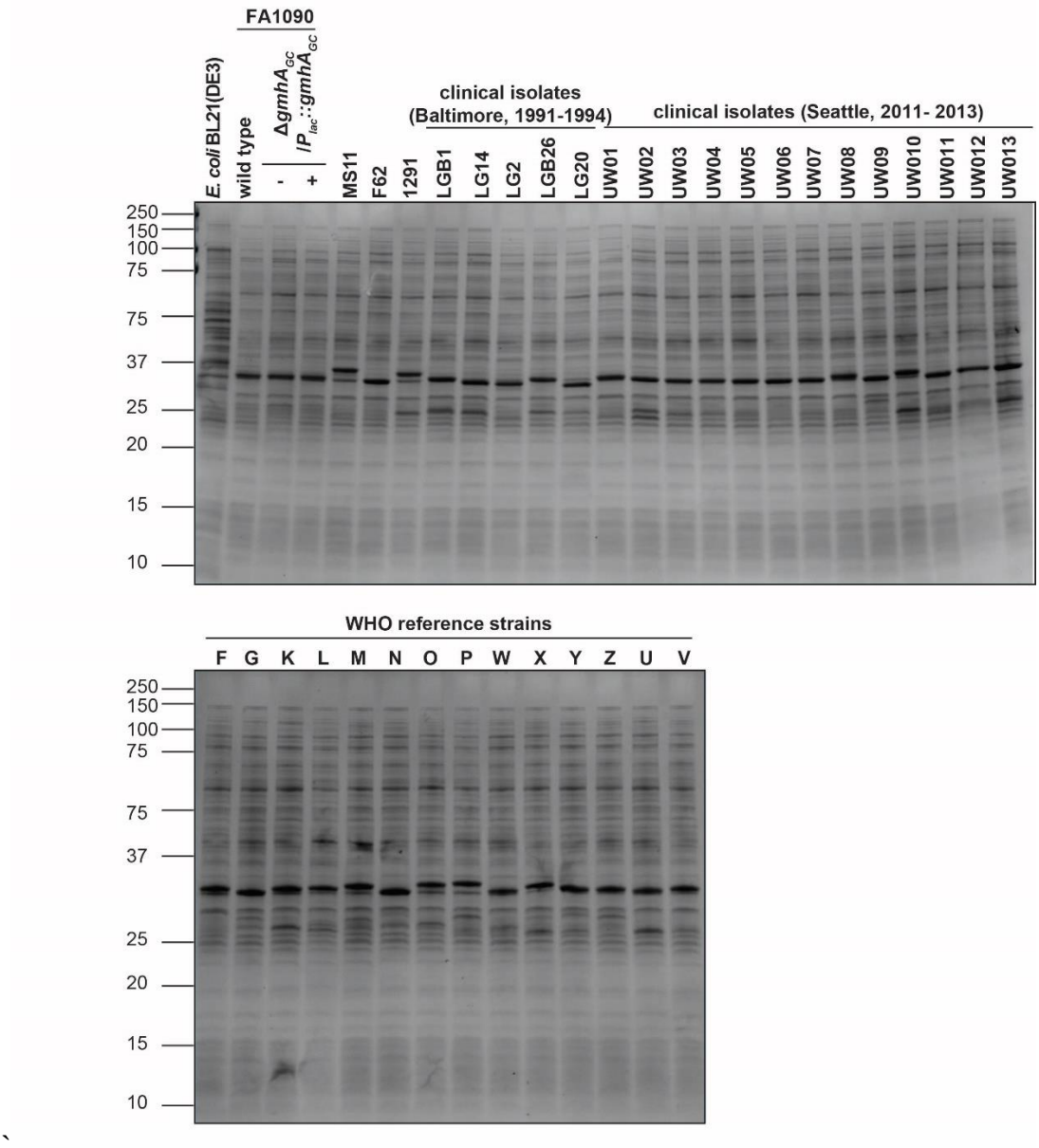
Table 2.3. Structural homologs of *N. gonorrhoeae* GmhA$_{GC}$.

Protein	Organism	r.m.s.d.[a]	Sequence identity[a] (%)	Ligands	PDB ID	Reference
GmhA	*P. aeruginosa*	0.5	57	D-glycero-α-D-manno-heptopyranose-7-phosphate	1X92	(Taylor *et al.*, 2008)
DiaA	*E. coli*	0.7	52	Apo	2YVA	(Keyamura *et al.*, 2007)
DiaA	*E. coli*	0.7	52	Apo	4U6N	unpublished
GmhA	*F. tularensis*	0.7	45	Apo	3TRJ	(Chaudhury *et al.*, 2013)
GmhA	*C.psychrerythraea*	0.7	48	Apo	5BY2	(Do *et al.*, 2015)
GmhA	*C. jejuni*	0.7	54	Apo	1TK9	(Seetharaman *et al.*, 2006)
GmhA	*P. aeruginosa*	0.8	56	Apo	3BJZ	(Taylor *et al.*, 2008)
GmhA	*B. pseudomallei*	0.8	43	Zn^{2+}	2X3Y	(Harmer, 2010)
GmhA	*B. pseudomallei*	0.9	43	Zn^{2+}, D-glycero-α-D-manno-heptopyranose-7-phosphate	2XBL	(Harmer, 2010)
GmhA	*E. coli*	1.4	45	Apo	2I2W	(Taylor *et al.*, 2008)
GmhA	*E. coli*	1.1	45	D-seduheptulose-7-phosphate	2I22	
GmhA	*V. cholerae*	1.1	43	Apo	1X94	(Seetharaman *et al.*, 2006)

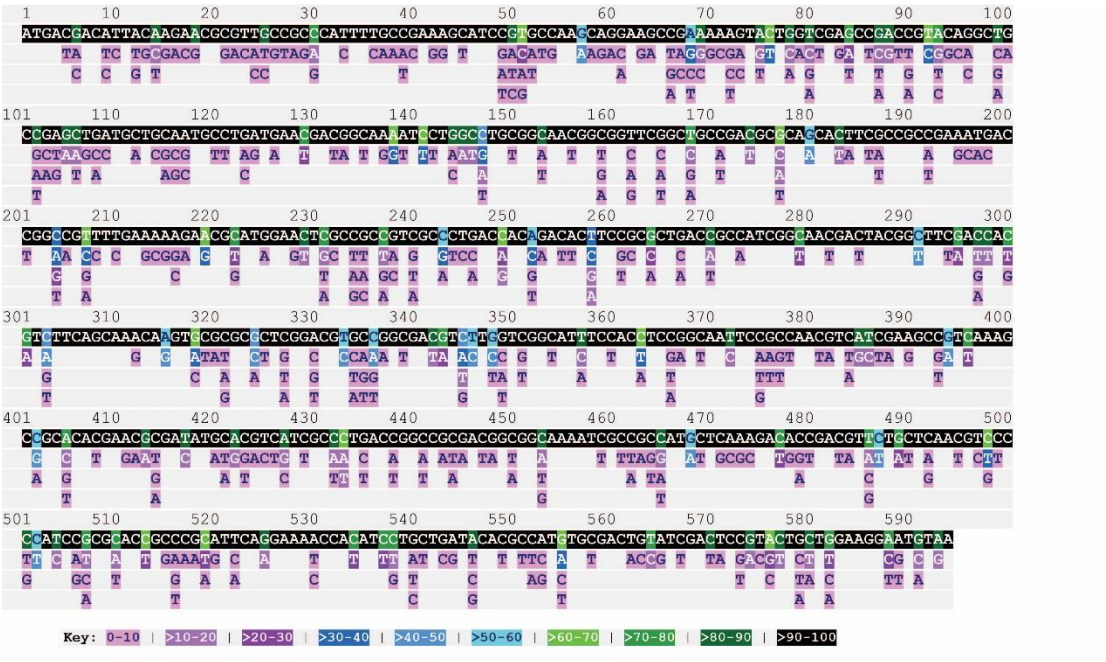
[a]As reported by the Dali server (Holm and Rosenstrom, 2010) for superposition of monomers. A mean value is listed for the structures containing more than one protein subunit in the asymmetric unit.



Supplementary Figure S2.1. Purification of rGmhA$_{GC}$. (A) rGmhA$_{GC}$ eluted mainly as tetramers during size exclusion chromatography. Purified rGmhA$_{GC}$ was separated by NGC Scout Chromatography system (Bio-Rad) with HiLoad 16/600 Superdex 75 pg column (GE Healthcare Life Sciences). Elution chromatogram of rGmhA$_{GC}$ is indicated by blue line. Predicted mass of a monomeric rGmhA$_{GC}$ is 21.1 kDa. Gel Filtration Standard (BioRad) is shown on the chromatogram as the red line. (B) Increasing amounts of rGmhA$_{GC}$ after removal of His-tag by TEV protease were resolved by SDS-PAGE and visualized by Coomassie Brilliant Blue G-250 staining. The migration of molecular mass markers (kDa) is indicated on the left.



Supplementary Figure S2.2. Loading controls for immunoblotting experiments. Samples of whole-cell lysates were prepared for SDS-PAGE as described in the text, separated in 10-20% Tris-Glycine gel and the protein profiles were visualized using Coomassie Brilliant Blue G-250. Loaded OD_{600} units matched the corresponding samples used in immunoblotting analyses of $GmhA_{GC}$ and Zwf. The migration of molecular mass markers (kDa) is indicated on the left.



Supplementary Figure S2.3. Analysis of single nucleotide polymorphisms in *gmhA*. Analysis of *gmhA* (locus NGO1986, NMB2090, NMC2070) in 39,182 *Neisseria spp* genomes deposited into PubMLST database (http://pubmlst.org/neisseria/ as of July, 20, 2016) showed that there are 340 *gmhA* alleles and 323 single nucleotide polymorphic sites.

Supplemental Table S2.1. Plasmids and oligonucleotides used in this study.

Plasmid Name	**Reference**
pRSF-NT	(Korotkov *et al.*, 2013)
pGCC4	(Skaar *et al.*, 2002)
pUC18	(Yanisch-Perron *et al.*, 1985)
pUC18K	(Menard *et al.*, 1993)
pRSF-NT-GmhA$_{GC}$	This study
pRSF-NT-Zwf	This study
pGCC4-GmhA$_{GC}$	This study
pUC18K-GmhA$_{GC}$	This study
pUC18-GmhA$_{GC}$	This study
pUC18-GmhA$_{GC}$E65A	This study
pUC18-GmhA$_{GC}$H183A	This study
pGCC4-GmhA$_{GC}$E65A	This study
pGCC4-GmhA$_{GC}$H183A	This study
pGCC4-GmhA$_{NM}$	This study
pUC18K-GmhA$_{NM}$	This study
pGCC4-GmhA$_{EC}$	This study
Oligonucleotide	**Sequence**[1]
Primers used for creating recombinant protein with N-terminal 6×His-tag:	
rNGO1986-F	GGATTTACCCATGGCGACATTACAAGAACGCG
rNGO1986-R	ACTCGGTCAAGCTTCATTCCTTCCAGCAGTACG
rZwf-F	GACTCCATGGGTACACAGACAAATTTTGATTTGG
rZwf-R	GATCAAGCTTGCATTACTGTTCTTCGTGC
Primers used for gene deletion and complementation:	
NGO1986-Up-F	GACTGATAGAATTCGCGCAGGGTAATGTCTG
NGO1986-Up-R	ATCGATGGTACCGCAACGCGTTCTTGTAATG
NGO1986-Down-F	GGATTTACGGATCCACTGTATCGACTCCGTACTGC
NGO1986-Down-R	ACTCGGTCAAGCTTTGATGCCCAGCAGCGTG
NGO1986-RBS-F	GAATATTACAGGTTGACGATATG
NGO1986-RBS-R	AAGCTTGGCCGGCCTTACATTCCTTCCAGCAG
pGCC4-Ver-F	AAATCGCCCTTGATACCG
pGCC4-Ver-R	ACACTTTATGCTTCCGGCTC
NGO1986-Ver-F	GCCGGCGTACCCGCAT
NGO1986-Ver-R	TGAAGGCGGTTCAGACGGC

NMB2090-Up-F	GACTGATAGAATTCCAATACCGCCAAAGCG
NMB2090-Down-R	ACTCGGTCAAGCTTTGATGCCCAACAGCGTG
ECBD3400-RBS-F	TTATGCTGAAGGATATCCTC
ECBD3400-RBS-R	AAGCTTGGCCGGCCTTACTTAACCATCTCTTTTT CAATC
Primers used for the mutagenesis of the $GmhA_{GC}$ conserved residues E65 and H183:	
E65A-F	CTTCGCCGCCGCAATGACCGGGC
E65A-R	TGTTGCGCGTCGGCAGCC
H183A-F	CCTGCTGATAGCCGCCATGTGCG
H138A-R	ATGTGGTTTTCCTGAATGC

1 Sequences recognized by restriction enzymes are underlined.

Chapter 3

NGO1985 is a surface-exposed lipoprotein interacting with BAM complex and a gonorrhea vaccine candidate

Igor H. Wierzbicki, Ryszard A. Zielke, Afrin A. Begum, Ann E. Jerse, Magnus Unemo, and Aleksandra E. Sikora

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Abstract

The sexually transmitted disease gonorrhea, caused by an obligate human pathogen, *Neisseria gonorrhoeae*, remains a significant burden on reproductive and neonatal health worldwide. Treatment of gonococcal infections has been continuously challenged by the rapid development of antimicrobial resistance. The development of a protective vaccine would be an ideal solution for containing gonorrhea and identification of new vaccine candidates is an important endeavor in this process great demand. Here we showed that NGO1985 is a surface exposed lipoprotein that is highly conserved and ubiquitously expressed among diverse *N. gonorrhoeae* strains. Deletion of *ngo1985* in recent gonococcal clinical isolates caused increase susceptibility to antibiotics currently and previously used in clinics. The Δ*ngo1985* mutant displayed decrease viability during conditions mimicking the host environment and was highly attenuated in the murine model of infection. Lack of NGO1985 results in compromised cell envelope integrity as well as altered protein profiles of cell envelopes and naturally released membrane vesicles. Interactome studies comprising of pull-down experiments coupled to mass spectrometry suggested that NGO1985 plays a role in the cell envelope homeostasis via its association with the β-Barrel Assembly Machinery (BAM) complex, antibiotic efflux pump(s) including Mtr, and several lipoproteins. We conclude that NGO1985 is crucial for proper cell envelope biogenesis and has great potential as a vaccine antigen.

Introduction

Cell envelopes are a crucial component of pathogenic bacterial species, functioning as an important interaction site with the host. Some components of the outer membrane are essential for bacterial viability, participation in nutrient acquisition, antibiotic resistance, and evasion of host defense mechanisms. Therefore, maintaining cell envelope homeostasis is indispensable for pathogen survival. Folding and assembly of β-barrel outer membrane proteins (OMPs) into to the outer membrane depends on the β-barrel assembly machinery (BAM) complex. The BAM complex has primarily been studied in *Escherichia coli* and *Neisseria meningitidis* and it is reported to be composed of five proteins: BamA-E (reviewed by Noinaj *et al*., 2015); however, BamB component was found to be absent in *Neisseria* species (Volokhina *et al*., 2009). Defects in individual BAM components result in different levels of impaired OMP assembly, leading to defects in cell envelope integrity and functionality.

Gonorrhea, a sexually transmitted disease caused by the by the Gram-negative bacterium *Neisseria gonorrhoeae* (GC) remains a global heath burden. Untreated gonococcal infections can lead to devastating health consequences including epididymitis in men and endometritis, salpingitis, ectopic pregnancy, or sterility in women (Edwards and Apicella, 2004). Neonates delivered from infected mothers are at high risk of conjunctivitis, which may result in blindness. Moreover, GC infections are associated with increased risk of acquisition and transmission of human immunodeficiency virus (HIV) type 1 (Bignell *et al*., 2013; Cohen *et al*., 1997; Tapsall *et al*., 2009; World Health Organization, 2012). Treatment of gonorrhea has been challenged by the rapid development of antibiotic resistance. The possibility of untreatable GC underscores the necessity of identification and validation of new molecular targets for development of new antibiotics and a protective gonorrhea vaccine(s).

To identify potential new vaccine candidates, a proteomic-driven antigen discovery approaches have recently been used (Zielke *et al*., 2014; Zielke *et al*., 2016). Among the proteins identified as ubiquitously expressed in four common laboratory strains – FA1090, MS11, F62, and 1291 – was NGO1985, a predicted outer membrane lipoprotein (Zielke *et al*., 2014). Additionally, this protein was similarly expressed by

FA1090 during *in vitro* conditions mimicking conditions encountered during infection of the human host (Zielke *et al*., 2016). Initial characterization of NGO1985 confirmed its localization to the cell envelopes and naturally released membrane vesicles (MVs). In addition, the Δ*ngo1985* strain was more susceptible than wild type (wt) GC FA1090 towards a variety of compounds, including host- and non-host derived detergents (bile salts, SDS) and antibiotics (polymyxin B, chloramphenicol). Based on these studies, NGO1985 was hypothesized to be involved in maintaining cell envelope integrity. The homologue of NGO1985 from *N. meningitidis* (NM), GNA2091, has been included as a fusion protein with factor H binding protein in the successful multicomponent meningococcal group B vaccine, the Bexsero vaccine (Serruto *et al*., 2012). The function of GNA2091 was associated with maintaining the cell envelope homeostasis, as a Δ*gna2091* mutant accumulated misassembled OMPs, was more susceptible to detergents, and exhibited reduced survival in whole human blood and serum (Bos *et al*., 2014; Seib *et al*., 2010).

Herein, we evaluated the potential of NGO1985 as a gonorrhea vaccine candidate and investigated its role in GC cell envelope homeostasis. We have found that NGO1985 is a surface exposed lipoprotein important for cell envelope permeability and virulence of GC. The NGO1985 interactome determined by Tandem Affinity Purification coupled with mass spectrometry revealed interactions between this protein and the BAM complex, in addition to efflux pumps and several other lipoproteins.

Materials and methods

Bacterial strains and growth conditions. Bacterial strains used in this study are listed in Supplemental Table S3.1. *Neisseria* species and *E. coli* were propagated using media and methods as described (Zielke *et al*., 2016).

Antibiotics were used at the following concentrations for GC: kanamycin 40 µg/mL, erythromycin 0.5 µg/mL, streptomycin 100 µg/mL, chloramphenicol 0.1 µg/mL, polymyxin B sulfate and ceftriaxone (as specified in the text); for NM: kanamycin 80 µg/mL, erythromycin 2 µg/mL, and ceftriaxone 0.0013 µg/mL; for *E. coli*: kanamycin 50 µg/mL, erythromycin 250 µg/mL, and tetracycline 20 µg/mL.

Genetic manipulations and site-directed mutagenesis. The genome sequences of GC FA1090 (NC_002946), NM MC58 (NC_003112), and *E. coli* BL21(DE3) (NC_012892) were used to design oligonucleotides with SnapGene software version 2.8 (GSL Biotech LLC). Genomic DNA from different GC isolates as indicated in the text, NM MC58, and *E. coli* BL21(DE3) were purified with the Wizard Genomic DNA Purification Kit (Promega). Plasmid DNA and PCR products were purified with QIAprep Spin Miniprep Kit (QIAGEN). Individual PCRs were performed with the appropriate primers synthesized by IDT, chromosomal or plasmid DNA as a template, and Q5® High-Fidelity DNA Polymerase (NEB). Genetic manipulations were performed using *E. coli* MC1061 strain. All constructs were verified by DNA sequencing at the Center for Genome Research and Biocomputing at Oregon State University (CGRB OSU). Transformation procedures for GC and NM were conducted as described previously (Alexander *et al*.,, 2004; Zielke *et al*., 2014).

A recombinant version of NGO1985 (rNGO1985) with C-terminal-6×His-tag was constructed by amplifying a fragment of NGO1985 (531 bp) lacking the *N*-terminal 34 amino acids with primer pair rNGO1985-F/rNGO1985-R and cloning into NdeI and XhoI cleaved pET28a. Analogously, the *C*-terminally-6×His-tagged BamE excluding the initial 19 amino acids (rBamE) was generation by amplifying a fragment of BamE (NGO1780) (342 bp) with primer pair rBamE-F and rBamE-R, and subsequent introduction into NcoI/HindIII digested pET28a.

To perform mutagenesis of the conserved cysteine (+1) in the predicted lipobox of NGO1985, the NGO1985 gene with its native ribosomal binding site (RBS) was

amplified with primers NGO1985-RBS-F and NGO1985-RBS-R. The 643 bp PCR product was sub-cloned into pUC18 digested with SmaI, resulting in pUC18-NGO1985. This plasmid served as a template in the site directed mutagenesis reactions as described in the instructions provided by the manufacturer using the QuikChange site-directed mutagenesis kit (Agilent Technologies) and appropriate primers listed in Supplemental Table S3.2. Subsequently, the individual mutated variants of NGO1985 were amplified with primers NGO1985-RBS-F and NGO1985-RBS-R, digested with FseI, and cloned into ScaI/FseI treated pGGC4, yielding pGCC4-NGO1985 C23A and pGCC4-NGO1985 C23S. The obtained constructs were individually introduced into the chromosome of FA1090Δngo1985 (Zielke *et al*., 2014).

For tandem affinity purification (TAP) experiments, strain FA1090 Δ*ngo1985*/P_{lac}*::ngo1985*-TAP expressing functional *C*-terminally TAP-tagged NGO1985 was created. First the genomic region encoding NGO1985 with native RBS and lacking the STOP codon was amplified with primers NGO1985-TAP-F and NGO1985-TAP-R. The 654 bp PCR product was digested with SacI/KpnI and cloned into similarly prepared pUC18, yielding pUC18-NGO1985(STOP). Vector pBS1479 (Puig *et al*., 2001) was used as a template for amplification of the TAP-cassette with primers NGO1985-TAP-F2 and NGO1985-TAP-R2. The 572 bp DNA fragment was subsequently digested with KpnI/BamHI and cloned into pUC18-NGO1985. The obtained pUC18-NGO1985-TAP was used to amplify the NGO1985-TAP with oligonucleotides NGO1985-RBS-F and NGO1985-TAP-R3. The resulting 1242 bp PCR product was digested with FseI and cloned into SacI/FseI treated pGCC4, yielding pGCC4-NGO1985-TAP. This plasmid was introduced to FA1090Δ*ngo1985* (Zielke *et al*., 2014). To provide a control for the pull-down experiments, the DNA sequence encoding the TAP cassette was amplified with primers TAP-F and TAP-R, fused to the RBS of *ngo1985* and placed under the control of the P_{lac} promoter. The 586 bp PCR product was treated with FseI and cloned into pGCC4. pGCC4-TAP was subsequently introduced into wt FA1090, yielding FA1090 P_{lac}*::*TAP. For verification of pull-down experiment results, additional strains were generated: Δ*bamE*, Δ*bamE*/P_{lac}*::bamE*, and Δ*bamE*/P_{lac}*::bamE*-TAP. In order to generate a deletion of *bamE*, the upstream region of the gene was amplified with primers BamE-Up-F and BamE-Up-R. The 757 bp

product was cleaved with SacI/KpnI enzymes and cloned to similarly treated pUC18K vector, yielding pUC18K-BamEUp. Subsequently, the downstream region from the gene encoding BamE was amplified with primer pair BamE-Down-F/BamE-Down-R and the obtained product (724 bp) was cloned into pUC18K-BamEUp with BamHI/HindIII enzymes. The final product, pUC18K-BamE, was used for an allelic exchange of *bamE* for the kanamycin resistance cassette, as described above. Deletion of *bamE* was confirmed by PCR with primers BamE-Ver-F and BamE-Ver-R using wt bacteria chromosomal DNA as controls and additionally by probing the whole cell lysates with antisera against BamE as described below. The strain complementing the deletion of BamE, Δ*bamE*/P_{lac}*::bamE* was generated by amplification of *bamE*, together with the RBS region, with primers BamE-RBS-F and BamE-RBS-R, and the 415 bp PCR product was digested with FseI and inserted into ScaI/FseI cleaved pGCC4 vector. pGCC4-BamE was introduced into the Δ*bamE* mutant as described above, and positive clones selected on GCB containing erythromycin were validated by PCR with pGCC4-Ver-F and pGCC4-Rev-R primers. The *C*-terminally TAP-tagged BamE was generated analogously as described for NGO1985. BamE with native RBS and without STOP codon was amplified with primers BamE-TAP-F and BamE-TAP-R, and the obtained 421 bp product was cloned into pUC18 with EcoRI/KpnI enzymes, yielding pUC18-BamE(STOP). Subsequently, TAP-tag was amplified with as described above with primers NGO1985-TAP-F2/ NGO1985-TAP-R2, digested with KpnI/BamHI, and introduced into pUC18-NGO1985. BamE-TAP was then amplified with BamE-TAP-F2 and NGO1985-TAP-R3, using pUC18-BamE-TAP vector as template, and cloned into the pGCC4 vector. The final construct, pGCC4-BamE-TAP, was introduced into the Δ*bamE* background.

The clean deletion of ngo1985 in different GC clinical isolates (Zielke *et al*., 2016) was constructed by homologous recombination using pUC18K-NGO1985 as described previously (Zielke *et al*., 2014). The absence of *ngo1985* was confirmed by PCR with primers NGO1985-Ver-F and NGO1985-Ver-R using chromosomal DNA isolated from corresponding wt bacteria as controls and additionally by probing the whole cell lysates with antisera against NGO1985 as described below.

The clean deletion of the *ngo1985* homologue in NM serogroup B strain MC58 (*gna2091*; locus tag NMB2091) was generated in the following steps. The 583 bp fragment of chromosomal DNA upstream from GNA2091 was amplified with primers GNA2091-Up-F and GNA2091-Up-R, digested with SacI/KpnI, and cloned into similarly treated pUC18K, yielding pUC18K-GNA2091Up. The downstream region of GNA2091 (546 bp) was amplified with oligonucleotides GNA2091-Down-F and GNA2091-Down-R, cleaved with BamHI/HindIII, and cloned into pUC18K-GNA2091Up. The obtained pUC18K-GNA2091 was used to transform MC58. Deletion of *gna2091* was confirmed by PCR with primers GNA2091-Ver-F and GNA2091-Ver-R using genomic DNA of wt MC58 as a control.

For trans-complementation studies, *ngo1985* from GC FA19, *gna2091* from NM MC58, and *yraP* from *E. coli* BL21(DE3) were amplified with primer pairs: NGO1985-RBS-F/NGO1985-RBS-R, GNA2091-RBS-F/NGO1985-RBS-R, and YRAP-RBS-F/YRAP-RBS-R, respectively. The obtained PCR fragments, comprised of 643 bp, 644 bp, and 610 bp respectively, were digested with FseI and cloned into SacI/FseI cleaved pGCC4 (Skaar *et al*., 2002) to yield pGGC4-NGO1985FA19, pGGC4-GNA2091, and pGCC4-YraP.

Assessment of conservation of NGO1985 at the nucleotide and amino acid levels was performed as described (Zielke *et al*., 2016). To sequence the *ngo1985* in 13 GC isolates collected at the Public Health–Seattle & King County Sexually Transmitted Disease clinic in 2011-2013 (Zielke *et al*., 2016) the chromosomal region encompassing ~700 bp upstream and downstream from the gene was amplified with primers NGO1985-Amp-F and NGO1985-Amp-R. The resulting individual 2 kb PCR products were sequenced from both DNA strands using NGO1985-Seq-F and NGO1985-Seq-R. Subsequently, the ClustalOmega available at the European Bioinformatics Institute (http://www.ebi.ac.uk/) was utilized to compare the sequences at both nucleotide and predicted amino acid levels between FA1090, NCCP11945 (http://www.ncbi.nlm.nih.gov/), the draft genome sequences of 14 different GC strains (downloaded from http://www.broadinstitute.org/), and WHO reference strains.

Purification of recombinant NGO1985 and generation of polyclonal antibodies. The rNGO1985 was purified using batch-gravity flow method. An

overnight culture of *E. coli* BL21(DE3) carrying pET28a-rNGO1985 was back diluted into 3.0 L of LB broth supplemented with kanamycin and incubated with aeration at 37 °C. The production of rNGO1985 was induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) during mid-logarithmic growth. Bacterial cells were collected by centrifugation 3 h after induction and suspended in lysis buffer comprised of 500 mM NaCl, 10 mM imidazole, 20mM Tris-HCl, pH 8.0, and complete EDTA-free Protease Inhibitor tablet (Roche). Bacteria were lysed by passaging six times through a French pressure cell at 12,000 psi. Unbroken cells and cell debris were removed by centrifugation at 16,000 × g for 30 min at 4 °C. The cell-free lysate was passed through a 0.22 μm filter unit (VWR International) and incubated with rotation at 4 ºC for 1 h with 1 mL of Ni-NTA agarose (Qiagen) equilibrated with lysis buffer. The sample/Ni-NTA slurry was applied onto a 5 mL polypropylene column (Thermo Scientific), washed with buffer containing 40 mM imidazole and rNGO1985 was eluted with 250 mM imidazole. The elution was dialyzed four times against 20 mM Tris-HCl pH 8.0. For protein storage, the buffer was supplemented with glycerol (10% w/v).

E. coli BL21(DE3) cultures harboring pET28-rBamE were treated the same way to allow expression of rBamE. After cell lysis the lysates were applied onto a 5 mL IMAC column (Bio-Rad) conditioned with the lysis buffer and connected to a NGC Purification System (Bio-Rad). The column was washed with 10 column volumes of the lysis buffer containing 3% of elution buffer (20 mM Tris pH 8.0, 500 mM NaCl, 250 mM imidazole) at a flow rate of 5 mL/min. Finally, the protein was eluted with 5 column volumes of elution buffer. Fractions containing rBamE were pooled together and dialyzed against 20 mM Tris pH 8.0, 10% glycerol.

Polyclonal antisera against purified rNGO1985 and rBamE were prepared by Pacific Immunology Corp. using a 13-week antibody production protocol and two New Zealand White rabbits under Animal Protocol #1 approved by IACUC and the NIH Animal Welfare Assurance Program (#A4182-01) in a certified animal facility (USDA 93-R-283).

Protein localization studies. Colonies of different GC strains, as specified in the text, were collected from GCB, suspended to OD_{600} of 0.1 in GCBL, and cultured in Fernbach flasks at 37 °C with shaking (220 rpm) until reached OD_{600} of 0.6-0.8.

Cells were separated from the supernatants by centrifugation (6,000 × g), lysed, and the cell envelopes fraction was extracted using sodium carbonate as described previously (Zielke and Sikora, 2014; Zielke *et al*., 2014). The supernatants were passed through the 0.22 µm filter units, a complete EDTA-free Protease Inhibitor tablet was added, and the naturally released membrane vesicles (MVs) were separated from the soluble protein fraction (SS) by ultracentrifugation at 210,000 × g, 3 h, 4 °C (Zielke and Sikora, 2014; Zielke *et al*., 2014). After ultracentrifugation, SS were concentrated using either trichloroacetic acid (TCA) or Pyrogallol Red (Sigma). In the first procedure, chilled 100 % TCA was added to the samples to 15% vol/vol. Following 1 h incubation at 4 °C, the samples were centrifuged at 14,000 × g, 20 min, 4 °C. Supernatant was decanted and the precipitated proteins were washed three times with ice-cold 100% acetone, air-dried, and finally suspended in phosphate buffered saline pH 7.5 (PBS, Li-Cor). For the second precipitation method, secreted proteins were mixed with the Pyrogallol Red in 1:1 ratio; the pH of the mixture was adjusted to 2.8, incubated 1 h at room temperature, and followed by overnight incubation at 4 °C. Following day, samples were centrifuged at 10,000 × g, 1 h, 4 °C. Supernatant was decanted, and pelleted proteins were washed with 100% acetone, and air-dried. The total protein concentration in obtained subproteome fractions [cytoplasm(C), cell envelopes(CE), MVs, and SS] was measured using DC Protein Assay (BioRad).

Immunodots. Assessment of gonococcal proteins surface localization by immunodots was performed as described previously (Zielke *et al*., 2016). Colonies of the GC wt FA1090, isogenic Δ*ngo1985*, Δ*ngo1985*/P_{lac}*::ngo1985*, Δ*ngo198*5 carrying either P_{lac}*::ngo1985*-C23A or P_{lac}*::ngo1985*-C23S, and NM MC58, isogenic Δ*gna2091*, Δ*gna2091*/P_{lac}*::gna2091* were suspended to OD_{600} of 0.1 in a pre-warmed to 37 °C GCBL. Bacteria were grown with aeration for 3 h. Cells were collected, suspended in GCBL to OD_{600} = 2.0 and 5 µL of the suspensions were applied to 0.45 µm nitrocellulose membrane. The nitrocellulose was air-dried for 30 min at room temperature and the immunoblotting was performed as described below.

Protease accessibility assays. Modified protocols described by Shewell *et al*. 2013 and Pinne *et al*., 2009 were used to examine surface accessibility of NGO1985. Colonies of wt FA1090 were suspended to OD_{600} of 0.1 in GCBL and incubated with

aeration at 37 °C for 3 h. Bacteria were collected by centrifugation for 10 min, 2,000 × g, 4 °C, and suspended to OD_{600} of 2.5 in PBS pH 8.0 - for trypsin treatment, or to OD_{600} of 2.0 in the same buffer supplemented with 5 mM $MgCl_2$ – for proteinase K treatment. Cell suspensions (500 µL) were incubated with 0, 40, 80 µg/mL of trypsin (NEB), or 0, 20, 40 µg/mL of proteinase K (NEB) for 1 h at 37 °C. Afterwards, 10 µL of 50 mM phenylmethanesulfonylfluoride (PMSF) was added, cells were washed with PBS, and collected by centrifugation.

Immunogold labeling. Parental wt strain of FA1090 and isogenic Δ*ngo1985* were inoculated to GCBL and incubated at 37 °C until mid-logarithmic phase. A 1 mL of each culture was gently pelleted and resuspended in 100 µL of filtered PBS. A drop of each culture was placed on a gold grid (Electron Microscopy Sciences), incubated for 30 min at room temperature, and an excess of the liquid was removed using blotting paper. Subsequently, cells were blocked for 1 h in 2% bovine serum albumin (BSA) in PBS following incubation with anti-NGO1985 antisera (1:50) prepared in the same buffer. Grids were washed three times for 5 min with filtered PBS containing Tween 20 at the final concentration of 0.05% (PBST), followed by 1 h incubation with 12 nm Colloidal Gold AffiniPure Goat Anti-Rabbit IgG (H+L) (EM Grade; Jackson ImmunoResearch Laboratories) at 1:100 dilution. Samples were washed with PBST, dried, and stained with 2% ammonium molybdate. Microphotographs of stained cells were captured using FEI Titan 80-200 TEM/STEM electron microscope at the Oregon State University Electron Microscope Facility.

Fitness assessments of Δ*ngo1985* cell during different *in vitro* growth conditions and cell envelopes purification. The colonies of wt FA1090, isogenic Δ*ngo1985*, and Δ*ngo1985*/P_{lac}*::ngo1985* were collected from GCB, suspended in GCBL to OD_{600} of 0.1, and incubated for 3 h at 37 °C with 220 rpm. Bacterial cultures were back-diluted to OD_{600} of 0.2 and either 100 or 5 µL aliquots (of undiluted and diluted bacteria) were spread or spotted, respectively, on solid media for concurrent growth under standard GCB, -Fe, +NHS, and $–O_2$ growth conditions, as described above. Bacteria that arose on solid media inoculated with 100 µL aliquots were harvested for isolation of the cell envelope fractions (Zielke and Sikora, 2014; Zielke *et al*., 2014). The CFUs were determined from spotted 5 µL aliquots after 18 and 48 h

for aerobic and anaerobic conditions, respectively. Experiments were performed in three biological replicates and mean values with corresponding SEMs are presented.

Sensitivity to ceftriaxone. Different GC strains, as specified in the text, were suspended in GCBL to OD_{600} of 0.1 and incubated for 3 h at 37 °C. Cultures were back-diluted to OD_{600} of 0.2 and 5 µL of undiluted and serially diluted bacteria were plated on GCB agar with different concentrations of ceftriaxone, and, for strains Δ*ngo1985*/P_{lac}*::ngo1985*, Δ*ngo1985*/P_{lac}*::ngo1985*-TAP, additionally with IPTG (0.04 mM or 1.0 mM, as specified). The CFUs were scored on following day and the relative viability was calculated by comparing CFUs/mL on GCB plates supplemented with ceftriaxone to the CFUs/mL on control plates. Experiments were performed three times on separate occasions and mean values with corresponding SEMs are presented.

Sensitivity to polymyxin B. FA1090 wt, Δ*bamE*, Δ*bamE*/P_{lac}*::bamE*, and Δ*bamE*/P_{lac}*::bamE*-TAP, were suspended in GCBL to OD_{600} of 0.1 and incubated for 3 h at 37 °C. Cultures were back-diluted to OD_{600} = 0.2 and 5 µL of undiluted and serially diluted bacteria were plated on GCB agar with or without polymyxin B 800 U/mL, and supplemented with IPTG (0.02 mM). The CFUs were scored on the following day and the relative viability was calculated by comparing CFUs/mL on GCB plates supplemented with polymyxin to CFUs/mL on control plates. Experiments were performed three times on separate occasions and mean values with corresponding SEMs are presented.

Antimicrobial susceptibility determined by E-tests. The Minimal Inhibitory Concentration (MIC) of cefpodoxime, cefotaxime, ceftriaxone, cefixime, cefuroxime, azithromycin, ciprofloxacin, polymyxin B, tetracycline, ampicillin, and benzylpenicilin was determined with the use of E-test reagent strips (Biomerieux) according to the manufacturer's recommendations.

Expression of NGO1985 during exposure to detergents and antibiotics. FA1090 cells were suspended in GCBL to OD_{600} = 0.1 and incubated for 3 h at 37 °C with aeration. Bacterial culture was then back-diluted to OD_{600} = 0.2 and 100 µL suspensions were plated on GCB supplemented with either SDS 0.01%, chloramphenicol 0.1 µg/mL, polymyxin B 400U/mL, or ceftriaxone 0.9 ng/mL. Cells were collected for immunoblotting analysis after 22 h. Expression of NGO1985 upon

exposure to these compounds was also examined during growth of GC in liquid media. The culture was back diluted to OD_{600} = 0.1 and maintained at 37 °C, with aeration, until reaching OD_{600} = 0.4. Bacteria were split into 1 mL aliquots and incubated for an additional 1 h at 37 °C, 220 rpm, in the presence of either SDS (0.0001%), chloramphenicol (0.1 μg/mL), polymyxin B (50 U), or ceftriaxone (0.9 ng/mL). The turbidity of the cultures was measured, bacterial cells were collected and subjected to SDS-PAGE as described below.

Experimental murine infection. Competitive infection studies were conducted as described previously (Jerse *et al*., 2002). Groups of 6 and 10 estradiol treated mice were inoculated intravaginally with a mixture containing either FA1090 wt and isogenic Δ*ngo1985* strain or FA1090 wt and Δ*ngo1985*/$P_{lac::}$*ngo1985*. The initial inoculum, per mouse, for wt/Δ*ngo1985* analysis was approximately 2.2×106 CFU/mL, while for wt/Δ*ngo1985*/P_{lac}*::ngo1985* was 2.1×106 CFU/mL. To minimalize possible transmission of antibiotic resistance cassettes during growth *in vivo*, non-piliated colonies were used for the experiment. Vaginal swabs were collected on days 1, 3, 5, and 7 post-inoculation, and equal amounts of undiluted and diluted bacterial suspensions were plated on GCB agar supplemented either with streptomycin alone, or streptomycin and kanamycin. The next day, CFUs were calculated, and the ratios of Δ*ngo1985* or Δ*ngo1985*/P_{lac}*::ngo1985* to the wt FA1090 strains were determined for input inoculum and output of the cultures obtained from vaginal swabs. Competitive index (CI) was calculated as described by Beuzon and Holden, 2001, by dividing the ratio of the derivative strain and the wt FA1090 in the output, by the ratio of these two strains in the input.

Isolation of NGO1985-associated complexes by Tandem Affinity Purification. Pull-down experiments were performed as described (Puig *et al*., 2001) with the following modifications. Equal amounts of cells derived from 1 L of mid-logarithmic cultures of FA1090 P_{lac}*::*TAP, Δ*ngo1985*/P_{lac}*::ngo1985*, and Δ*ngo1985*/P_{lac}*::ngo1985*-TAP were resuspended in 5 mL of IPP150 buffer (10 mM Tris pH 8, 300 mM NaCl, 0.1% NP-40) and lysed by passing three times through a French press. Unbroken cells and cell debris were pelleted at 12,000 × g for 20 min. The obtained cell lysates were incubated with 400 μL of IgG resin (GE Healthcare) for

4 h at 4 °C. Subsequently, the beads were washed six times with 6 mL of IPP150 buffer followed by a wash with 6 mL of TEV Cleavage buffer (10 mM Tris pH8, 300 mM NaCl, 0.1% NP-40, 0.5 mM EDTA, 1 mM DTT). Protein A tag was cleaved overnight in 1 mL of TEV Cleavage buffer using 50 U of acTEV protease (Invitrogen). The liberated complexes were mixed with 6 mL of Calmodulin binding buffer (10 mM Tris pH 8, 300 mM NaCl, 1 mM magnesium acetate, 1 mM imidazole, 2 mM CaCl2, 10 mM β-mercaptoethanol) and incubated with Calmodulin resin for 1 hour at 4 °C. The beads were washed 6 times with 6 mL of Calmodulin binding buffer and bound proteins were eluted from the Calmodulin resin by addition of 1 mL of Calmodulin elution buffer (10 mM TrispH8, 300 mM NaCl, 1 mM magnesium acetate, 1 mM imidazole, 20 mM EGTA, 10 mM 2-mercaptoethanol, 0.1% NP-40) followed by secondary elution with the same buffer. Protein fractions were combined and precipitated for 4 h at 4°C by addition of TCA to a final concentration of 15%. Protein pellets were washed twice with ice-cold acetone and reconstituted in SDS loading buffer, separated by SDS-PAGE and either silver stained (Invitrogen, SilverQuest™ Silver Staining Kit, according to manufacturer's recommendations) or subjected to Western blotting with selected sera, as indicated in the text.

Assessment of NGO1985 interactome. For mass spectrometry (MS) analysis, eluted proteins were precipitated overnight in 90% acetone at -20 °C, washed with ice-cold acetone and reconstituted in 100 µL of 0.5 M triethylammonium bicarbonate and 0.1% SDS. To reduce disulfide bonds, 5 µL of 50 mM Tris-(2-carboxyethyl) phosphine was added to each sample and the samples were incubated for 1 h at 60 °C. Cysteine residues were blocked by addition of 200 mM methyl methane thiosulfonate (MMTS). Digestion of proteins was performed with 5 µg of trypsin (Promega) for 24 h at 37 °C. Obtained peptides were analyzed at Fred Hutchinson Cancer Research Center Proteomic Core.

Data analysis was performed using Proteome Discoverer 1.4 (Thermo Scientific). The data were searched against the GC FA1090 database (downloaded on December 12, 2012) that included common contaminants. Trypsin was set as the enzyme with maximum missed cleavages set to 2. The tolerance for precursor ion and the fragment ion were set to 10 ppm and 0.6 Da, respectively. The variable

modifications were set as follows: oxidation on methionine (+15.995 Da) and carbamidomethyl on cysteine (+57.021 Da). Data were searched using Sequest HT. Only proteins identified with 1% FDR based on at least two unique peptides with ≥95% confidence were recorded.

The Clusters of Orthologous Groups (COG) functional categories were assigned to the identified proteins using WebMGA (Tatusov *et al*., 1997; Wu *et al*., 2011).

SDS-PAGE and immunoblotting. As described previously (Zielke *et al*., 2016)(Chapter 2). Samples containing gonococcal whole cell lysates were obtained, as specified in the text, from either colonies collected from GCB after 22 h of aerobic or 48 h of anaerobic growth, from bacteria cultured in GCBL, or recovered from infected mice. The whole cell lysates of *E. coli* ER2738 derived from overnight cultures of bacteria maintained in LB at 37 °C. Samples of purified rNGO1985 and isolated subproteome fractions were normalized as specified in the text by either the amount of protein (µg) or the same OD_{600} units, while whole cell lysates were loaded onto SDS-PAGE based on OD_{600} values; cell lysates of bacteria recovered from infected mice were standardized either by equal bacterial counts (3,180 CFUs) or by sample volume (15 µL, approximate CFUs as indicated, day 1: 90,000; day 3: 23,175; day 5: 3,180). Samples were boiled in SDS sample buffer containing 50 mM DTT and were separated in 15% polyacrylamide gels or in 4-12% NuPAGE® Bis-Tris Precast Gels (Life Technologies). After electrophoresis proteins were stained in Coomassie Briliant Blue G-250 or transferred onto GE Amersham Protran nitrocellulose membrane (0.2 µm or 0.45 µm for proteins with molecular weight below or above 20 kDa, respectively) using Trans-blot Turbo (Bio-Rad) system. Membranes were incubated in 5% milk in PBS with 0.1 % Tween 20, followed by incubation with primary antibodies, then in HRP conjugate goat anti-Mouse IgG (H+L) or HRP conjugate goat anti-Rabbit IgG (H+L) Secondary Antibodies (1:10,000) (ThermoFisher Scientific). Immunoblots were developed using Clarity Western ECLSubstrate (BioRad) and visualized with Chemi-DocTM MP System (BioRad). Primary antibodies were used in following concentrations: anti-NGO1985 (1:30,000), anti-BamE (1:20,000), anti-BamA (1:20,000), anti-BamD (1:20,000), anti-LptD (1:5,000), anti-TamA (1:5,000), anti-

AniA (1:10,000), anti-Laz (1:20,000); anti-NGO2054 (1:10,000), anti-NGO2139 (1:30,000), anti-Obg (1:5,000), anti-Zwf (1:10,000), anti-GmhA (1:10,000), anti-SurA (1:10,000), anti-DsbA (1:10,000), anti-MtrE (1:5,000), anti-Ng-MIP (1:20,000).

Isolation and silver staining of lipooligosaccharide. As described in Chapter 2. Lipooligosaccharide was isolated from GC isolates based on the protocol described by Hitchcock and Brown, 1983. FA1090, isogenic Δ*ngo1985* mutant, and Δ*ngo1985*/P_{lac}*::ngo1985* were reconstituted in GCBL to OD_{600} of 0.2 and 1.5 mL of the cell suspensions were pelleted down by 1.5 min centrifugation at 15,000 x g. Cells were suspended in 50 µL of lysis buffer composed of 2% SDS, 4% β-mercaptoethanol, 10% glycerol, 1M Tris-HCl pH 6.8, 0.01% bromophenol blue and incubated for 10 min at 100 °C. After the samples were to cool down to room temperature, 25 µg of proteinase K in 10 µL of lysis buffer was added and incubated for 1 h at 60 °C. Isolated lipooligosaccharide was resolved on 16.5% Mini-PROTEAN® Tris-Tricine Gel (BioRad) and visualized by silver staining method described by Tsai and Frasch, 1982.

Densitometry analysis. Protein abundance was determined by densitometry as described previously (Park *et al*., 2015; Zielke *et al*., 2015). To quantify the protein abundance, western blots, developed as described above, were subjected to densitometry analysis using Image Lab 5.0 software (Bio-Rad). Intensities of un-processed and processed NGO1985 were quantified by volume tool (rectangle), local background subtraction, and linear regression method. Experiments were performed three times on separate occasions and mean values with corresponding SEMs are presented.

Statistical analysis. GraphPad Prism's build-in t-test was used for determination of statistically significant differences between obtained experimental results. A confidence level of 95% was used for all analyses.

Results

NGO1985 is a surface exposed lipoprotein conserved among GC isolates. The domain architecture of NGO1985 includes a signal peptide containing the lipobox motif LALGGCF (predicted by LIPO algorithm http://services.cbu.uib.no/tools/lipo) with a conserved cysteine residue (C23), and two BON (Bacterial OsmY and Nodulation) domains, which are characteristic for *E. coli* protein OsmY involved in osmotic shock resistance, a group of nodulation proteins, haemolysins, as well as secretory channels (Yeats and Bateman, 2003) (Fig. 3.1 A).

Lack of NGO1985, similar to its homologue GNA2091 in NM (Seib *et al*., 2010), did not result in increased sensitivity to osmotic stress (Fig. S3.1), indicating that it does not share functional similarity with OsmY. To study and understand the role of NGO1985, a recombinant, *C*-terminally His-tagged protein, excluding the initial 34 amino acids (rNGO1985; 21.81 kDa) was purified and used to raise polyclonal rabbit antibodies (Fig. 3.1 A and B). Anti-NGO1985 antisera cross-reacted with cell lysates of wt FA1090 and Δ*ngo1985*/P_{lac}*::ngo1985* strains (size of mature protein excluding signal peptide predicted to be 19.42 kDa) as well as with rNGO1985, and no protein was detected in the Δ*ngo1985* strain (Fig. 3.1 C).

NGO1985 is a predicted outer membrane (OM) lipoprotein, and initial studies suggested that it associates with cell envelopes and MVs. We confirmed the localization of NGO1985 to the cell envelope and membrane vesicles by probing sub-proteome fractions of wt FA1090 with anti-NGO1985 antibodies (Fig. 3.1 D). OM lipoproteins are described in the literature to either localize to the inner membrane, periplasmic leaflet of the OM or to associate with the cell surface (reviewed by Konovalova and Silhavy, 2015). Surface accessibility is an important consideration for a potential vaccine target; therefore, the orientation of NGO1985 within the OM was evaluated by multiple approaches. Immunogold labeling coupled with Transmission Electron Microscopy of wt FA1090 showed golden particles distributed throughout the surface of the diplococcus while in contrast, scarce particles were present of the Δ*ngo1985* strain (Fig. 3.1 E). The ability of antibodies to recognize NGO1985 on the surface of the GC cells was further verified by immunodot experiments. Anti-NGO1985 antibodies cross-reacted with intact cells of wt FA1090 and

Δ*ngo1985*/P_{lac}*::ngo1985*. Similarly, antibodies against surface exposed protein BamA bound to these strains as well to the Δ*ngo1985* mutant (Fig. 3.1 E) (Zielke *et al*., 2016). Neither BamD nor Obg antisera bound intact cells, as anticipated, because their protein targets are localized to the inner leaflet of the outer membrane and the cytoplasm, respectively (Zielke *et al*., 2016). Antisera against both proteins cross-reacted with lysed cells and recombinant proteins. Finally, surface exposure of NGO1985 was verified by assessing protein accessibility to trypsin or proteinase K. Western blotting analysis of treated intact cells indicated that NGO1985 was accessible for protease processing, similarly to BamA (Fig. 3.1 G). Unaltered levels of BamD and Obg, as well as overall unaffected protein profiles in the presence of either of the enzymes indicated that cells were not lysed during handling (Fig. 3.1 G and H).

The invariant cysteine within the lipobox motif of lipoproteins is involved in protein maturation and serves as the site for addition of a lipid membrane anchor (reviewed by Konovalova and Silhavy, 2015). The importance of C23 for NGO1985 maturation was examined by site directed mutagenesis, replacing this residue with either alanine (C23A) or serine (C23S; Fig 3.1A). Alanine was chosen because of the lack of any charge or functional group on its side chain, while serine was selected because the side chain is similar in shape to cysteine. The two variants of NGO1985 were individually cloned under the control of an IPTG inducible promoter and introduced into FA1090 Δ*ngo1985*. Western blotting analysis revealed that changing cysteine to either alanine or serine led to the presence of two forms of NGO1985, ~ 21 and 18 kDa, which likely corresponded to the unprocessed- and mature-lipoprotein, respectively (Fig. 3.1 I). Densitometry analysis showed that there was no statistically significant difference between the average abundance of either form of the protein (Fig. 3.1 J). To test whether alterations of the conserved cysteine affect the surface localization of NGO1985, immunodot experiments were performed. The anti-NGO1985 antibodies failed to react with intact cells of GC expressing either NGO1985 C23A or NGO1985 C23S (Fig. 3.1 K). In contrast, the protein was detected when lysed gonococci were probed with antibodies.

Together, these studies demonstrated that C23 within the predicted lipobox motif of NGO1985 is critical for protein maturation and localization to the outer surface of the GC cell envelope.

A suitable vaccine antigen should be expressed by a wide range of gonococcal isolates. Therefore, we examined the level of NGO1985 expression in a panel of temporally and geographically diverse GC isolates, including the 2016 WHO reference strains (Unemo *et al*., 2016). Anti-NGO1985 antisera cross reacted with cell lysates derived from all 36 strains examined, and similar levels of NGO1985 were detected in all strains (Fig. 3.1 L). No signal was detected for the *E. coli* cell lysate.

The nucleotide- and amino acid level conservation of NGO1985 among GC isolates was also investigated. Analysis of 118 NGO1985 sequences derived from available draft genomes (NCBI, Broad Institute), isolates from the Seattle STD Clinic, contemporary isolates from Europe, and the 2016 WHO reference strains indicated only 22 single nucleotide polymorphic sites (4.3%), and 11 amino acid changes (5.4%).

Together, these studies demonstrate that NGO1985 is a surface exposed lipoprotein, ubiquitously expressed and highly conserved among distinct GC isolates.

NGO1985 and GNA2091 compensate each other's function despite different orientation within the outer membrane. NGO1985 and GNA2091 share 96% similarity at the amino acid level. As a result, rabbit antisera raised against NGO1985 cross-reacted with GNA2091 (Fig. 3.2 A). Despite the high degree of similarity, GNA2091 localized mainly to the periplasmic side of the OM (Bos *et al*., 2014). Therefore, we decided to investigate whether these proteins function interchangeably. For these studies, we generated FA1090 Δ*ngo1985*/P_{lac}*::gna2091* and in NM MC58 background: Δ*gna2091*, Δ*gna2091*/P_{lac}*::gna2091*, and Δ*gna2091*/P_{lac}*::ngo1985*. Additionally, FA1090 Δ*ngo1985* strains expressing the homologue of NGO1985 from *E. coli*, YraP (34% amino acid identitity with NGO1985); as well as NGO1985 from GC strain FA19, bearing a V to A substitution at position 31, were engineered. In our initial studies regarding NGO1985, we observed that absence of this protein resulted in increased susceptibility to membrane perturbing agents and chloramphenicol, suggesting an overall alteration in the cell envelope integrity. Similarly, the Δ*ngo1985* mutant had a severe growth defect on media

containing ceftriaxone, which was fully complemented by the reintroduction of wt allel *ngo1985* (Fig. 3.2 B). We further used the ceftriaxone sensitivity phenotype as means to assess the functionality of NGO1985. Expression of NGO1985 A31V from the FA19 strain and GNA2091 in the Δ*ngo1985* strain reversed the ceftriaxone-sensitive phenotype (Fig. 3.2 B). In contrast, *E. coli* YraP did not rescue the antibiotic susceptibility of the Δ*ngo1985* strain. Further, the growth of the NM MC58 Δ*gna2091* strain was impaired in the presence of ceftriaxone (Fig. 3.2 B) and as with GC, expression of either GNA2091 or NGO1985 in the Δ*gna2091* background led to restoration of ceftriaxone resistance to the wild type level.

Together, these findings provide evidence that, despite the difference in subcellular localization, NGO1985 and GNA2091 function interchangeably. Additionally, we have determined that the single amino acid polymorphism in NGO1985 in the FA19 strain did not affect the protein's function.

NGO1985 contributes to GC antibiotic resistance. Following the ceftriaxone-sensitivity phenotype of Δ*ngo1985* in FA1090, we have investigated the importance of NGO1985 in recent GC isolates with decreased susceptibility to antibiotics. Δ*ngo1985* derivatives of strains isolated from the Public Health–Seattle & King County Sexually Transmitted Disease clinic (2011-2013) were generated and validated by western blotting (Fig. S3.2). All Δ*ngo1985* mutants displayed a statistically significant decrease in viability compared to the wt strains in the presence of ceftriaxone (Fig. 3.3 B). Antibiotic susceptibility of the isogenic Δ*ngo1985* progenies was further analyzed by E-test strips containing 11 antibiotics with distinct mechanisms of action: inhibition of peptidoglycan, translation, and DNA synthesis. Overall, we observed the decrease of MICs ranged from 2 up to 8-fold between original and Δ*ngo1985* derivatives (Fig. 3.3 A). Moreover, FA1090 Δ*ngo1985*/P_{lac}*::ngo1985* mutant overexpressing NGO1985 (1 mM IPTG) was more resistant to higher concentrations of ceftriaxone compared to wt and Δ*ngo1985*/P_{lac}*::ngo1985* expressing native levels of NGO1985 (0.04 mM IPTG) (Fig. 3.3 C).

These results showed that NGO1985 was involved in susceptibility to antibiotics of contemporary GC isolates.

NGO1985 expression patterns and importance for GC survival during murine model of infection. In NM, lack of GNA2091 caused decreased doubling time (Bos *et al*., 2014). The absence NGO1985 in GC did not affect growth under standard aerobic conditions, as there was no significant difference in cell doubling time between the wt and Δ*ngo1985* strain [63 and 67 minutes respectively (Fig. 3.4 A)]. Analysis of NGO1985 abundance during different stages of bacterial growth revealed that expression increased upon entering and during stationary phase, suggesting growth-phase dependent regulation of NGO1985 expression (Fig. 3.4 B). Next, Δ*ngo1985* was exposed to growth conditions mimicking some of the microniches within the human body to test the importance of NGO1985 for survival within the host. The Δ*ngo1985* mutant exhibited an overall decrease in colony forming units (CFUs) under standard growth conditions and during iron limitation (Fe-) on solid media, and a dramatic loss of viability when exposed to normal human serum (NHS) and during anoxia (O_2-) (Fig. 3.4 C). The levels of NGO1985 remained unaltered (Fig. 3.4 D) corroborating our iTRAQ experiments (Zielke *et al*., 2016). Moreover, NGO1985 abundance didn't change when bacteria were probed with different compounds in either solid or liquid media (Fig. 3.4 D).

Next, we wanted to determine whether NGO1985 plays an important role during GC growth *in vivo* by employing a murine model of GC infection. Initially, we investigated NGO1985 expression during colonization of the estradiol-treated mouse vaginal tract (Jerse *et al*., 2002). Immunoblot analysis of GC recovered from vaginal swabs showed that NGO1985 is expressed throughout infection, albeit at higher levels at later days from inoculation (Fig. 3.4 E). Subsequently, competitive infections between wt and Δ*ngo1985* or wt and Δ*ngo1985*/P_{lac}*::ngo1985* strains were performed. Competitive index (CI) values for every mouse were calculated at days 1, 3, 5, and 7 post-infection for each tested pair of strains (Fig. 3.4 F). The low CI values (ranging from 0.061 to 0.000003) observed in the wt and Δ*ngo1985* co-infection indicated that the mutant was highly (100 to 1000-fold) attenuated. In comparison, the CI values for wt and Δ*ngo1985*/P_{lac}*::ngo1985* ranged from 1.85 to 0.037 and only on the last day the values were very similar to the wt/Δ*ngo1985* competition.

These experiments demonstrated that NGO1985 plays a crucial role GC viability *in vivo* as well as under in vitro conditions related to host infection.

Absence of NGO1985 causes alterations in cell envelope and membrane vesicle protein profiles. In NM, absence of GNA2091 was associated with disruption of membrane integrity, resulting in increased release of MVs and elevated protein content in culture supernatants. To investigate whether absence of NGO1985 causes similar phenotypes in GC, culture supernatants were collected at mid-logarithmic (3h) and stationary (6h) phases of growth, and fractionated into soluble protein fraction (SS) and MVs. Coomassie staining of culture supernatants showed that at the 3h time point, the Δ*ngo1985* strain released substantially more SS and ~ 3-fold more MVs (Fig. 3.5 A and B, respectively). Increased vesiculation among bacteria has been related to cell envelope stress and as a means of removing misfolded proteins (McBroom and Kuehn, 2007). Analysis of LOS showed no significant differences between the strains, suggesting that the compromised integrity of the cell envelope was not due to defects in LOS (Fig. 3.5 C). Supernatants from stationary phase, however, showed much fewer differences. Nevertheless, further analysis of supernatants by western blotting demonstrated that the Δ*ngo1985* mutant released from both time points contained more periplasmic (SurA, DsbA), inner membrane-cytoplasmic (Obg), and cytoplasmic (Zwf, GmhA) protein contents (Fig. 3.5 D). Simultaneous analysis of whole cell lysates (WC) of the Δ*ngo1985* strain revealed decreased abundance of the periplasmic chaperones SurA and DsbA, while levels of Glucose-6-phosphate 1-dehydrogenase, Zwf, were increased (Fig. 3.5 E). SurA facilitates transport of OMPs in the periplasm and prevents their aggregation, while DsbA participates in proper protein folding by introducing disulfide bonds (Heras *et al*., 2009; Sklar, Wu *et al*., 2007). Zwf was found to be upregulated during membrane stress caused by bile salts, as well as in the presence of reactive oxygen species, and was shown to be important for resistance to reactive oxygen and nitrogen intermediates (Bernstein *et al*., 1999; Merritt and Donaldson, 2009; Lundberg *et al*., 1999; Sandoval *et al*., 2011). The lower levels of periplasmic chaperones in the absence of NGO1985 suggested OMPs misfolding that could result in cell envelope stress.

To further analyze consequences of NGO1985 absence on GC membrane integrity, cell envelopes and MVs collected during mid-logarithmic growth were analyzed. The overall cell envelope protein profiles visualized by Coomassie Blue staining did not differ between analyzed strains (Fig. 3.5 F). Western blotting analysis indicated, however, decreased amounts of a major component of the LOS transport system, LptD (Bos *et al*., 2004; Braun and Silhavy, 2002; Ruiz *et al*., 2009), as well as part of the autotransporter secretion system, TamA (Selkrig *et al*., 2012) in the Δ*ngo1985* strain (Fig. 3.5 G). The decrease in TamA amounts was also consistently observed in cell envelopes derived from colonies of Δ*ngo1985* collected from solid media during standard conditions, in the presence of NHS, and from anaerobic growth (Fig. S3.3 C and D). In contrast, lower levels of LptD were observed only during anaerobiosis (Fig. S3.3 C and D).

The Δ*ngo1985* MVs protein profile exhibited apparent alterations in comparison to wt (Fig. 3.5 F) and among 11 proteins analyzed by western blotting, only MtrE abundance was not changed (Fig. 3.5 G).

Taken together, our results suggested that absence of NGO1985 caused decreased periplasmic chaperone abundance affecting the composition of certain OMPs (e.g. TamA and LptD) and functionality of the GC cell envelopes.

NGO1985 co-purifies with Bam machinery, Mtr, and several lipoproteins. Bacterial cell envelope homeostasis is a coordinated process mediated by many proteins, including those that often form large protein complexes of unique composition and structure. To better understand the function of NGO1985 in the cell envelope, we aimed to characterize the NGO1985 interactome by tandem affinity purification (Fig. 3.6 A). For this purpose, a strain expressing functional NGO1985 *C*-terminally fused with a TAP tag was generated (Fig. S3.4 A and B). SDS-PAGE and silver staining of purified protein complexes revealed multiple bands for the Δ*ngo1985*/P_{lac}*::ngo1985*-TAP while few or no bands were detected for the two control strains, Δ*ngo1985*/P_{lac}*::ngo1985* and P_{lac}*::*TAP (Fig. 3.6 B). Tandem mass spectrometry of purified complexes identified 332 proteins for Δ*ngo1985*/P_{lac}*::ngo1985*-TAP, 128 proteins for P_{lac}*::*TAP and 5 proteins for Δ*ngo1985*/P_{lac}*::ngo1985* (Fig. 3.6 C) with 246 proteins uniquely identified in the

Δ*ngo1985*/P_{lac}*::ngo1985*-TAP fraction (Table S3.3). Cluster of Orthologous Groups (COG) analysis revealed that the identified proteins belonged to many classes. Excluding the proteins within the Poorly Characterized group, the most represented classes were 1) Cell wall/membrane/envelope biogenesis (31 proteins), 2) Energy production and conversion (20 proteins), and 3) Amino acid transport and metabolism (17 proteins) (Fig. 3.6 E). Western blot analysis with available antibodies confirmed that among the proteins co-purified in complex with NGO1985 were components of membrane biogenesis β-barrel assembly machinery (BAM), BamA, BamD, and BamE; a member of the Mtr efflux pump, MtrE; and OM lipoproteins: Laz, AniA, Ng-MIP, NGO2139 and NGO2054 (Fig. 3.6 D).

The BAM complex contributes to the maintenance of cell envelope integrity due to its involvement in folding and assembly of β-barrel OMPs into to the outer membrane (reviewed by Noinaj *et al*., 2015). To validate the potential interaction of NGO1985 with the BAM complex, a strain expressing functional TAP-tagged BamE was generated (Fig. S3.4 C and D). Multiple protein bands were detected for protein complexes purified from Δ*bamE*/P_{lac}*::bamE*-TAP by silver staining, and, as expected, immunoblotting revealed that BamE co-purified with other components of the BAM complex as well as with NGO1985. Moreover, proteins found in complex with BamE included Laz, AniA, Ng-MIP, NGO2139, NGO2054, as well as LptD and TamA. Thus, the role of NGO1985 in cell envelope integrity is possibly mediated through interaction with the BAM complex.

Discussion

In this work, we characterized the predicted OM lipoprotein NGO1985 as a potential vaccine candidate and investigated its role in cell envelope homeostasis. Our data showed that NGO1985 is exposed on the surface of GC cells (Fig. 3.1 E-H). In contrast, the NM homologue GNA2091 was proposed to localize to the periplasmic side of the OM (Bos *et al*., 2014). However, later studies showed that GNA2091 is recognized by a component of the innate immune system, PTX3 (Bottazzi *et al*., 2015), suggesting that it could be at least partially exposed to the extracellular milieu. Nevertheless, the literature describes several examples of GC and NM homologous proteins, including NspA and fHbp that display differences in localization between species, despite high amino acid coverage (Jongerius *et al*., 2013; Moe *et al*., 1999).

Cysteine residues within lipobox motifs are essential for maturation of lipoproteins. Mutations of C23 in NGO1985 led to expression of two protein forms, ~21 and ~18 kDa, corresponding to full length and mature NGO1985, respectively (Fig. 3.1 I). Supporting these findings, in *Listeria monocytogenes* and *Streptococcus agalactiae*, cysteine was shown not to be necessary for signal peptide cleavage (Baumgartner *et al*., 2007; Henneke *et al*., 2008). Additionally, it has been shown that the RseP protein of *E. coli* (NGO1800 in GC) can be responsible for the proteolytic cleavage of diverse signal peptides (Saito *et al*., 2011).

Absence of NGO1985 manifested itself through increased susceptibility to different classes of antibiotics, decreased viability during in vitro conditions relevant to GC infection sites, and attenuation during the *in vivo* model of infection (Figs. 3.3 A and B; and 3.4 C and F). Maintenance of steady state levels of NGO1985 during *in vitro* growth conditions as well in the presence of chemical probes suggested that the phenotypes observed in Δ*ngo1985* are the result of a general cell envelope defect. Compromised membrane integrity was suggested by increased protein content in Δn*go1985* supernatants (Fig. 3.5 A). Moreover, increased vesiculation and upregulation of Zwf in the Δ*ngo1985* mutant suggested cell envelope stress (Fig. 3.5 B, D and E) (Bernstein *et al.*, 1999; McBroom and Kuehn, 2007; Merritt and Donaldson, 2009). However, protein profiles of cell envelopes were not dramatically altered in the absence of NGO1985, and among analyzed proteins, only TamA was

consistently lower in abundance, while LptD was downregulated during certain growth conditions (Fig. 3.5 G, Fig. S3.3 C and D). The decrease in periplasmic chaperones SurA and DsbA observed in the Δ*ngo1985* background could cause impaired OMP folding and aggregation (Fig. 3.5 D and E). Proper folding of LptD depends on both SurA and DsbA (Denoncin *et al*., 2010). Because vesiculation is a way of relieving cells of misfolded and toxic proteins (McBroom and Kuehn, 2007), elevated levels of all three analyzed OMPs (BamA, TamA, LptD) in Δ*ngo198*5 MVs could result from their improperly folded state (Figs. 3.5 G, and S3.3 A). Moreover, MVs of the Δ*ngo1985* mutant contained high levels of several OM lipoproteins, implying that their transport and/or assembly might be affected as well.

Analysis of NGO1985 and co-purified proteins revealed a broad network of interactions including the BAM complex, multidrug efflux pumps, and several surface exposed lipoproteins (Table S3.3, Fig. 3.6 D). NGO1985 interaction with the BAM complex was validated by the detection of NGO1985 in pull down experiments with BamE (Fig. 3.6 G). BAM is involved in folding and inserting OMPs into the OM, thus it is essential for cell envelope biogenesis and integrity. In *E. coli*, this complex is composed of the integral membrane protein BamA and four accessory lipoproteins, BamB, C, D, and E. *Neisseria* genomes, however, do not encode the BamB homologue. In NM, an additional component of BAM was identified. The RmpM protein was required for stability, but not folding, of OMP complexes (Volokhina *et al*., 2009). GNA2091 was suggested to be associated with BAM complex, although this interaction could not be shown (Bos *et al*., 2014). BamB was recently shown in *E. coli* to be crucial for BamA folding and biogenesis *in vivo* (Misra *et al*., 2015). Our observation of high levels of BamA, presumably aggregates, in Δ*ngo1985* MVs suggest that in GC, NGO1985 could be involved in regulating BamA stability.

In conclusion, we reported that lipoprotein NGO1985, is a novel component of the BAM complex, participating in biogenesis and homeostasis of the GC cell envelope. The importance of NGO1985 in antibiotic resistance and during infection of the female mouse model, as well as its surface exposure and conservation across gonococcal isolates, makes it a promising candidate for gonorrhea vaccine development.

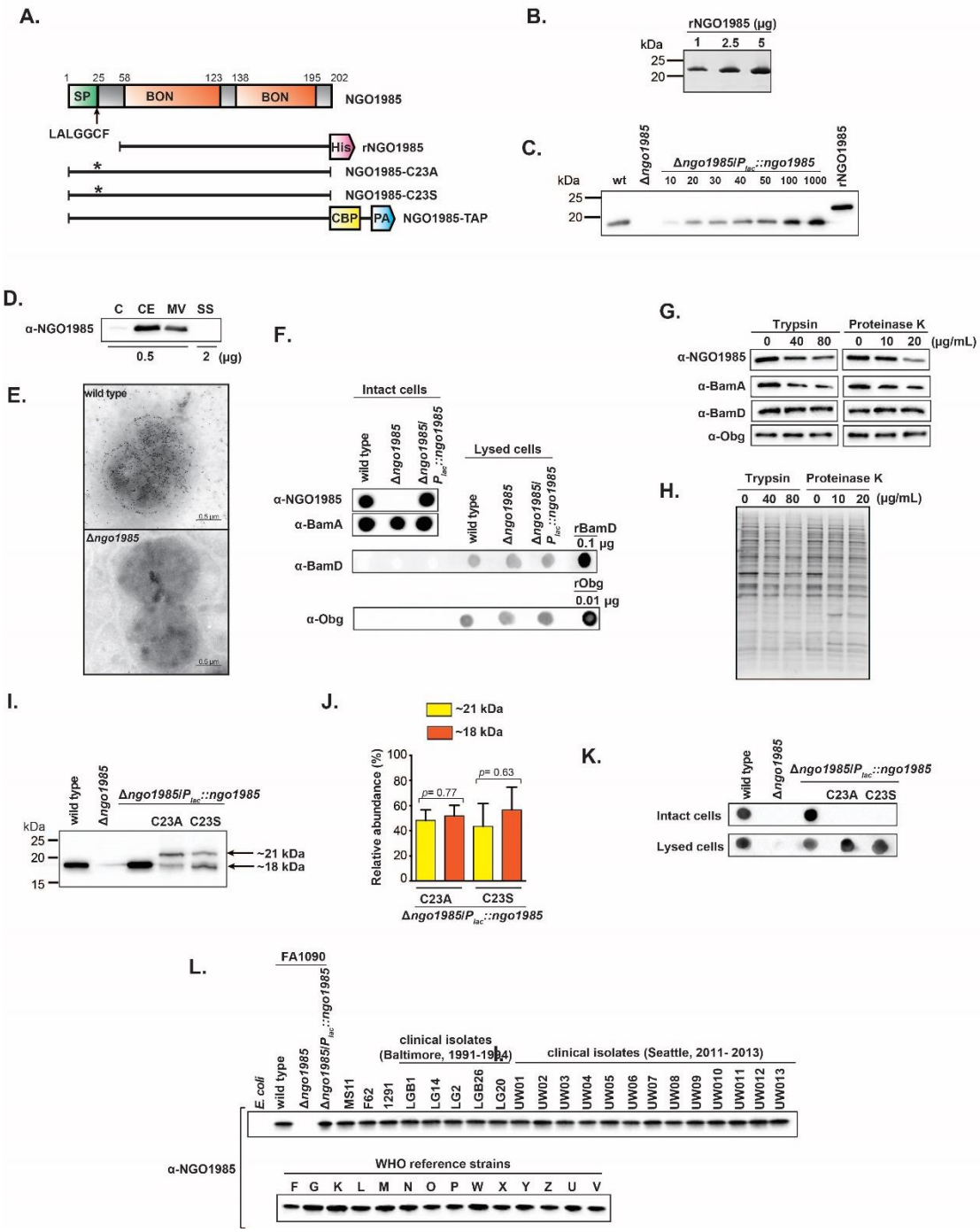


Figure 3.1. NGO1985 is a surface localized lipoprotein conserved among GC isolates. (A) Schematic of predicted domains and motifs of native NGO1985 (SP – signal peptide, LALGGCF – lipobox motif, BON - bacterial OsmY and nodulation domain) and outline of recombinant NGO1985 constructs used: for protein purification (rNGO1985), for assessment of important of cysteine in the putative lipobox, and for

interactome studies (C-terminally TAP-tagged NGO1985; CBP - calmodulin binding protein, PA – Protein A). (B) rNGO1985 was resolved by SDS-PAGE and visualized with Coomassie Brilliant Blue G250 (CBB). (C) Whole cell lysates normalized based on OD_{600} units and rNGO1985 (0.02 µg) were resolved in 15% Tris-glycine gel and probed with polyclonal rabbit anti-NGO1985 antisera. Migration of the protein weight marker is indicated on the left (kDa). (D) Analysis of NGO1985 subcellular localization. Subproteome fractions: cytoplasm/periplasm (C), cell envelopes (CM), membrane vesicles (MVs), and soluble secreted proteins (SS) were probed with anti-NGO1985 antisera. (E) Examination of NGO1985 surface localization using immunogold labeling coupled with transmission electron microscopy. Wt FA1090 and Δ*ngo1985* strains were cultured in GCBL medium till mid-log phase of growth (OD_{600}≈0.8). Cultures were harvested and reconstituted in PBS. Cell suspensions were placed onto a gold grid and incubated at room temperature. Grids were blocked in BSA, and incubated with anti-NGO1985 antisera. Following washing, grids were probed with secondary antibodies conjugated with 12 nm gold particles, stained with 2% ammonium molydbate, and visualized by TEM. (F) Immunodot analysis. Intact cells of wt, Δ*ngo1985*, and Δ*ngo1985*/P_{lac}*::ngo1985* were spotted onto nitrocellulose and probed with different antisera, as indicated. Controls include: BamA, BamD, Obg, lysed GC cells, and purified recombinant proteins Obg and BamD. (G, H) Intact GC cells were incubated in the presence or absence of trypsin/proteinase K. Whole cell lysates were normalized based on OD_{600} units, separated by SDS-PAGE, and either probed with different antibodies (G) or visualized with CBB (H). (I) Whole cell lysates of different GC strains, as indicated, were normalized based on OD_{600} units, separated by SDS-PAGE, and probed with anti-NGO1985 antisera. (J) The abundance of unprocessed (21 kDa) and mature (18 kDa) forms of NGO1985 C23A and NGO1985 C23S was assessed by densitometry (n=3 ± SEMs). (K) Intact and lysed cells of indicated GC strains were spotted on nitrocellulose and probed with anti-NGO1985 antisera. (L) Whole cell lysates of GC were normalized by OD_{600} units, separated on a 4-20% Tris-glycine gel, and probed with anti-NGO1985 antibodies. As positive controls, wt FA1090 and Δ*ngo1985*/P_{lac}*::ngo1985* were used, while *E. coli* ER2738 and Δ*ngo1985* served as negative controls.

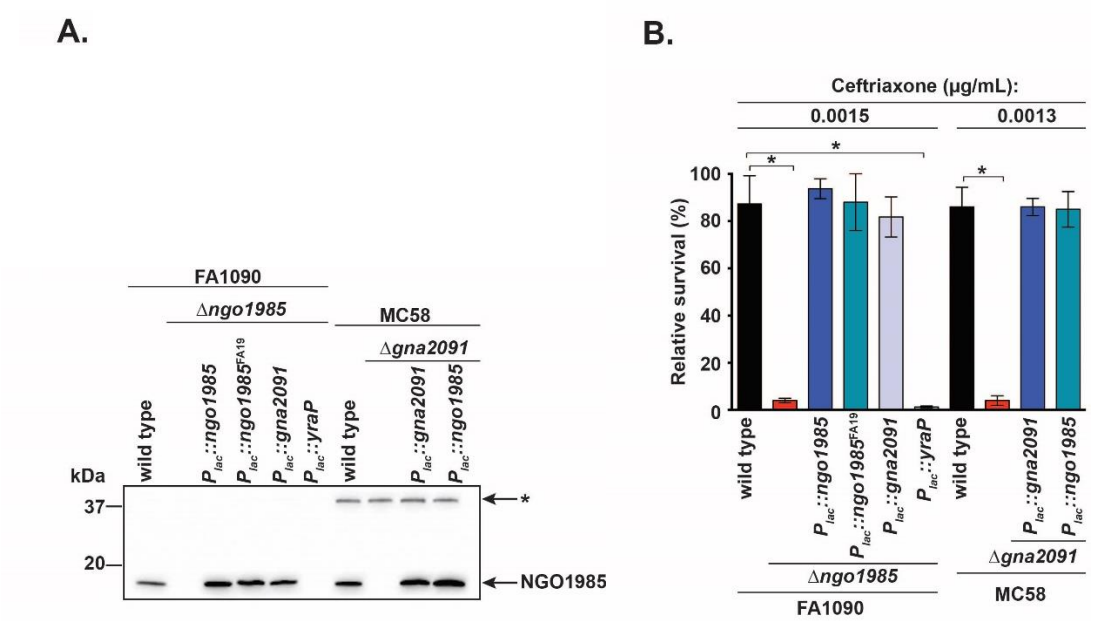


Figure 3.2. NGO1985 and GNA2091 function interchangeably. (A) Whole cell lysates of GC and NM, as indicated, were normalized based on OD_{600} units, separated by SDS-PAGE, and probed with anti-NGO1985 antisera. Nonspecific protein band detected in NM samples is indicated (*). Migration of the protein molecular weight marker is indicated on the left (kDa). (B) GC or NM strains, as indicated, were grown for 3 h in GCBL at 37 °C with aeration, back-diluted to OD_{600} = 0.2, and plated on GCB agar supplemented with 0.04 mM IPTG and without or with ceftriaxone (concentration as indicated in the text). After 22 h of incubation at 37 °C, 5% CO_2, relative survival values were calculated (as described in materials and methods section) (n=3 ± SEMs). Statistically significant differences ($p < 0.05$) are indicated (*).

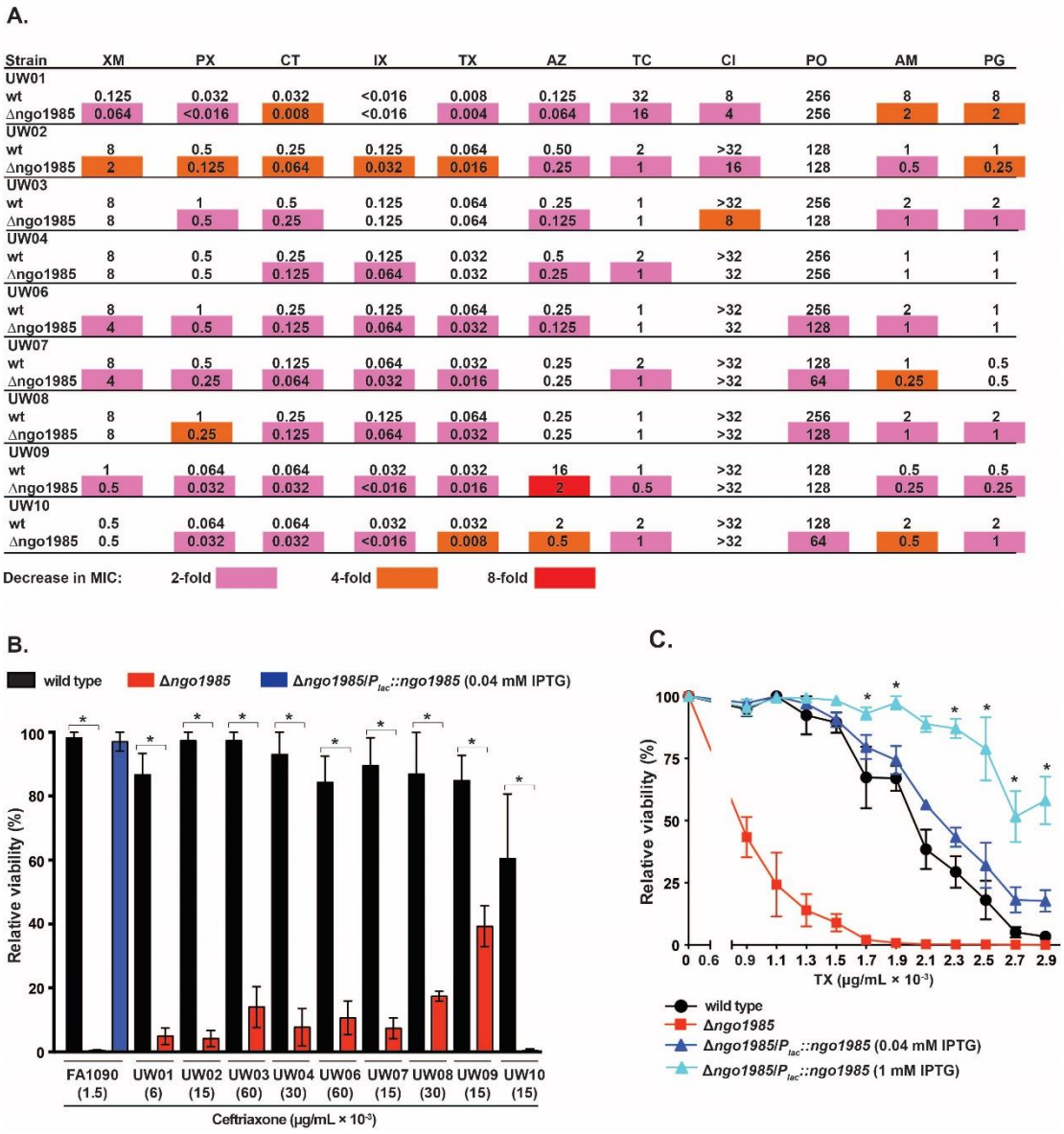


Figure 3.3. Involvement of NGO1985 in resistance to antimicrobial compounds. (A) Gonococcal strains, as specified in the text, were suspended in Brain-heart infusion (BHI) broth to equivalent turbidity of 0.5 McFarland turbidity standard and were streaked with a cotton swab onto GCB agar plates. After allowing the surface of the agar to dry, E-test antimicrobial gradient test strips were applied. Following 22 h, antibiotic minimal inhibitory concentrations (MICs) (µg/mL) were determined based on the zone of bacterial growth inhibition. The presented numbers are the MIC values that repeated at least twice in three biological replicates of the experiment. Antibiotic

abbreviations as indicated by manufacturer (BioMerieux): XM, cefuroxime; PX, cefpodoxime; CT, cefotaxime; IX, cefixime; TX, ceftriaxone; AZ, azithromycin; TC, tetracycline; CI, ciprofloxacin; PO, polymyxin B; AM, ampicillin; PG, benzylpenicillin. CDC GISP alert criteria: cefpodoxime and cefixime MIC ≥ 0.250 μg/mL; ceftriaxone MIC ≥ 0.125 μg/mL; azithromycin MIC ≥ 2.0 μg/mL. (B) GC strains, as indicated in the text, after 3 h of growth in GCBL at 37 °C with aeration were suspended to OD_{600} = 0.2, serially diluted, and plated on solid media with or without different concentrations of ceftriaxone, as indicated. The following day, relative survival for each strain was calculated (as described in materials and methods section) (*n*=3 ± SEMs). (C) Wt FA1090, isogenic Δ*ngo1985*, and Δ*ngo1985*/P_{lac}*::ngo1985* strain following 3 h of cultivation in liquid media were back-diluted to OD_{600} = 0.2, serially diluted, and plated on agar supplemented with either 0.04 mM or 1.0 mM IPTG, and with or without ceftriaxone. Relative survival for each strain was calculated for each strain and each ceftriaxone concentration (*n*=3 ± SEMs). Statistically significant differences ($p < 0.05$) are indicated (*).

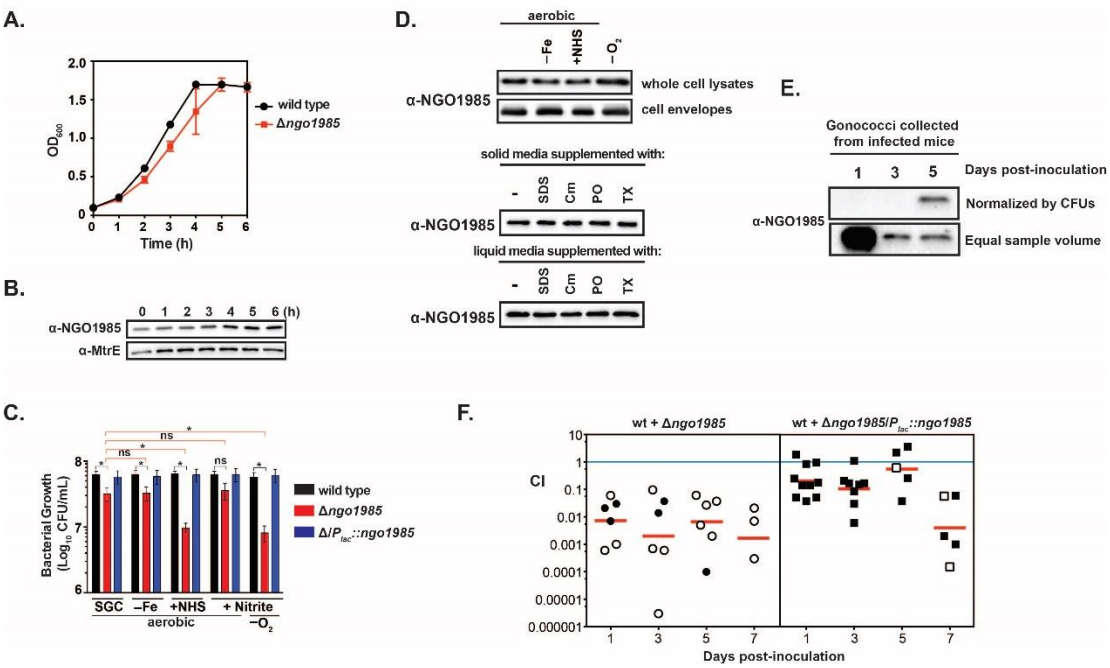


Figure 3.4. NGO1985 is important for GC survival during *in vitro* host relevant conditions and in gonorrhea mouse model of infection. (A) Proliferation of FA1090 wt and Δn*go1985* strains was assessed by OD_{600} measurements during 6 h of growth with aeration in GCBL medium (*n*=3 ± SEMs). (B) The expression of NGO1985 throughout GC growth was examined in whole cell lysates of wt FA1090, normalized based on OD_{600} units, by immunoblotting analysis with anti-NGO1985 antisera. Anti-MtrE antibodies were used as a loading control. (C) FA1090 wt, Δ*ngo1985*, and Δ*ngo1985*/P_{lac}*::ngo1985* were cultured in GCBL with aeration for 3 hours, diluted to OD_{600} = 0.2, serially diluted, plated on solid media, and cultured under different growth conditions: standard aerobic (SGC), iron deprivation (Fe-), presence of normal human serum (+NHS), aerobically in the presence of nitrite, and anaerobically in the presence of nitrite as a terminal electron acceptor (O_2-). Following 22 h of aerobic or 48 h of anaerobic growth, CFUs were scored (*n*=3 ± SEMs). (D) FA1090 whole cell lysates (normalized by OD_{600} units) or cell envelopes (0.25 µg) cultured under the conditions described above, or whole cell lysates (normalized by OD_{600} units) of FA1090 exposed to different compounds (sodium dodecyl sulfate, SDS; chloramphenicol, Cm; Polymyxin B, PO; ceftriaxone; TX) in solid or liquid media were separated by SDS-PAGE and probed with anti-NGO1985 antisera. (E) Whole cell lysates of FA1090

recovered from mice at days 1, 3, and 5 post-infection and normalized by CFUs (3,180) or equal sample volumes (15µL) were resolved by SDS-PAGE page and probed with anti-NGO1985 antisera. (F) Mixtures containing similar CFUs of wt and Δ*ngo1985* as well as wt and Δ*ngo1985*/P_{lac}*::ngo1985* were inoculated into two groups of estradiol-treated female BALB/c mice. Bacteria were recovered at days 1, 3, 5, and 7 post infection and enumerated on GCB agar containing streptomycin (total number) and GC agar with streptomycin and kanamycin (Δ*ngo1985* or Δ*ngo1985*/$P_{lac::}$*ngo1985*). Competitive indices (CI) for individual mice at each time point are shown. Red lines represent the geometric mean. The CI values below 1 represent decreased ratios of the mutant to the wt strain. Mice from which no Δ*ngo1985* or Δ*ngo1985*/P_{lac}*::ngo1985* bacteria were recovered are designated by open symbols. To calculate CI in these cases, the limit of detection (1 CFU in 100 µL of vaginal swab suspension) was used as CFU value.

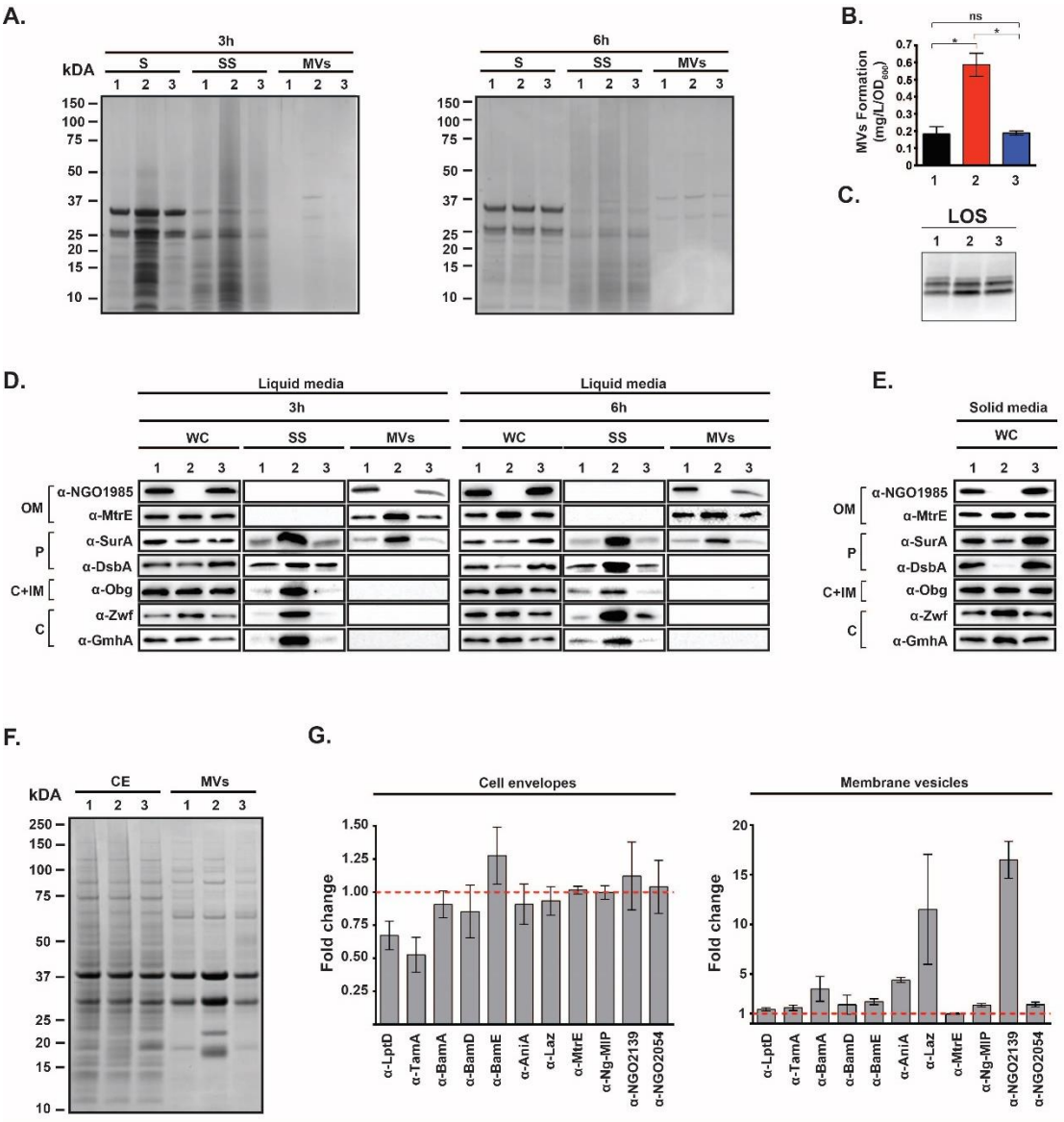


Figure 3.5. Lack of NGO1985 causes loss of cell envelope homeostasis demonstrated by increased leakage of protein content and vesiculation, as well as altered protein profiles of cell envelopes and membrane vesicles. (A) Protein contents of culture supernatants (S), soluble protein fraction (SS), and natural membrane vesicles (MVs), of FA1090 wt (1), Δ*ngo1985* (2), and Δ*ngo1985*/P_{lac}::*ngo1985* (3), collected at indicated time points, were analyzed by resolving samples on 4-12% NuPAGE Novex Bis-Tris Gels, normalized based on OD_{600} value, followed by Coomassie Brilliant Blue G250 staining. (B) Amount of released MVs of strains indicated above was calculated (as described in materials and

method section) (*n*=3 ± SEMs). (C) Equal amounts (0.75 µL) of LOS derived from GC isolates were resolved on a 16.5% Tris-Tricine gel and subjected to silver staining. (D, E) Whole cell lysates (WC), soluble protein fraction (SS), and naturally released membrane vesicles (MVs) of GC strains determined in the text were isolated from liquid culture at determined time points (D), or from solid media, as indicated (E). Samples normalized by OD_{600} units were resolved by SDS-PAGE and probed with antibodies. Subcellular localization of analyzed proteins is indicated: outer membrane, OM; periplasm, P; inner membrane and cytoplasm, C+IM; cytoplasm, C. (F) Cell envelopes (CE) and MVs were isolated from indicated GC strains cultured until mid-logarithmic growth (OD_{600} = 0.8-0.9). Equal amounts (3 µg) of each sub-proteome fraction were resolved by SDS-PAGE and visualized by Coomassie Brilliant Blue G250 staining. (G) Relative abundance of indicated proteins within cell envelopes and membrane vesicles of Δ*ngo1985* strain, compared to wt FA1090 (*n*=3 ± SEMs). Migration of the protein weight marker is indicated on the left (kDa).

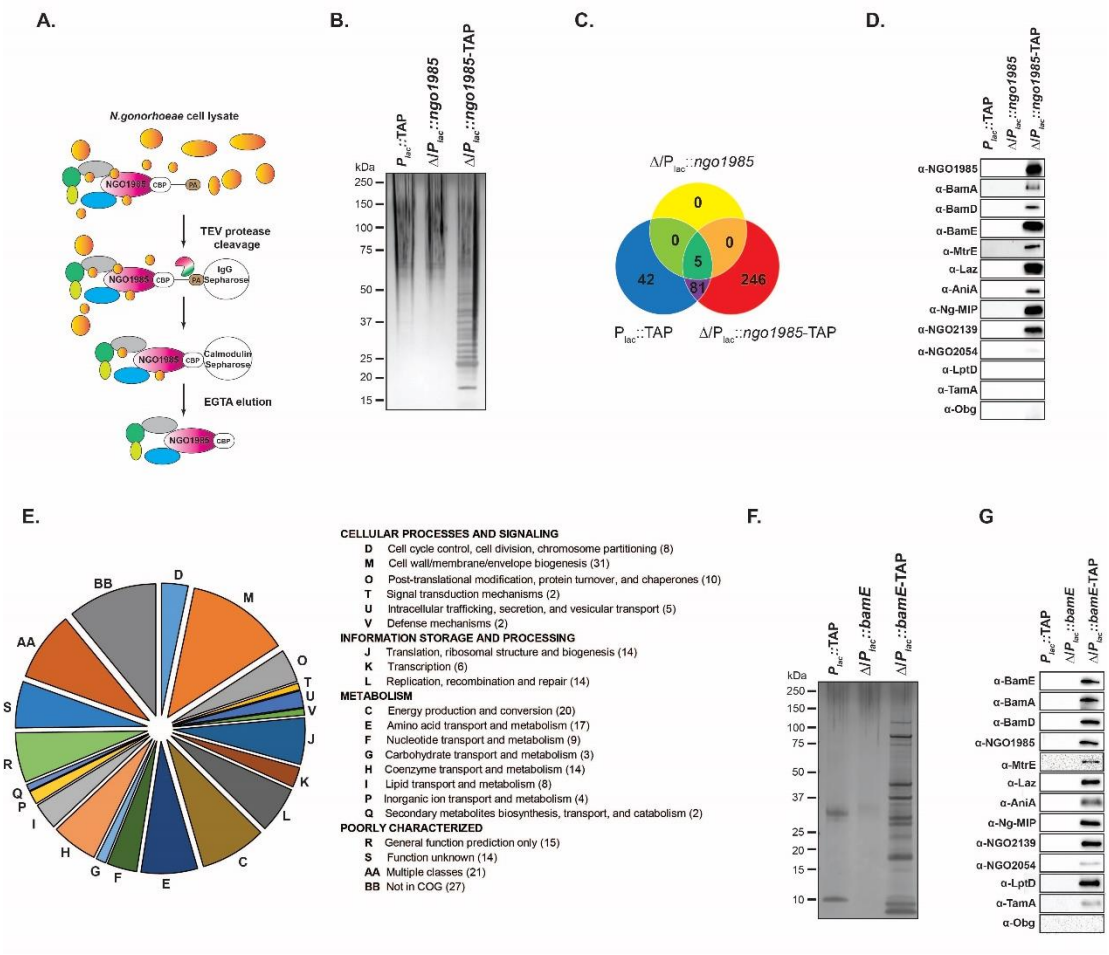
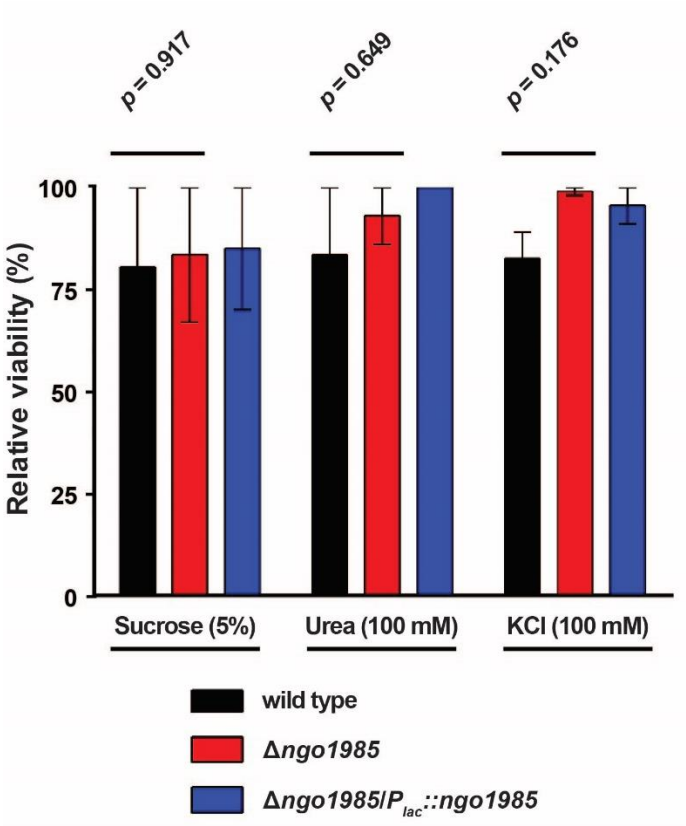
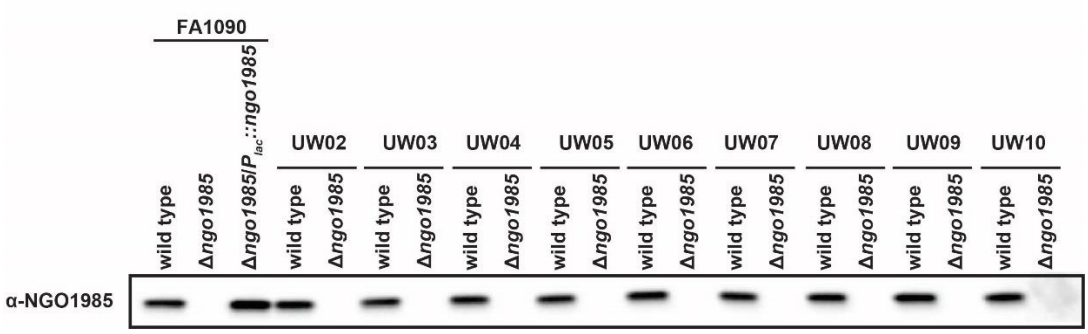


Figure 3.6. Interactome of NGO1985. (A) Outline of experimental design. NGO1985 was tagged with TAP-tag, placed under P_{lac} promoter and expressed in Δ*ngo1985* background. GC FA1090 wt cells expressing empty TAP-tag and Δ*ngo1985* complemented with wt NGO1985, as well as Δ*ngo1985* expressing NGO1985-TAP-tag placed under P_{lac} promoter were harvested at mid-log phase of growth. Bacteria were lysed and the protein lysates were incubated with IgG Sepharose followed by the TEV protease cleavage of the multiprotein complexes. Samples were applied to Calmodulin Beads and after extensive washing proteins were eluted by the addition of EGTA. (B) Equal amounts of eluate were resolved on a 4-15% Tris-glycine gel and visualized by silver staining. (C) Venn diagram of proteins identified by mass spectrometry analysis in pulldown experiment. Total of 246 individual proteins were identified uniquely in the Δ*ngo1985*/P_{lac}::*ngo1985*-TAP strain. (D) Mass spectrometry

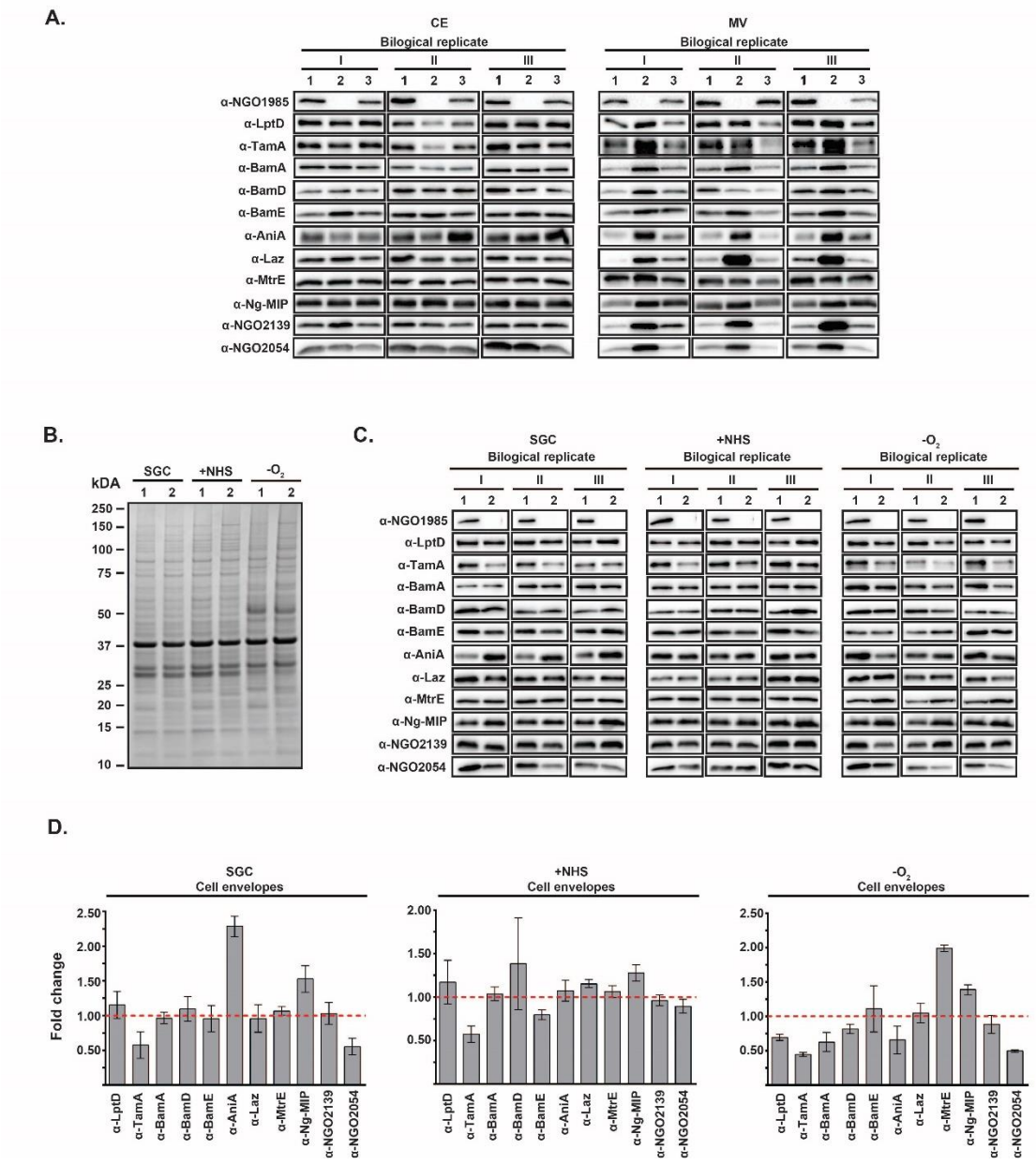
analysis was validated by resolving equal amounts of eluted protein on a 4-15% gel, transferring to a nitrocellulose membrane and probing with antibodies, as indicated. Immunoblotting with anti-LptD, anti-TamA, and anti-Obg antisera served as negative controls. (E) Identified proteins that interact with NGO1985 were categorized into functional classes according to Clusters of Orthologous Groups (COGs) analysis. The pie chart presents the numbers of identified proteins in each COG cluster. COG categories are listed on the right. (F, G). Association with BAM complex was verified by performing tandem affinity purification with Δ*bamE*/P_{lac}*::bamE*-TAP and control strains Δ*bamE*/P_{lac}*::bamE* and P_{lac}*::*TAP. Isolated protein complexes were resolved on a 4-15% gel and visualized by silver staining or probed with antisera. Migration of the protein weight marker is indicated on the left (kDa).



Supplementary Figure S3.1. Absence of NGO1985 does not increase GC susceptibility to osmotic shock. FA1090 wt, Δ*ngo1985*, and Δ*ngo1985*/P_{lac}*::ngo1985* strains were grown for 3 h in GCBL media, back-diluted to OD_{600} = 0.2, serially diluted, and plated on GCB agar supplemented with 0.04 mM IPTG, and without or with sucrose (5 %), urea (100 mM), or KCl (100 mM). After 22 h of incubation, relative survival values were calculated (as described in materials and methods section) (n=3 ± SEMs).

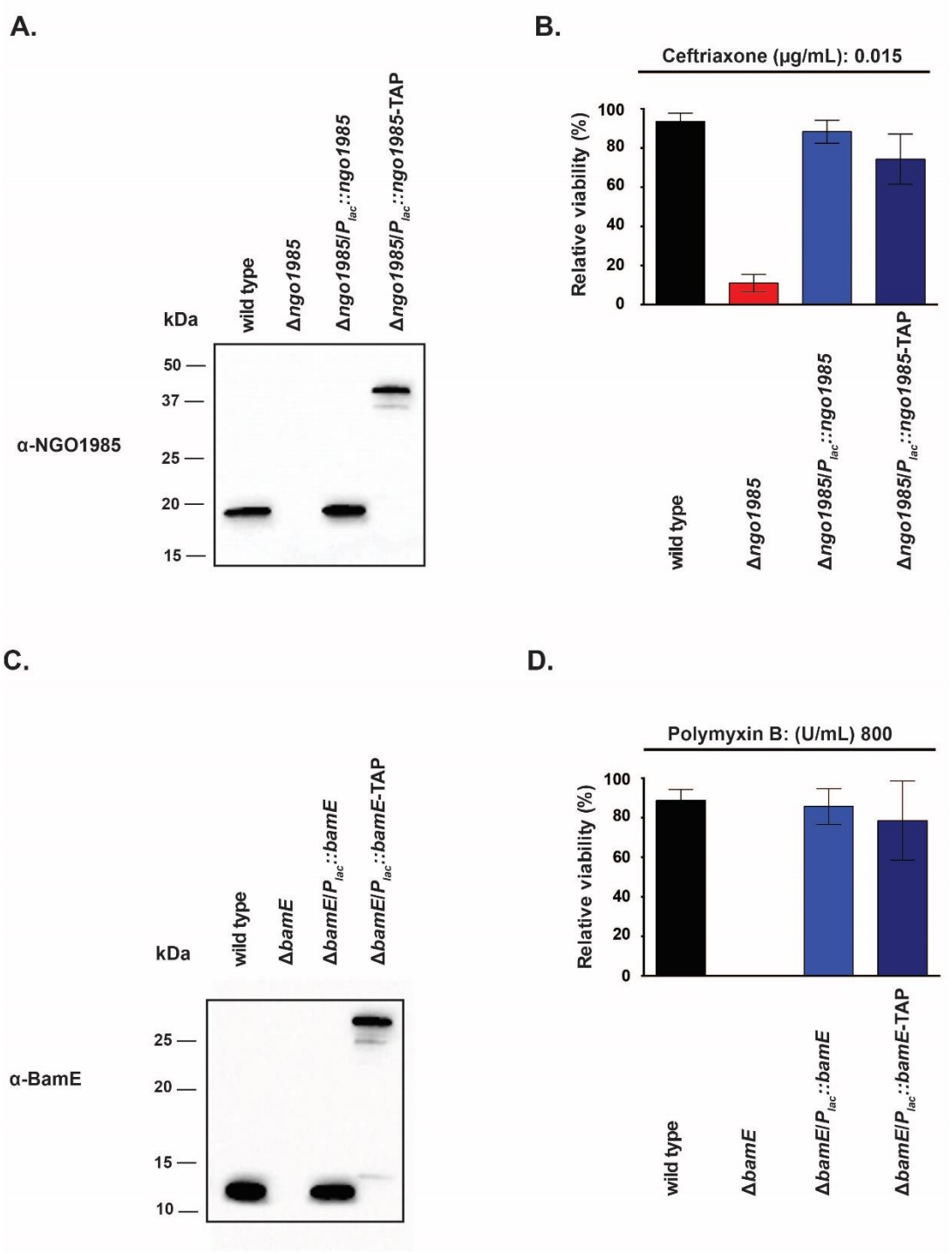


Supplementary Figure S3.2. Western blotting validation of ngo1985 deletion in GC clinical isolates (Seattle STD Clinic 2011-2013). Whole cell lysates of GC isolates were normalized by OD_{600} units, separated in a 4-20% Tris-glycine gel, and probed with anti-NGO1985 antibodies. Wt FA1090 and Δ*ngo1985*/P_{lac}*::ngo1985* were included as the positive controls, while Δ*ngo1985* mutant was used as a negative control.



Supplementary Figure S3.3. Changes in protein composition of cell envelopes and membrane vesicles in the absence of NGO1985. (A) Equal amounts (µg) of cell envelopes (CE) and membrane vesicles (MV) isolated from FA1090 wt, Δ*ngo1985* mutant, and Δ*ngo1985*/P_{lac}::*ngo1985* grown to mid-logarithmic stage (OD_{600} = 0.8-0.9) were resolved by SDS-PAGE and probed with antisera. Biological replicates of the experiments are indicated. (B, C) Cell envelopes were isolated from wt FA1090 and Δ*ngo1985* grown under the following growth conditions: standard conditions

(SGC), aerobically in the presence of normal human serum (NHS), and anaerobically in the presence of $NaNO_2$ as a terminal electron acceptor (O_2-). Equal amounts (µg) of protein fractions were resolved by SDS-PAGE, and either stained with Coomassie Brilliant Blue G250 (representative samples) (B), or probed with antibodies (C). Biological replicates of the experiments are indicated. (D) Relative abundance of indicated cell envelope proteins of Δ*ngo1985* strain cultivated under indicated growth conditions, compared to wt FA1090 (*n*=3 ± SEMs).



Supplementary Figure S3.4. Functional NGO1985-TAP-tag and BamE-TAP-tag fusions. (A and C) Whole cell lysates of GC strains, normalized based on OD_{600} values, were resolved by SDS-PAGE and probed with anti-NGO1985 or anti-BamE antisera, as indicated. Migration of the protein weight marker is indicated on the left (kDa). (B)

GC strains, grown for 3 h in GCBL, were diluted to OD_{600} = 0.2, serially diluted, and plated on GCB agar supplemented with 0.04 mM IPTG and in the absence or presence of ceftriaxone (0.0015 µg/mL). After 22 h of incubation, colony forming units were scored and relative survival values were calculated (as described in materials and methods) (*n*=3 ± SEMs). (D) GC strains were cultured and handled as described above and plated on GCB agar supplemented with 0.02 mM IPTG, and in the absence or presence of Polymyxin B (800 U/mL). After 22 h of incubation, colony forming units were scored and relative survival values were calculated (as described in materials and methods) (*n*=3 ± SEMs).

Table S3.1. Bacterial strains used in this study.

Bacterial strain	Reference
N. gonorrhoeae	
FA1090	Connell *et al*., 1988
MS11	Meyer *et al*., 1982
1291	Apicella *et al*., 1978
F62	Sparling, 1966
FA19	Maness and Sparling, 1973
FA1090 Δ*ngo1985*	Zielke *et al*., 2014
FA1090 Δ*ngo1985*/P_{lac}*::ngo1985*	Zielke *et al*., 2014
FA1090 Δ*ngo1985*/P_{lac}*::ngo1985* C23A	This study
FA1090 Δ*ngo1985*/P_{lac}*::ngo1985* C23S	This study
FA1090 Δ*ngo1985*/P_{lac}*::ngo1985* (FA19)	This study
FA1090 Δ*ngo1985*/P_{lac}*::gna2091*	This study
FA1090 Δ*ngo1985*/P_{lac}*::yraP*	This study
FA1090 Δ*ngo1985*/P_{lac}*::ngo1985*-TAP	This study
FA1090 P_{lac}*::*TAP	This study
FA1090 Δ*bamE*	This study
FA1090 Δ*bamE*/P_{lac}*::bamE*	This study
FA1090 Δ*bamE*/P_{lac}*::bamE*-TAP	This study
Baltimore collection 1991-1994: LGB1, LG14, LG2, LGB26, LG20	Garvin *et al*., 2008; Zielke *et al*., 2014
Public Health–Seattle & King County Sexually Transmitted Disease clinic isolates: UW01, UW02, UW03, UW04, UW05, UW06, UW07, UW08, UW09, UW10, UW11, UW12, UW13	Zielke *et al*., 2016
WHO 2015 reference strains: F, G, K, L, M, N, O, P, W, X, Y, Z, U, V	Unemo *et al*., 2016
UW02 Δ*ngo1985*	This study
UW03 Δ*ngo1985*	This study
UW04 Δ*ngo1985*	This study
UW05 Δ*ngo1985*	This study
UW06 Δ*ngo1985*	This study
UW07 Δ*ngo1985*	This study
UW08 Δ*ngo1985*	This study
UW09 Δ*ngo1985*	This study
UW10 Δ*ngo1985*	This study
N. meningitides	
MC58	McGuinness *et al.*, 1991

MC58 Δ*gna2091*	This study
MC58 Δ*gna2091*/P_{lac}::*gna2091*	This study
MC58 Δ*gna2091*/P_{lac}::*ngo1985*	This study
E. coli	
MC1061	Casadaban and Cohen, 1980
BL21(DE3)	Studier and Moffatt, 1986

Table S3.2. Plasmids and oligonucleotides used in this study.

Plasmid Name	**Reference**
pET28a	Novagen
pGCC4	Skaar *et al*., 2002
pUC18	Yanisch-Perron *et al*., 1985
pUC18K-NGO1985	Zielke *et al*., 2014
pET28a-rNGO1985	This study
pET28a-rBamE	This study
pUC18-NGO1985(STOP)	This study
pBS1479	Puig et al., 2001
pUC18-NGO1985-TAP	This study
pGCC4-NGO1985-TAP	This study
pGCC4-TAP	This study
pUC18-BamE(STOP)	This study
pUC18-BamE-TAP	This study
pGCC4-BamE-TAP	This study
pGCC4-NGO1985(FA19)	This study
pGCC4-GNA2091	This study
pGCC4-YraP	This study
pUC18K-GNA2091	This study
pUC18-NGO1985	This study
pUC18-NGO1985 C23A	This study
pUC18-NGO1985 C23S	This study
pGCC4-NGO1985 C23A	This study
pGCC4-NGO1985 C23S	This study
pUC18K-BamE	This study
pGCC4-BamE	This study
Oligonucleotide	**Sequence[1]**
Primers used for creating recombinant proteins with C-terminal 6×His-tag:	
rNGO1985-F	TTCATCCATATGGCAAAATCCGTCATCGACC
rNGO1985-R	TTCATCCTCGAGGCGTTGGACGTAGTTTTGGT
rBamE-F	GAATTCCCATGGTCGAACGCGTCTCGCTGT
rBamE-R	GGATCCAAGCTTTTGTTTGTCTGCGTTTTGTTTCGC
Primers used for the mutagenesis of the conserved cysteine (+1) in a predicted lipobox of NGO1985	
NGO1985-RBS-F	CCCAAGAAAGGAAGTCCC
NGO1985-RBS-R	AAGCTTGGCCGGCCTCAGCGTTGGACGTAGTT
NGO1985-C23A-F	CGGCGGCGCCTTCAGCG

NGO1985-C23A-R	CGCTGAAGGCGCCGCCG
NGO1985-C23S-F	CCCTCGGCGGCAGCTTCAGCGCA
NGO1985-C23S-F	TGCGCTGAAGCTGCCGCCGAGGG
pGCC4-Ver-F	AAATCGCCCTTGATACCG
pGCC4-Ver-R	CTTCCGGCTCACACTTTATG

Primers used for creating TAP-tagged NGO1985, TAP-tag expressed under NGO1985 RBS, and TAP-tagged BamE:

NGO1985-TAP-F	CTATCGATGAGCTCCCCAAGAAAGGAAGTCCC
NGO1985-TAP-R	ATCCGCGGTACCGCGTTGGACGTAGTTTTG
NGO1985-TAP-F2	ATCCGCGGTACCAAGAGAAGATGGAAAAAGAATTTCA
NGO1985-TAP-R2	GGATTTACGGATCCTCAGGTTGACTTCCCCG
NGO1985-TAP-R3	AAGCTTGGCCGGCCCTACTTGTCATCGTCATCCTTG
TAP-F	CCCAAGAAAGGAAGTCCCCGATGGAAAAGAGAAGATGG AAAAA
TAP-R	AAGCTTGGCCGGCCTCAGGTTGACTTCCCC
BamE-TAP-F	GACTGATAGAATTCTTGCACAGAAAGGTTCTCC
BamE-TAP-R	ATCGATGGTACCTTGTTTGTCTGCGTTTTGTTTC
BamE-TAP-F2	TTGCACAGAAAGGTTCTCC

Primers used for gene deletion:

NGO1985-Ver-F	CCCGCCCGAGTGGATAAAG
NGO1985-Ver-R	CATAATGCTTCCTTGGATTGCG
GNA2091-Up-F	CTATCGATGAGCTCTGCCGACGCGCAACACTT
GNA2091-Up-R	ATCCGCGGTACCCGGTGTGCGGTTTGGGTTTC
GNA2091-Down-F	GCTCAGGGATCCCCAAAACTACGTCCAACGC
GNA2091-Down-R	GATTCGAAGCTTGGACGTTTGTGGGCAGTA
GNA2091-Ver-F	CGTGCCAAGCAGGAAGC
GNA2091-Ver-R	TCCGGTTTGGTATGGTTTTCTTTG
BamE-Up-F	AGGCCTGAGCTCGGCAGTTCTCCAAAAACACAGA
BamE-Up-R	CCGCGGTACCCACGGGAGAACCTTTCTGTG
BamE-Down-F	CGATCGGGATCCGCGAAACAAAACGCAGACAA
BamE-Down-R	GTTAACAAGCTTGAAGAGGGCGGTGTGGT
BamE-Ver-F	AAAGCATAGGCAGGATCGGG
BamE-Ver-R	CCTGCATATCGTACAAACCCG

Primers used for the complementation/trans-complementation studies:

GNA2091-RBS-F	CCCAAGAAAGGAAGCACC
YRAP-RBS-F	AGGATGATTAAGGAGAATACATGAAG
YRAP-RBS-R	AAGCTTGGCCGGCCCTATTTAATAAACGTAAACGCTGTG G
BamE-RBS-F	TTGCACAGAAAGGTTCTCCC
BamE-RBS-R	AAGCTTGGCCGGCCTCCTTATTGTTTGTCTGCGTTT

Primers used for sequencing of NGO1985 from the recent GC clinical isolates:

NGO1985-Amp-F	CCCGCCCGAGTGGATAAAG
NGO1985-Amp-R	CATAATGCTTCCTTGGATTGCG
NGO1985-Seq-F	GCCGCCATGCTCAAAGAC
NGO1985-Seq-R	TGTCGCCGTTCGGGTTG

[1] Sequences recognized by restriction enzymes are underlined.

Table S3.3. Proteins identified in pull-down experiments by mass spectrometry.

Accession	Description	Gene	Score	Coverage	Number of Unique Peptides
Amino acid transport and metabolism					
59801193	Homoserine dehydrogenase	NGO0779	75.7	49.66	14
59800659	ABC transporter periplasmic binding protein, polyamine	NGO0206	65	31.48	10
59801562	Anthranilate phosphoribosyltransferase	trpD, NGO1203	64	30.41	8
59800553	3-dehydroquinate synthase	aroB, NGO0092	59	35.38	8
59800701	Tryptophan synthase subunit alpha	trpA, NGO0248	57.4	40.61	7
59801253	2-isopropylmalate synthase	leuA, NGO0848	46.6	30.37	11
59801612	ABC transporter periplasmic binding protein, polyamine	NGO1253	35.1	25.53	8
59801419	Aminotransferase	NGO1047	30.4	22.52	8
59801298	3-phosphoshikimate 1-carboxyvinyltransferase	aroA, NGO0900	22.9	19.4	5
59801825	Arginine decarboxylase	speA, NGO1487	22.7	10.16	5
59800898	Succinyldiaminopimelate transaminase	NGO0460	15.4	8.35	3
59801601	Imidazoleglycerol-phosphate dehydratase	hisB, NGO1242	10.6	12.3	2
59801073	Bifunctional N-succinyldiaminopimelate-aminotransferase/acetylornithine transaminase	argD, NGO0646	8.1	11.08	3
59801674	GDSL family lipase	NGO1323	8.1	14.08	3
59800818	ABC transporter ATP-binding protein, amino acid	NGO0374	7.7	15.94	4
59800672	Argininosuccinate lyase	argH, NGO0219	7.2	14.19	4
59802089	Oligopeptidase A	prlC, NGO1770	0	4.42	2
Carbohydrate transport and metabolism					
59802227	6-phosphogluconate dehydrogenase	gnd, NGO1914	16.1	13.07	5
59802095	Glyceraldehyde-3-phosphate dehydrogenase	epd, NGO1776	13	16.03	5
59800819	Phosphoglucomutase	pgm, NGO0375	9.2	11.52	4
Coenzyme transport and metabolism					
161572979	S-adenosylmethionine synthetase	metK, NGO0106	39.8	28.28	7
59800768	Ubiquinone/menaquinone biosynthesis methyltransferase	ubiE, NGO0321	38.3	37.96	7
59801748	Glutamyl-tRNA reductase	hemA, NGO1403	23.9	23.37	8
59801695	3-octaprenyl-4-hydroxybenzoate carboxy-lyase	ubiD, NGO1345	19	10.16	4
59802307	Bifunctional 5,10-methylene-tetrahydrofolate dehydrogenase/ 5,10-methylene-tetrahydrofolate cyclohydrolase	folD, NGO1999	17.8	27.82	5
59801904	Thiamine monophosphate kinase	thiL, NGO1575	14.1	9.12	2
59801764	Thiamine biosynthesis protein ApbE	apbE, NGO1420	11.9	13.68	4
59800508	Glutamate-1-semialdehyde aminotransferase	hemL, NGO0040	7.3	7.75	3
59801900	Quinolinate synthetase	nadA, NGO1567	7.3	10	3
59802375	3-demethylubiquinone-9 3-methyltransferase	ubiG, NGO2074	6.9	11.57	3
59800804	Heme biosynthesis operon protein HemX	hemX, NGO0360	5.1	11.08	3
59802000	Octaprenyl diphosphate synthase	ispB, NGO1675	5	8.02	2
59802327	Molybdopterin/thiamine biosynthesis adenylyltransferase	thiF, NGO2019	4.8	8.59	2
59801501	GTP cyclohydrolase II	ribA, NGO1134	4.1	8.12	2

Nucleotide transport and metabolism					
59800964	Phosphoribosylaminoimidazole synthetase	purM, NGO0526	78.4	36.92	10
59800797	Uracil phosphoribosyltransferase	upp, NGO0353	64.6	77.88	12
59800789	Nucleoside phosphorylase	mtnN, NGO0345	42.4	48.07	9
59801041	Ribonucleotide-diphosphate reductase subunit alpha	nrdA, NGO0614	18.3	9.35	7
59802032	Adenylosuccinate lyase	purB, NGO1711	9.1	8.33	4
59802282	Uridylate kinase	pyrH, NGO1973	8.3	15.06	3
59802251	Phosphoribosylamine--glycine ligase	purD, NGO1939	8	8.75	3
59801571	CTP synthetase	pyrG, NGO1212	5.2	3.68	2
59800526	Formate--tetrahydrofolate ligase	fhs, NGO0062	5	5.91	2
Cell cycle control, cell division, chromosome partitioning					
59801865	Cell division protein FtsA	ftsA, NGO1529	115.4	59.9	21
59802109	Chromosome partitioning protein ParA	parA,NGO1790	92.4	59.14	10
59801255	Cell-division protein FtsK	ftsK, NGO0851	42.8	16.57	15
59801885	Cell division protein FtsN	ftsN, NGO1549	38.9	36.68	8
59801021	FtsK-like cell division/stress response protein	NGO0590	20.8	11.57	8
59802132	Cell division topological specificity factor MinE	minE, NGO1814	9.3	35.63	3
59801785	Chromosome segregation ATPase	smc, NGO1443	6	3.96	3
59800521	MRP family ATPase	NGO0056	5.6	7.24	2
Inorganic ion transport and metabolism					
59801548	Magnesium transporter	mgtE, NGO1188	22.1	14.26	5
59802088	Cytochrome-c peroxidase	ccpR, NGO1769	8.2	8.27	3
59802393	Ferric enterobactin periplasmic binding protein	fetB, NGO2092	6.3	10.22	3
59800626	Zinc ABC transporter ATP-binding protein	znuC, NGO0170	6.1	9.96	2
Lipid transport and metabolism					
59801240	Squalene/phytoene synthase	crtB, NGO0830	49.7	42.07	11
59802464	Glycerol-3-phosphate acyltransferase PlsX	plsX, NGO2171	29.7	30.17	8
59801024	4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase	ispG, NGO0594	27.8	20.67	7
59801366	Squalene/phytoene synthase	NGO0980	19.1	16.73	4
59802117	1-deoxy-D-xylulose 5-phosphate reductoisomerase	dxr, NGO1799	8.4	6.6	3
59801565	Phosphatidylserine decarboxylase	psd, NGO1206	8.2	16.22	4
59800825	Cyclase	NGO0381	6.9	17.93	3
59800702	Acetyl-CoA carboxylase subunit beta	accD, NGO0249	2.9	6.9	2
Secondary metabolites biosynthesis, transport and catabolism					
59801633	Nitrite reductase AniA	aniA, NGO1276	19	14.29	5
59802414	ABC transporter ATP-binding protein	NGO2116	6.4	11.89	2
Cell wall/membrane/envelope biogenesis					
59801339	Hypothetical lipoprotein NGO0948	NGO0948	121	45.98	16
59801427	Peptidase	NGO1056	102.4	36.48	13
59801851	OpaD protein	opaD, NGO1513	102.3	44.6	1

59801713	Antibiotic resistance efflux pump component	mtrC, NGO1365	80.5	46.36	14
59802119	Outer membrane protein assembly factor BamA	bamA, NGO1801	68.6	28.16	18
59801875	UDP-murnac-pentapeptide synthetase	murF, NGO1539	56.8	41.37	12
229220794	UDP-N-acetylmuramate--L-alanine ligase	murC, NGO1532	53.4	33.97	13
651851645	Opacity protein	NGO1277a	41.3	35.04	3
59802356	Membrane-bound lytic murein transglycosylase A	ltgC, NGO2048	39.5	32.2	9
59802432	Transglycosylase LtgA	ltgA, NGO2135	32.2	20.45	8
651851643	Opacity protein	NGO1040a	31.6	31.86	2
59801871	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate synthetase	murD, NGO1535	23.1	18.88	6
59800658	LolA protein	lolA, NGO0205	22.9	22.22	4
59800547	Hypothetical protein NGO0086	NGO0086	19.4	18.3	6
59800788	Elongation factor 4	lepA, NGO0344	19.3	13.57	7
59800880	Outer membrane lipoprotein LolB	lolB, NGO0439	19.3	20.73	3
59800731	LPS-assembly lipoprotein	lptE, NGO0282	18	27.04	4
59801036	Murein transglycosylase	ltgE, NGO0608	17.9	14.89	8
59800686	Opacity family porin protein	nspA, NGO0233,	14.5	21.14	4
59801866	Cell division protein FtsQ	ftsQ, NGO1530	11.8	14.46	4
59801054	Murein hydrolase LtgD	ltgD, NGO0626	11.8	13.5	5
59800560	Penicillin-binding protein 1	mrcA, NGO0099	11.7	5.01	3
59801867	D-alanine--D-alanine ligase	ddl, NGO1531	10.3	13.49	3
59801892	Hypothetical protein NGO1559	NGO1559	9.3	19.11	3
59802101	Lipid-A-disaccharide synthase	lpxB, NGO1782	8.7	7.18	2
59800548	Hypothetical protein NGO0087	NGO0087	8.4	6.21	2
59801880	rRNA small subunit methyltransferase H	rsmH, mraW, NGO1544	7.1	11.32	2
59801782	Macrolide transport protein MacA	macA, NGO1440	6.5	6.12	2
59802353	Glucosamine--fructose-6-phosphate aminotransferase	glmS, NGO2045	6.3	3.92	2
59802419	Hypothetical protein NGO2121	NGO2121	5.7	9.03	2
59801830	Phospholipase	pldA, NGO1492	4.9	6.81	2
Defense mechanisms					
59801126	Type I restriction-modification system methyltransferase	hsdM, NGO0702	31.6	27.2	12
59801781	ABC transporter ATP-binding protein	macB, NGO1439	7	7.14	4
Energy production and conversion					
59802249	Electron transfer flavoprotein subunit alpha	etfA, NGO1936	103.6	43.09	8
59802444	ATP synthase F0F1 subunit delta	atpH, NGO2147	49.9	71.19	11
59801289	D-lactate dehydrogenase	NGO0890	49.6	28.24	16
161572971	Phosphoenolpyruvate carboxylase	ppc, NGO2020	40.7	15	11
59801304	Hypothetical protein NGO0906	NGO0906	34.1	22.93	8
59802446	ATP synthase F0F1 subunit gamma	atpG, NGO2149	33.5	25.77	6
59802339	Cytochrome C1 precursor	petC, NGO2031	19	32.06	6
59801810	NAD(P) transhydrogenase subunit alpha	pntA, NGO1470	14.3	7.23	3

59801721	Cbb3-type cytochrome c oxidase subunit II	ccoO, NGO1373	12.4	25.62	5
59802329	NAD(P)H-dependent glycerol-3-phosphate dehydrogenase	gpsA, NGO2021	11.5	8.51	4
59801313	2-oxoglutarate dehydrogenase E1	sucA, NGO0917	8.4	3.93	3
59800562	Cytochrome precursor	NGO0101	7	13.04	2
59801719	Cytochrome c oxidase subunit	ccoP, NGO1371	6.4	4.78	2
59801447	C-type cytochrome	NGO1080	6.3	25.66	4
59801312	Dihydrolipoamide succinyltransferase	sucB, NGO0916	5.6	6.36	2
59802070	NADH dehydrogenase subunit C	nuoC, NGO1749	5.4	11.17	2
59801762	Na(+)-translocating NADH-quinone reductase subunit F	nqrF, NGO1418	5.3	5.93	2
59801757	Na(+)-translocating NADH-quinone reductase subunit A	nqrA, NGO1413	4.2	6.26	2
59802069	NADH dehydrogenase subunit D	nuoD, NGO1748	2.8	9.09	3
59801431	Succinate semialdehyde dehydrogenase	gabD, NGO1061	2.7	4.19	2
Secretion and transport					
59802021	Signal recognition particle protein	ffh, NGO1700	87	31.14	14
59802367	Signal recognition particle protein	ftsY, NGO2060	81.7	45.13	14
59800643	Hypothetical protein NGO0188	NGO0188	17.1	45.45	4
59800638	Twin arginine translocase A	tatA, NGO0183	10.1	46.27	3
59800644	Preprotein translocase subunit secd	secD, NGO0189	4.9	3.07	2
Multiple classes					
59800557	Type IV pilus assembly protein PilO	pilO, NGO0096	71	59.53	12
59801519	ABC transporter periplasmic histidine-binding protein precursor	hisJ, NGO1152	57.3	54.48	10
59801025	Type IV pilus assembly protein PilF	pilF, NGO0595	36.4	27.67	6
59800882	Ribose-phosphate pyrophosphokinase	prs, NGO0441	33.8	30.28	7
59801660	Guanosine-3',5'-bis(diphosphate) 3'-pyrophosphohydrolase	spoT, NGO1308	30.1	20.75	11
59801998	Type IV pilus assembly protein PilF	pilF, NGO1673	23.2	15.23	8
59800558	Pilus assembly protein	pilN, NGO0097	22.3	45.73	7
59800893	Type IV pilus assembly protein PilW	pilW, NGO0454	18.6	22.39	8
59801077	ATP-dependent RNA helicase	rhlE, NGO0650	18.5	18.4	7
59800894	Type IV pilus assembly protein PilX	pilX, NGO0455	18.4	17.73	3
59801413	Exopolyphosphatase	NGO1041	18.3	18.53	8
59800607	ATP-dependent RNA helicase	rhlE, NGO0149	14.2	13.13	4
59800892	Type IV pilus assembly protein PilV	pilV, NGO0453	11.8	19.7	3
59800891	Type IV pilus assembly protein FimT	fimT, NGO0452	10.2	10.86	2
59802274	Protease	NGO1963	10.1	13.41	4
59801595	Acetolactate synthase 3 catalytic subunit	ilvI, NGO1236	9.5	4.35	2
59802003	Ubiquinone biosynthesis hydroxylase	NGO1678	6.8	7.61	3
59800895	Type IV pilin-like protein	NGO0456	6.7	15.43	3
59801605	Periplasmic protease	NGO1246	6.6	7.43	2
59802412	Aminodeoxychorismate synthase	NGO2112	3.9	3.5	2
59802029	ATP-dependent DNA helicase DinG	dinG, NGO1708	1.6	2.09	2

Posttranslational modification, protein turnover, chaperones					
59802038	Thiol:disulfide interchange protein	dsbA, NGO1717	85	49.57	10
59800826	Hypothetical protein NGO0382	ftsH, NGO0382	33.3	20.61	11
59801072	ATP-dependent protease ATP-binding subunit ClpX	clpX, NGO0645	17.7	17.39	7
59800811	DNA repair protein RadA	radA, NGO0367	14.3	14.6	5
59802216	Molecular chaperone DnaJ	dnaJ, NGO1901	13.1	16.62	5
59801780	Thiol:disulfide interchange protein DsbC	dsbC, NGO1438	11.4	14.56	4
59801202	Genome-derived Neisseria antigen 1220	NGO0788	9	7.3	2
59801239	Chaperone protein HscA	hscA, NGO0829	8	3.71	2
59800843	Heat shock protein HtpX	htpX, NGO0399	6.3	10.39	2
59801549	Hsp33-like chaperonin	hslO, NGO1189	5.7	7.62	2
Replication, recombination and repair					
59802091	DNA gyrase subunit B	gyrB, NGO1772	145	34.42	25
59801158	Recombinase A	recA, NGO0741	121	34.2	10
59801160	DNA polymerase III subunits gamma and tau	NGO0743	71.3	23.06	10
59800890	Replicative DNA helicase	dnaB, NGO0451	22.9	18.59	8
59800791	DNA polymerase III subunit delta'	NGO0347	22.4	42.46	10
59802243	DNA mismatch repair protein MutS	mutS, NGO1930	18.4	7.87	6
59802404	DNA polymerase I	polA, NGO2103	15.2	8.6	8
59800474	Chromosomal replication initiation protein	dnaA, NGO0001	14.5	10.04	5
59800732	DNA polymerase III subunit delta	NGO0283	14.1	13.86	4
59800539	DNA polymerase III subunit alpha	NGO0078	11	3.32	4
59801156	ATP-dependent DNA helicase	rep, NGO0739	8	5.22	3
59801186	Exodeoxyribonuclease V subunit alpha	recD, NGO0771	6.1	4.3	2
161572978	Holliday junction resolvase	ruvC, NGO0153	4.4	10.67	2
59801373	Methylated-DNA--protein-cysteine methyltransferase	NGO0988	3.2	8.65	2
Signal transduction mechanisms					
59801908	Anaerobic transcriptional regulator	NGO1579	31.7	29.51	7
59802186	Two-component system transcriptional response regulator	NGO1866	19.2	19.29	6
Transcription					
59801382	RNA polymerase sigma factor rpod	rpoD, NGO0999	99.8	27.41	17
59800652	Transcription termination factor Rho	rho, NGO0199	37.3	35.32	12
59801138	RpiR family transcriptional regulator	NGO0718	26.7	16.67	4
59801895	ArsR family transcriptional regulator	NGO1562	13.1	22.58	2
59800837	TetR family transcriptional regulator	NGO0393	10.5	12.5	2
59802027	LysR family transcriptional regulator	NGO1706	8.6	9.57	3
Translation, ribosomal structure and biogenesis					
59802104	Ribonuclease E	rne, NGO1785	108.9	32.86	23
59801613	Alanyl-trna synthetase	alaS, NGO1254	77.5	25.74	18
59801095	SUN-family protein	NGO0668	70.7	36.36	13

59802450	Glycyl-trna synthetase subunit beta	glyS, NGO2154	65.7	39.01	20
59802099	Outer membrane protein assembly factor BamE	bamE, NGO1780	38.6	52	6
59802352	Methionyl-trna synthetase	metG, NGO2044	21.4	8.63	5
59802189	16S RNA methyltransferase	NGO1869	19.4	12.41	6
59801089	Aspartyl/glutamyl-trna amidotransferase subunit A	gatA, NGO0662	17.1	11.23	4
59801012	30S ribosomal protein S6	rpsF, NGO0581	16.1	36.07	3
59800723	Dimethyladenosine transferase	rsmA, ksgA, NGO0272	10.5	22.78	4
59802127	Valyl-trna synthetase	valS, NGO1809	8.9	5.61	4
59802333	30S ribosomal protein S9	rpsI, NGO2025	6.9	28.46	2
59802449	Glycyl-trna synthetase subunit alpha	glyQ, NGO2153	6.3	10.4	3
59800751	Phenylalanyl-trna synthetase subunit beta	pheT, NGO0304	3.8	3.43	3
Function unknown					
59800994	Hypothetical protein NGO0561	NGO0561	46.8	30.23	12
651851633	Hypothetical protein NGO1551	NGO1551	43.5	44.34	4
59800868	Hypothetical protein NGO0425	NGO0425	39.7	52.63	8
59802340	DNA recombination protein RmuC	NGO2032	35	20.64	10
59800835	Hypothetical protein NGO0391	NGO0391	24.2	28.72	4
59801088	Hypothetical protein NGO0661	NGO0661	17.1	17.42	5
59802206	Hypothetical protein NGO1889	NGO1889	11.2	10.95	4
59802257	Hypothetical protein NGO1945	NGO1945	10.6	13.31	3
59802030	Hypothetical protein NGO1709	NGO1709	10.4	17.16	3
59801350	Hypothetical protein NGO0961	NGO0961	10	12.32	3
59802266	Hypothetical protein NGO1955	NGO1955	8.3	3.24	3
59801672	Hypothetical protein NGO1321	NGO1321	6.3	15.7	2
59801841	Hypothetical protein NGO1503	NGO1503	4.8	9.66	2
59800735	Hypothetical protein NGO0286	NGO0286	3.2	6.8	2
General function prediction only					
59800727	ComL	bamD, NGO0277	77.6	56.93	14
59801026	Dual-specificity RNA methyltransferase RlmN	rlmN, NGO0596	21.9	18.13	5
59801400	2-nitropropane dioxygenase	NGO1024	21.6	17.39	7
59801596	Hypothetical protein NGO1237	NGO1237	20.3	28.11	4
59801902	ABC transporter ATP-binding protein	NGO1573	14.4	10.75	5
59801028	Hypothetical protein NGO0598	NGO0598	13.9	15.4	4
59802261	Hypothetical protein NGO1949	NGO1949	12.9	16.33	4
59802037	Hypothetical protein NGO1716	NGO1716	11.7	13.47	5
59802288	Malate:quinone oxidoreductase	mqo, NGO1980	9.9	11.07	4
59801241	Oxidoreductase	NGO0831	9.5	7.22	3
59800588	7-cyano-7-deazaguanine synthase	queC, NGO0129	9.1	7.14	2
59802046	BioH - biotin biosynthesis protein	bioH, NGO1725	7.5	8.14	2
59801111	GTP binding protein	hflX, NGO0684	5.4	10.57	3

59802452	Glycosyl transferase	lgtD, NGO2158	5.3	8.28	3
59800762	RNase adaptor protein	NGO0315	3.2	10.21	3
Not in COG					
59801500	Hypothetical protein NGO1133	NGO1133	47.2	35.71	6
59801610	Hypothetical protein NGO1251	NGO1251	41.7	37.5	6
59800790	Twitching motility-like protein	NGO0346	40.3	22.7	8
59800689	Hypothetical protein NGO0236	NGO0236	38.6	26.87	9
59801448	Alpha-2,3-sialyltransferase	NGO1081	28.1	26.72	9
59801263	Hypothetical protein NGO0861	NGO0861	27.5	40.24	4
59801433	Hypothetical protein NGO1063	NGO1063	27.3	23.02	2
59801741	MafA-like protein	mafA2, NGO1393;, mafA3, NGO1584	23.9	27.48	5
59800516	Hypothetical protein NGO0049	NGO0049	19	19.21	4
59800681	Hypothetical protein NGO0228	NGO0228	17.3	34.38	4
59801416	Hypothetical protein NGO1044	NGO1044	16.7	37.57	5
59801437	MafA adhesin protein	mafA1, NGO1067;, mafA4, NGO1972	15.8	16.25	4
59802361	Hypothetical protein NGO2054	NGO2054	15.5	50	4
59800647	ABC transporter ATP-binding protein, polyamine	NGO0192	14.7	8.14	3
59801557	Hypothetical protein NGO1197	NGO1197	14.5	10.58	6
59800807	DEAD/DEAH box helicase	NGO0363	13.6	10.17	6
59800678	MafB-like protein	NGO0225	11.5	7.32	4
59802292	Hypothetical protein NGO1984	NGO1984	8.5	20.83	3
59800478	Leucyl-trna synthetase	leuS, NGO0006	8.4	2.96	2
59801181	Hypothetical protein NGO0766	NGO0766	7.8	5.86	3
59800733	Hypothetical protein NGO0284	NGO0284	7.3	18.71	3
59801141	Phage associated protein	scpA, NGO0721	6.8	11.28	4
59800711	GTP-binding protein Era	era, NGO0260	6.3	9.32	3
59801117	Hypothetical protein NGO0690	NGO0690	4.5	14.75	2
59801075	Hypothetical protein NGO0648	NGO0648	3	18.48	3
59801731	Hypothetical protein NGO1383	NGO1383	2.9	14.4	1
59801918	Hypothetical protein NGO1590	NGO1590	2.6	13.64	1

Chapter 4

Dissecting the signal of lipoprotein sorting and function of BON domains in *Neisseria gonorrhoeae* lipoprotein NGO1985

Igor H. Wierzbicki

Abstract

NGO1985 is an outer membrane, surface exposed lipoprotein of *Neisseria gonorrhoeae* involved in maintaining cell envelope integrity, is important for bacterial antibiotic resistance, survival in the animal model of infection, and was proposed as a vaccine development candidate. NGO1985 is composed of a signal peptide, containing a lipobox motif and two BON domains, which contain conserved glycine residues of unknown function and are hypothesized to have a role in membrane binding through interaction with phospholipids. Current knowledge regarding lipoprotein sorting signals to either inner or outer membrane in *N. gonorrhoeae*, as well as the general function of BON domains and their conserved residues is limited. In this work we expand our understanding of the amino acid components involved in NGO1985 protein localization and function. We demonstrated that NGO1985 does not undergo lipoprotein sorting according to the +2 residue of the lipobox motif as characterized for *Escherichia coli*. However, mutation of the NGO1985 lipobox +2 from phenylalanine to either aspartic acid or tyrosine negatively affected protein function, suggesting that this residue plays an important structural role. Analyses of BON domains have indicated that the presence of both BON domains in their native orientation is required for NGO1985 functionality and stability. Finally, site directed mutagenesis of the predicted conserved glycine residues of the NGO1985 BON domains have shown that these amino acids play an important role in protein stability. This study provides new insights into the sorting of lipoproteins in *N. gonorrhoeae* and addresses for the first time the importance of individual BON domains, their orientation, and their conserved residues in protein function.

Introduction

Bacterial lipoproteins are a class of cell envelope proteins characterized by the presence of acyl groups covalently attached to the invariant cysteine residue during post-translational modification. The acyl moieties function as a membrane anchor for the soluble protein exposed to the aqueous environment (Kamalakkannan *et al*., 2004). This diverse group of proteins participates in various physiological roles, including cell envelope biogenesis, signal transduction, nutrient acquisition, adhesion, antibiotic resistance, and evasion of host defense mechanisms (reviewed by Kovacs-Simon *et al*., 2011). Lipoproteins are synthetized in the cytoplasm as pre-prolipoproteins with N-terminal signal peptides containing a characteristic lipobox motif with an invariant cysteine residue (von Heijne, 1989; Hayashi and Wu, 1990) (Fig. S4.1 A). Pre-prolipoproteins are translocated across the inner membrane by either the Sec or Tat translocon (Shruthi *et al*., 2010; Kudvam *et al*., 2013). On the periplasmic side of the inner membrane, lipoproteins undergo a maturation process. The first modification is catalyzed by the phosphatidylglycerol/prolipoprotein diacylglyceryl transferase (Lgt) which adds a diacylglycerol moiety to the sulfhydryl group of the lipobox cysteine side chain (Sankaran and Wu, 1994) (Fig. S4.1 A). Subsequently, the lipoprotein signal peptidase (LspA, signal peptidase II) cleaves off the amino acids of the signal peptide preceding the cysteine residue of the lipidated prolipoprotein (Tokunaga *et al*., 1982; Yamagata *et al*., 1983; Dev and Ray, 1984) (Fig. S4.1 A). After cleavage, the cysteine of the lipobox becomes the new N-terminus of the nascent apolipoprotein and therefore it is referred to as the +1 (Inouye *et al*., 1983). Finally, the phospholipid/apolipoprotein transacylase (Lnt) attaches a third acyl chain to the free amino group of the cysteine (Gupta and Wu, 1991; Gupta *et al*., 1993) (Fig. S4.1 A). Mature lipoproteins are either transported to the outer membrane by the Lol pathway or remain attached to the inner membrane (Fig. S4.1 B). Export to the outer membrane begins with extraction of the lipoprotein from the inner membrane by the LolCDE complex, followed by coupling the released protein with the periplasmic chaperone LolA (Matsuyama *et al*., 1995; Yakushi *et al*., 2000). The LolA-cargo complex is recognized by the outer membrane acceptor protein LolB and the delivered lipoprotein is released while LolA is recycled to the LolCDE complex (Matsuyama *et al*., 1997). According to the dogma, in

Escherichia coli, lipoproteins destined for the inner membrane contain an aspartic acid residue in the +2 position of the mature protein, which functions as a Lol avoidance signal, otherwise known as the +2 rule (Yamaguchi *et al*., 1988). This sorting mechanism tends to be conserved among enterobacteria; however, it was shown not to apply to other bacterial families and species (reviewed by Konovalova and Silhavy, 2015).

NGO1985 is a surface exposed outer membrane lipoprotein of *N. gonorrhoeae* and a new gonorrhea vaccine candidate (Chapter 3). This protein was found to be crucial for virulence in the estradiol-treated female BALB/c mice model of *N. gonorrhoeae* infection. Functional analyses indicated that NGO1985 is involved in maintaining cell envelope integrity, possibly as a novel component of the β-barrel assembly machinery (BAM) complex. Despite its importance during infection and cell envelope integrity, the precise function and mechanism of action remains unknown. NGO1985 domain architecture include a signal peptide containing a lipobox motif with an invariant cysteine (+1) followed by a phenylalanine residue (+2), and two BON domains. BON stands for "bacterial OsmY and nodulation", and this conserved protein region has been identified in the bacterial osmotic-shock-resistance protein OsmY, hemolysis, nodulation specificity, secretory channels, putative regulatory proteins, and a group of proteins with unknown function (Yeats and Bateman, 2003). BON domains contain several hydrophobic regions and a conserved glycine residue with a currently undefined role. This amino acid organization suggests a binding or structural role of these domains (Yeats and Bateman, 2003). Present understanding of BON domain function comes mainly from *E. coli* OsmY protein studies. Based on the hypothesis, which was never experimentally verified, these domains bind to phospholipids surrounding the periplasmic space of the cell envelope.

Lipoprotein amino acid membrane localization signals in *Neisseria* species, as well as the role and function of BON domains remain underexplored fields of study. In the current work, we utilized NGO1985 as a tool for gaining insight into the +2 rule of lipoprotein sorting in *N. gonorrhoeae* and the involvement of individual BON domains and their predicted conserved glycine residues for protein function.

Materials and methods

Bacterial strains and growth conditions. Bacterial strains used in this study are listed in the Table 4.1. *Neisseria gonorrhoeae* strains were cultured on gonococcal base solid medium (GCB, Difco), or in gonococcal base liquid (GCBL) medium. Kellogg's supplement I and II, were used in 1:100 and 1:1000 ratios, respectively (Spence *et al*., 2008). GCBL was additionally supplemented with sodium bicarbonate (final concentration 0.042%). Gonococci were grown on solid medium for 18-22 h at 37 °C with 5% atmospheric CO_2. Piliated or non-piliated variants were propagated on fresh GCB and incubated for 18-22 h. Piliated bacteria were used for DNA transformation, while colonies with non-piliated morphology were used in all other experiments. *Escherichia coli* was grown on Luria-Bertani agar (LBA, Difco) or in Luria-Bertani broth (LB, Difco) at 37 °C.

Concentrations of isopropyl β-D-1-thiogalactopyranoside (IPTG) were used as indicated: for *N. gonorrhoeae*: 40 or 1000 μM (specified in the text); for *E. coli*: 100 μM. Antibiotics were used in the following concentrations: for *N. gonorrhoeae*: kanamycin 40 μg/mL, erythromycin 0.5 μg/mL; ceftriaxone 0.0009 or 0.0015 μg/mL (specified in the text); for *E. coli*: kanamycin 50 μg/mL, erythromycin 250 μg/mL.

Genetic manipulations. Plasmids and primers used in this study are listed in Table 4.2. Oligonucleotides were designed based on the genomic sequence of *N. gonorrhoeae* FA1090 (NC_002946) using SnapGene software version 2.8 (GSL Biotech LLC) and synthesized by Integrated DNA Technologies. Genomic DNA of *N. gonorrhoeae* FA1090 was isolated with the Wizard Genomic DNA Purification Kit (Promega). PCR products and plasmid DNA were purified using QIAprep Spin Miniprep Kit (QIAGEN). PCR reactions were performed using chromosomal or plasmid DNA as template, appropriate oligonucleotides, and Q5® High-Fidelity DNA Polymerase (NEB). *E. coli* MC1061 was used as the host during molecular cloning procedures. All obtained constructs were verified by Sanger Sequencing at the Center for Genome Research and Biocomputing (CGRB) at Oregon State University. Transformation of *N. gonorrhoeae* was performed as described previously (Zielke *et al*., 2014).

The Δ*ngo1985* mutant strains expressing *ngo1985* bearing mutations in position +2 of the lipobox (phenylalanine 24) or alanine substitutions in conserved glycine residues (91 and 169) were performed using pUC18-NGO1985 vector as template, proper primer pairs (Table 4.2), and Q5 Site-Directed Mutagenesis Kit (NEB), according to the manufacturer's recommendations. Subsequently, variants of *ngo1985* with the desired mutations were amplified with NGO1985-RBS-F and NGO1985-RBS-R primers, and 643 bp products treated with FseI were cloned into ScaI/FseI digested pGCC4 vectors. Obtained constructs were introduced into the FA1090 Δ*ngo1985* background as described above. After transformation, gonococci were selected on solid medium with 0.5 μg/mL erythromycin and verified by PCR with primers pGCC4-Ver-F and pGCC4-Ver-R.

Strains carrying *ngo1985* ΔBON1, ΔBON1, or BON2↔1 were generated as indicated in the following steps. The Gibson Assembly method (Gibson *et al*., 2009) was used to create ΔBON1, ΔBON1, or BON2↔1 variants of NGO1985. Individual variants were amplified with primer pairs BON1-F1/BON1-R1, BON1-F2/BON1-R2; BON2-F1/BON2-R1, BON2-F2/BON2-R2; and BON21-F1/BON21-R1, BON21-F2/BON21-R2, BON21-F3/BON21-R3, and BON21-F4/BON21-R4. NEBuilder HiFi DNA Assembly Master Mix (NEB) was used to ligate individual components according to the manufacturer's recommendations. Subsequently, the product were used as a DNA template for amplification of individual NGO1985 variants with NGO1985-RBS-F and NGO1985-RBS-R primers. Obtained PCR products were introduced into the pGCC4 and introduced into the Δ*ngo1985* strain as described above.

Ceftriaxone sensitivity assay. Strains of *N. gonorrhoeae* (as indicated in the text) were collected from plates, suspended in GCBL to optical density (OD_{600}) of 0.1, and incubated at 37 °C with aeration (220 r.p.m.) for 3 h. Following incubation, bacteria were diluted to OD_{600} of 0.2, serially diluted, and plated on GCB supplemented with IPTG, with or without ceftriaxone. Following 22 h of incubation, Colony Forming Units (CFUs) were scored and bacterial relative viability was calculated as a ratio of CFUs/mL on GCB plates with ceftriaxone to CFUs/mL on plates without the antibiotic. Experiments were performed in three independent biological replicates and mean values and SEMs are presented.

Immunodots. Cells of *N. gonorrhoeae* collected from plates were suspended in GCBL to OD_{600} of 0.1. Overnight culutres of *E. coli* were back diluted 1:100 in LB. Both GCBL and LB broth were supplemented with 40 and 100 µM IPTG, respectively. Following 3 h of incubation with shaking (220 r.p.m.) at 37 °C, cells were harvested by centrifugation (10 min at 4,000 × *g*), and suspended in media to OD_{600} of 2.0. Bacterial suspensions were (5 µL) spotted on 0.45 µm nitrocellulose membrane (GE Life Sciences), allowed to air dry for 30 min, and subjected to immunoblotting as described below.

SDS-PAGE and immunoblotting. *N. gonorrhoeae* whole cell lysates were collected from GCB after 22 h of growth. *E. coli* cell lysates were collected from LB agar plates after overnight incubation. Samples normalized based on OD_{600} values were prepared in SDS sample buffer in the presence of 50 mM dithiothreitol and resolved on 15% Tris-Glycine gels. Following separation, proteins were either visualized by Coomassie Brilliant Blue G-250 or transferred onto 0.2 µm nitrocellulose membrane (GE Life Sciences) using a Trans-Blot Turbo (Bio-Rad). A 5% milk solution in phosphate buffered saline pH 8.0 (PBS, Li-Core, Lincoln, NE) supplemented with Tween 20 at final concentration of 0.1 % (PBST) was used for blocking. After 1 h of incubation, polyclonal rabbit anti-NGO1985 antisera diluted 1:10,000 in 5% milk was added, Goat anti-rabbit IgG antiserum conjugated with horseradish peroxidase secondary antibodies (BioRad) were used at 1:10,000 dilution. Western blots were developed and visualized with Clarity Western ECL-Substrate (BioRad) and a Chemi-Doc^{TM} MP System (BioRad).

Statistical analyses. Statistically significant differences among experimental results were determined by GraphPad Prism's build-in t-test. A 95% confidence level was used for all analyses.

Results

NGO1985 protein architecture and conserved residues of BON domains. NGO1985 is a relatively small (202 amino acids) surface exposed lipoprotein involved in homeostasis of *N. gonorrhoeae* cell envelopes (Chapter 3). Based on predictions (SignalP 4.1 Server; http://www.cbs.dtu.dk/services/SignalP/), this protein is composed of an initial 25 amino acid signal peptide containing a lipobox motif, and two BON (Bacterial OsmY and Nodulation) domains (Fig. 4.1). The NGO1985 lipobox contains an invariant for lipoproteins cysteine residue (position +1; C23) which is followed by phenylalanine (position +2; F24). Cysteine is essential for lipoprotein maturation and lipidation, and in the case of NGO1985, it was shown to be essential for protein surface localization (Chapter 3, Fig. 3.1 K). BON domains are on average 60 amino acids in length with a single invariant glycine (Gly, G) of unknown function (Yeats and Bateman, 2003). In NGO1985, BON domain 1 and 2 span residues 58 to 123 (46 amino acids) and 138 to 195 (56 amino acids), and contain five and four glycine residues, respectively. To identify the conserved glycines, individual BON domains of NGO1985 and several other BON-containing proteins, including *E. coli* OsmY and YraP, were aligned (Fig. 4.2S). Based on this analysis we have concluded that G91 and G169 are the conserved residues of BON domain 1 and 2 of NGO1985, respectively.

Mutation of lipobox +2 Phe into Asp affects NGO1985 function but not sorting to the outer membrane. Residues following the lipobox invariant cysteine have been shown to be important for determining lipoprotein localization to either the inner or outer membrane. In *E. coli*, the aspartate residue (Asp, D) in position +2, as well as Asp, glutamate (Glu, E), or glutamine (Gln, Q) in position +3 serve as retention signals for lipoproteins to the inner membrane (Yamaguchi, *et al*., 1988; Gennity and Inouye, 1991; Seydel *et al*., 1999). However, this dogma does not apply to *Pseudomonas aeruginosa* and *Borrelia* species (Narita and Tokuda, 2007; Lewenza *et al*., 2008; Schulze and Zuckert, 2006). To determine if sorting of NGO1985 to the proper cell envelope compartment depends on position +2 of the lipobox, site directed mutagenesis was applied. The phenylalanine (F24) adjacent to the cysteine 23 was changed into an Asp, valine (Val, V), or tyrosine (Tyr, Y). NGO1985 and its homologue from *N. meningitidis*, GNA2091, display remarkable homology with only

7 divergent amino acids, including valine in the +2 position of the lipobox. Our previous studies have shown that these two proteins function interchangeably between the two species, indicating that the variable amino acid residues do not interfere with their function and, presumably, localization. Tyrosine substitution of phenylalanine was chosen as an additional mutation due to the similarity of amino acid side chain shape. The lipobox +2 variants (F24D, F24V, F24Y) of NGO1985 were placed under an IPTG-inducible promoter and introduced into the *N. gonorrhoeae* FA1090 Δ*ngo1985* background. NGO1985 should be only functional in maintaining cell envelope integrity when it is localized to the surface of the outer membrane. To test if mutations within the +2 position of NGO1985 lipobox will have adverse effect on the antibiotic susceptibility of *N. gonorrhoeae*, the wild type (wt), an isogenic Δ*ngo1985* mutant, and Δ*ngo1985* expressing either native or F24D, F24V, and F24Y variants of NGO1985 were exposed to increasing levels of ceftriaxone in solid medium. In the presence of low antibiotic concentrations (0.0009 μg/mL), the Δ*ngo1985* mutant displayed 59.96% ± 13.08 SEM relative viability, while wt and complemented mutants did not have any growth defect (Fig. 4.2 A). The presence of 0.0015 μg/mL ceftriaxone caused a further growth defect in the *ngo1985* deletion mutant (0.3% ± 0.08 SEM relative viability) but no significant loss of viability was observed in wt or Δ*ngo1985* strains expressing either the native or F24V variant of NGO1985. In contrast, the mutants expressing NGO1985 F24D or F24Y had 12.83% ± 3.02 SEM and 50.49% ± 2.97 survival rate during non-permissive conditions, respectively. These results indicated that the aspartate and tyrosine +2 mutants partially retained their function.

To determine if the observed decrease in viability of the F24D and F24Y mutants were related to the decreased protein stability, whole cell lysates of bacteria collected from solid media with and without 40 μM IPTG were subjected to immunoblotting with anti-NGO1985 antisera. No apparent difference in NGO1985 abundance between Δ*ngo1985* strains expressing wt or mutated protein variants was observed (Fig. 4.2 B). To examine whether introduction of aspartate in place of phenylalanine caused retention of NGO1985 in the bacterial inner membrane, the surface exposure of NGO1985 F24D was assessed by immunodotting. Intact cells of wt, Δ*ngo1985*, Δ*ngo1985*/P_{lac}*::ngo1985*, and Δ*ngo1985*/P_{lac}*::ngo1985* F24D grown in

liquid media supplemented with IPTG were spotted onto nitrocellulose membrane and probed with antibodies against NGO1985. NGO1985 F24D was identified on the gonococcal cell surface similarly as the wt protein (Fig. 4.2 C). Collectively, our data showed that substitution of NGO1985 F24 to either D or Y, but not V, affected protein function. However, the observed defect in the maintenance of cell envelope integrity was not due to decreased protein abundance and transport to the cell surface.

To examine transport of NGO1985 to the cell surface of *E. coli* and to test whether F24D mutations could interfere with that process, plasmids with either wt or the F24D mutant NGO1985 placed under IPTG-inducible promoters were introduced into *E. coli* MC1061. As control, MC1061 carrying an empty vector was also utilized. Whole cell lysates derived from the obtained *E. coli* strains were analyzed for NGO1985 expression by western blotting. Wt NGO1985 was detected as two protein bands of approximately 21 and 19 kDa, corresponding to the pre-prolipoprotein and mature lipoprotein, respectively (Fig. 4.2 D). The NGO1985 F24D variant was detected solely as a 19 kDa protein band which corresponds to mature protein. A subsequent immunodot experiment indicated that both NGO1985 lipobox mutants were detected on the surface of intact *E. coli* cells. These results show that aspartate in position +2 of NGO1985 does not function as an inner membrane retention signal in either *N. gonorrhoeae* or *E. coli*.

Presence of BON domains and their native order are essential for NGO1985 function. BON domains are hypothesized to bind phospholipid interfaces in the periplasmic space (Yeats and Bateman, 2003). This mechanism was proposed based on the function of *E. coli* OsmY protein, which, similarly to NGO1985, contains two BON domains. However, studies validating these or other binding properties of individual BON domains were never conducted. Moreover, because NGO1985 have been shown to localize to the surface of the *N. gonorrhoeae* cell (Chapter 3, Fig. 3.1 E-H) as well as interact with the BAM complex (Chapter 3, Fig. 3.6 D and G, Table S3.3), the individual BON domains could be associated with different roles. To investigate the importance of individual BON domains on NGO1985 function, Δ*ngo1985* strains expressing NGO1985 with either of the BON domains deleted (ΔBON1 and ΔBON2), as well as NGO1985 with the BON domains interchanged (BON2↔1) were generated.

The survival of wt, Δ*ngo1985*, the complemented strain, and the BON domain mutants was examined on solid media supplemented with ceftriaxone. Introduction of either ΔBON1, ΔBON2, or the BON2↔1 variant of NGO1985 failed to rescue the ceftriaxone sensitivity phenotype of the Δ*ngo1985* mutant (Fig. 4.3. A). The NGO1985 ΔBON1 variant (predicted migration ~14.5 kDa) was detected at a similar level as the native NGO1985 (Fig. 4.3 B). In contrast, no protein band was detected for the NGO1985 ΔBON2 construct (at expected migration ~13.5 kDa). The NGO1985 BON2↔1 migrated as three protein bands, all of which were low in abundance, suggesting that the protein was prone to degradation (Fig. 4.3 B). In conclusion, these results revealed that the presence of both BON domains was indispensable for the function of NGO1985 in maintaining cell envelope integrity. Additionally, the native order of these domains was vital for proper protein folding and stability.

Conserved glycine residues in BON domains are crucial for NGO1985 stability. Amino acid alignment of BON domains from various bacterial species revealed the existence of an invariant glycine residue (Yeats and Bateman, 2003). The implication of these residues in the function of BON domain-containing proteins was never experimentally examined. To examine if predicted conserved glycine residues G91 and G169 of NGO1985 BON domains 1 and 2, respectively, play a role in proper protein function, site directed mutagenesis was used. The G91A and G169A versions of NGO1985 were obtained and introduced under the P_{lac} promoter into the chromosome of the Δ*ngo1985* knockout. Subsequently, obtained strains were tested for cell envelope integrity defects using a ceftriaxone viability experiment. At the 0.0009 ng/mL concentration of the antibiotic, Δ*ngo1985* and Δ*ngo1985*/P_{lac}*::ngo1985* G91A displayed similar decreases in viability (59.96% ± 13.08 SEM and 65.13% ± 10.12 SEM relative viability, respectively), while growth of strains expressing wt and the G169A variant was not significantly affected (Fig. 4.4 A). Concentrations of 0.0015 ng/mL ceftriaxone caused further, decreases in viability of the Δ*ngo1985* and Δ*ngo1985*/P_{lac}*::ngo1985* G91A strains (0.3% ± 0.08 SEM and 1.69% ± 0.59 SEM relative viability, respectively), as well as a significant growth defect in Δ*ngo1985*/P_{lac}*::ngo1985* G169A (33.15% ± 8.84 SEM relative viability) (Fig. 4.4 A).

These results revealed that both G91 and G169 residues were important for NGO1985 function. To examine potential changes in the abundance of the mutated proteins, western blot analysis of wt FA1090 and NGO1985 mutants was performed. The NGO1985 G169A level was lower than in the cells expressing wt protein, while NGO1985 G91A was not detected at all (Fig. 4.4 B). Elevating the IPTG induction level to 1000 µM allowed for detection of the NGO1985 G91A, although still at dramatically low abundance (Fig. 4.4 C). These results suggested that the lack or partial reversion of the antibiotic susceptibility phenotype of Δ*ngo1985* by NGO1985 G91A and G169A, respectively, is caused by the decreased stability of these mutated proteins.

Finally, the stability of NGO1985 variants with glycine mutations in the BON domains was assessed in the heterologous *E. coli* MC1061 host strain. Whole cell lysates of MC1061 bearing vector expressing wt, G91A, and G169A mutants of NGO1985, as well as empty vector as a control, were analyzed for NGO1985 abundance. Similarly, as in *N. gonorrhoeae*, substantially lower levels of NGO1985 G169A were detected in comparison to the wt protein while G91A was not detected (Fig. 4.4 D).

In conclusion, conserved glycine residues G91 and G169 of NGO1985 BON domains are involved in maintaining protein stability and therefore are crucial for the function of NGO1985 in maintaining cell envelope integrity.

Discussion

NGO1985 of *N. gonorrhoeae* is a BON domain containing outer membrane surface exposed lipoprotein, which participates in cell envelope biogenesis through potential interaction with the BAM complex (Chapter 3). In *E. coli*, lipoproteins directed to either the inner or outer membrane can be distinguished based on their amino acid residue following the cysteine (+1) of the lipobox motif. Asp residue in position +2 of the lipobox motif functions as a Lol avoidance signal, resulting in retention of the lipoprotein in the inner membrane. The lipobox +2 sorting rule was shown to be conserved among enteric bacteria (Lewenza *et al*., 2006). However, representatives of other bacteria, including *Pseudomonas aeruginosa* and *Borrelia* species, do not follow this rule (Narita and Tokuda, 2007; Lewenza *et al*., 2008; Schulze and Zuckert, 2006). We have tested whether an artificially introduced Asp residue in the +2 position of NGO1985 would function as an inner membrane retention signal (Fig. 4.1). Our results showed that the NGO1985 F24D was expressed at a similar level as the wt protein, and, despite the introduced Asp mutation, it was detected on the surface of *N. gonorrhoeae* cells (Fig. 4.2 B and C). Moreover, when expressed in the heterologous *E. coli* MC1061 host, both wt and the F24D variant of NGO1985 were also identified on the cell surface (Fig. 4.2 E). Immunoblotting analysis of NGO1985 variants expressed in *E. coli* showed that wt protein was detected in two forms, possibly corresponding to pre-prolipoprotein and mature lipoprotein, while the F24D mutant was detected solely in the mature form (Fig. 4.2 D). These results suggest that the inner membrane proteins and Lol machinery of *E. coli* recognized and processed this gonococcal lipoprotein. Existence of two forms of NGO1985 in the heterologous host suggests that the maturation process is not as efficient in *E. coli*. However, the reason NGO1985 F24D was more efficiently processed and exported to the outer membrane by *E. coli* remains puzzling.

Although NGO1985 carrying the F24D mutation was detected on the gonococcal surface, expression of this variant of the protein failed to fully rescue the antibiotic susceptibility of the FA1090 Δ*ngo1985* strain (Fig. 4.2 A). Moreover, mutation of the +2 F24Y also partially abolished the NGO1985 function. A possible explanation for our observed results could be that the side chains of the introduced

amino acids affect the steric interaction between NGO1985 and components of the cell envelope. The phenotype of the Asp residue substitution could be additionally explained by the chemical properties of its side chain. The negative charge of Asp located immediately to the lipid anchor of the NGO1985 might interact with negatively charged phosphate groups of the lipooligosaccharide in the outer leaflet of the outer membrane. This could result in electrostatic repulsion of NGO1985 from the cell envelope, thereby abolishing its interaction with other proteins and function.

The role and function of BON domains in bacterial physiology remains a relatively underexplored field. Most of the current knowledge regarding conserved residues and function of these domains is based on computer modeling and predictions (Yeats and Bateman, 2003). The phospholipid-binding mechanism of BON domains was proposed based on the role of *E. coli* OsmY protein in resistance to inner membrane shrinkage during osmotic shock (Liechty *et al*., 2000; Oh *et al*., 2000; Yeats and Bateman, 2003). According to this model, one BON domain binds the phospholipid layer of the inner membrane while the other binds phospholipids of the inner leaflet of the outer membrane. However, this association was never experimentally validated. Moreover, this model assumes the necessity of two BON domains, while different proteins among various bacterial species tend to have between one to three tandemly repeated copies (Yeats and Bateman, 2003). Because NGO1985 is a surface exposed protein and the Gram-negative outer leaflet of the outer membrane is composed predominantly of lipopolysaccharide/lipooligosaccharide (LPS/LOS), the binding of phospholipid by this protein is not likely. This suggests that BON function might be much more complex than solely phospholipid binding. In our work we have found that deletion of either of the two BON domains, as well as their rearrangement results in complete abolishment of NGO1985 function (Fig. 4.3 A). The ΔBON1 variant of NGO1985 was detected by immunoblotting to migrate accordingly to the predicted mass (~14.5 kDa) and its abundance was similar to the wt protein (Fig. 4.3 B). In contrast, the NGO1985 ΔBON2 construct, which was predicted to migrate at ~13.5 kDa, was not detected at all, while three low abundance forms of NGO1985 BON2↔1 were observed. Absence of BON domain 2 or its mislocalization could affect the protein stability, ultimately leading to the degradation of NGO1985. In our study we

have used polyclonal rabbit anti-NGO1985 antisera. It is possible that our antibodies recognize epitopes outside the BON domain 2 region, which could provide additional explanation why NGO1985 ΔBON2 was not detected during immunoblotting. Further, for the first time we have addressed in our work the importance of predicted conserved glycine residues of BON domains. Bacteria expressing either of the G91A or G169A NGO1985 variants had decreased levels of detected protein, and as a result they displayed a growth defect on solid media supplemented with ceftriaxone (Fig. 4.4 A, B, and C). Subsequent analysis of NGO1985 G91A and G169A expressed in *E. coli* showed a similar decrease in protein abundance, further suggesting that the conserved glycine residues are crucial for NGO1985 protein stability (Fig. 4.4 A).

In conclusion, we report that the *N. gonorrhoeae* outer membrane lipoprotein NGO1985 does not follow the +2 lipoprotein sorting rule; however, phenylalanine in position +2 of the lipobox is potentially important for proper protein topology or interaction with cell envelope components. Both of the NGO1985 BON domains are indispensable for protein function in maintaining cell envelope integrity, as is the native orientation of the two domains. Finally, conserved glycine residues of NGO1985 BON domains are crucial structural components maintaining the stability of the protein.

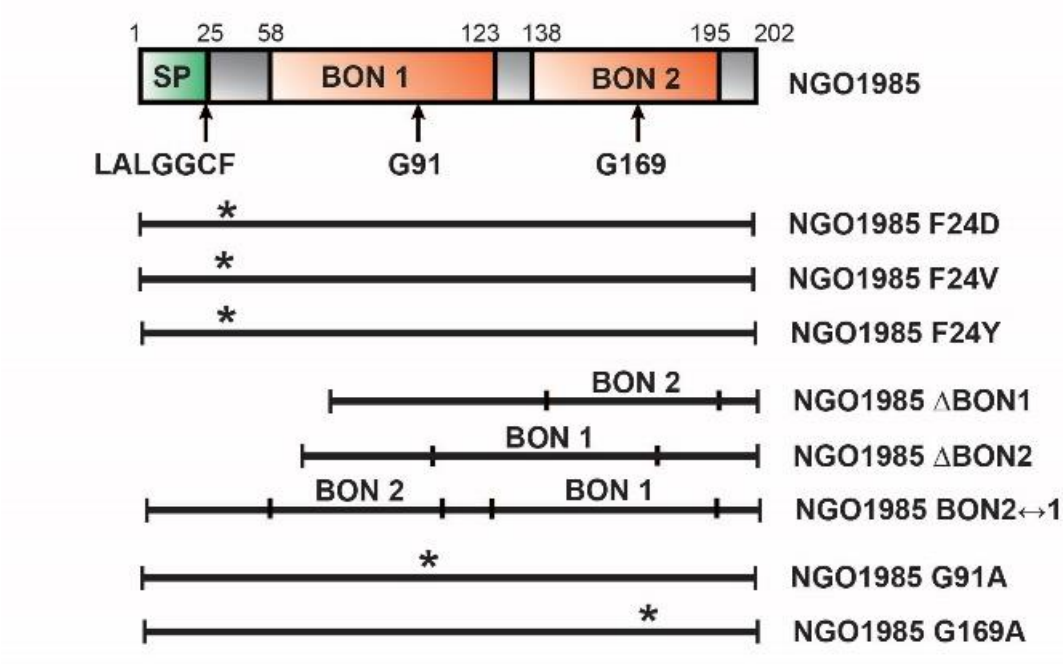


Figure 4.1. Predicted domain organization and characteristic features of NGO1985. Features of NGO1985 are indicated: SP – signal peptide, LALGGCF – lipobox motif, BON1 and 2 - bacterial OsmY and nodulation domain 1 (aa 58-123) and 2 (aa 138-195), G91 and G169 – invariant glycine residues of BON domain 1 and 2, respectively. Different constructs with indicated point mutations, deletions, and rearranged domains used in the current study are presented.

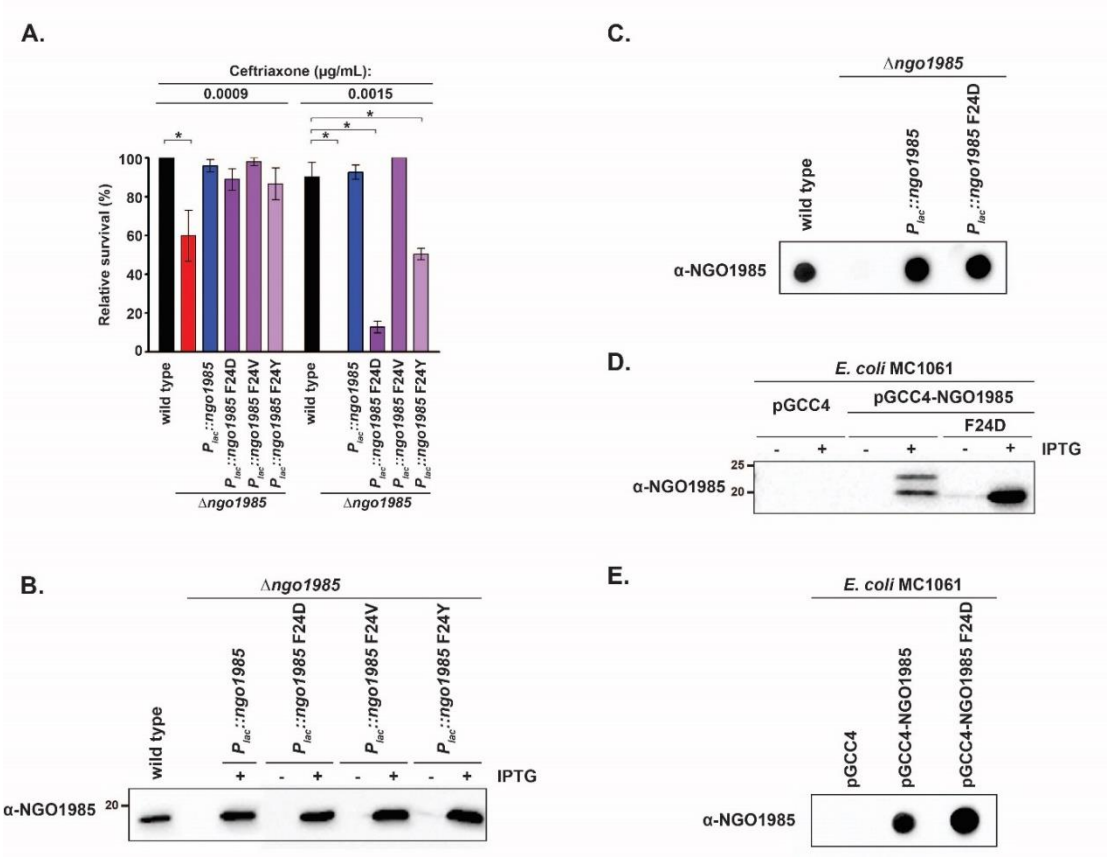


Figure 4.2. Aspartate in lipobox +2 position does not direct NGO1985 to the inner membrane. (A) *N. gonorrhoeae* strains indicated in the text were collected from solid media supplemented with 40 μM IPTG and suspended in liquid media to OD_{600} of 0.1. Bacteria were cultured for 3 h, back-diluted to OD_{600} of 0.2, serially diluted, and spotted on solid media supplemented with IPTG with or without ceftriaxone. On the following day, CFUs were scored and relative viabilities were calculated. Graphs present average values and SEM of three independent experiments. Statistically significant differences ($p<0.05$) are indicated with (*). (B) Whole cell lysates of strains specified in the text were normalized by equivalent OD_{600} units, resolved by SDS-PAGE, transferred onto nitrocellulose membrane, and probed with anti-NGO1985 antisera. (C) Gonococcal isolates were collected from GCB with 40 μM IPTG, suspended in GCBL to OD_{600} of 0.1, and cultured for 3 h. Bacteria were then harvested, suspended in GCBL to OD_{600} of 2.0, spotted on nitrocellulose and probed with antibodies against NGO1985. (D)

Whole cell lysates of *E. coli* MC1061 bearing pGCC4, pGCC4-NGO1985, or pGCC4-NGO1985 F24D plasmids, collected from LB agar plates, were normalized based on OD_{600} values, resolved on a 15% gel, and probed with anti-NGO1985 antibodies. (E) *E. coli* overnight cultures were back diluted 1:100 in fresh media with IPTG and incubated for 3 h. Cells were harvested, suspended in LB broth to OD_{600} of 2.0, spotted on nitrocellulose and probed with antibodies against NGO1985. Migration of a molecular mass marker (kDa) is indicated on the left.

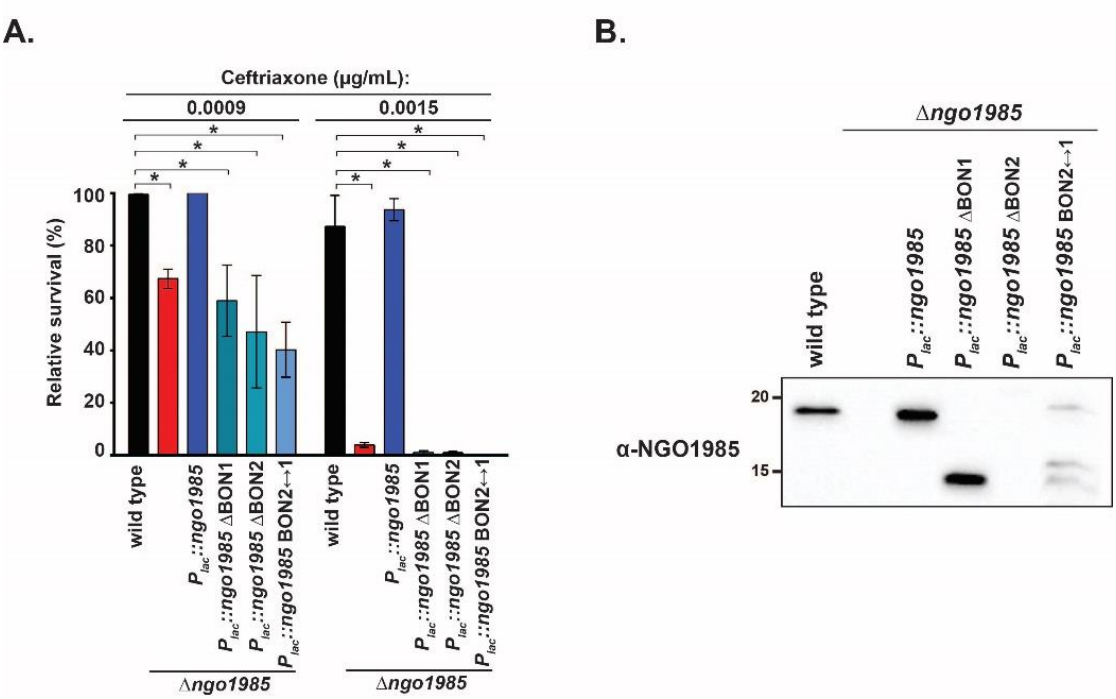


Figure 4.3. Presence of individual BON domains and their order is crucial for NGO1985 function. (A) Wt FA1090 and indicated mutants were collected from GCB supplemented with 40 µM IPTG, suspended in GCBL to OD_{600} of 0.1, incubated for 3 h, back-diluted to OD_{600} of 0.2, serially diluted, and spotted on solid media supplemented with IPTG and either with or without ceftriaxone. CFUs were scored and relative viabilities were calculated the following day. Average values and SEM are presented (*n*=3). Statistically significant differences ($p<0.05$) are indicated with (*). (B) Bacterial whole cell lysates, normalized by equivalent OD_{600} units, were resolved by SDS-PAGE, transferred onto nitrocellulose membrane, and probed with anti-NGO1985 antibodies. Migration of a molecular mass marker (kDa) is indicated on the left.

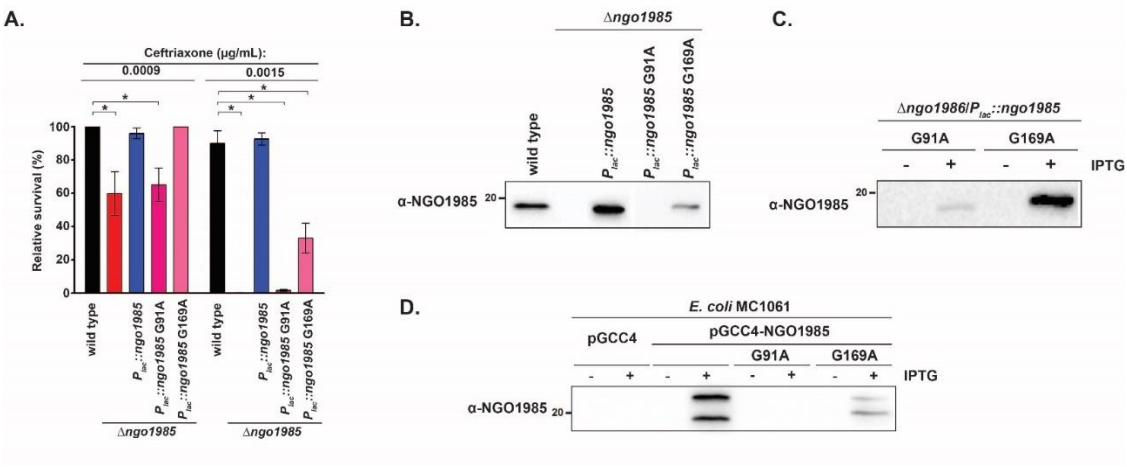


Figure 4.4. Conserved glycine residues of NGO1985 BON domains are pi for protein stability. (A) FA1090 wt, Δ*ngo1985*, and Δ*ngo1985* strains expressing either wt or mutated variants of NGO1985 were collected from GCB, suspended in GCBL to OD_{600} of 0.1 and incubated for 3 h. Subsequently, strains were back diluted to OD_{600} of 0.2, serially diluted, and spotted for CFU scoring on GCB with IPTG and either supplemented or not with ceftriaxone. Relative viability values were calculated based on the CFU numbers the following day. Average values and SEM of three independent experiments are presented. (B) Whole cell lysates of *N. gonorrhoeae* isolates indicated in the text, normalized based on OD_{600} values, were resolved by SDS-PAGE, transferred onto nitrocellulose membrane and probed with antibodies against NGO1985. (C) Δ*ngo1985*/P_{lac}*::ngo1985* G91A and G169A mutants were collected either from plates without or supplemented with 1000 µM IPTG. Whole cell lysates, loaded based on $OD_{600,}$ were resolved on 15% gel and subjected to immunoblotting with anti-NGO1985 antisera. (D) Equal amounts of whole cell lysates of *E. coli* MC1061 strains, described in the text, were resolved by SDS-PAGE and probed with anti-NGO1985 antibodies.

Table 4.1. Bacterial strains used in this study.

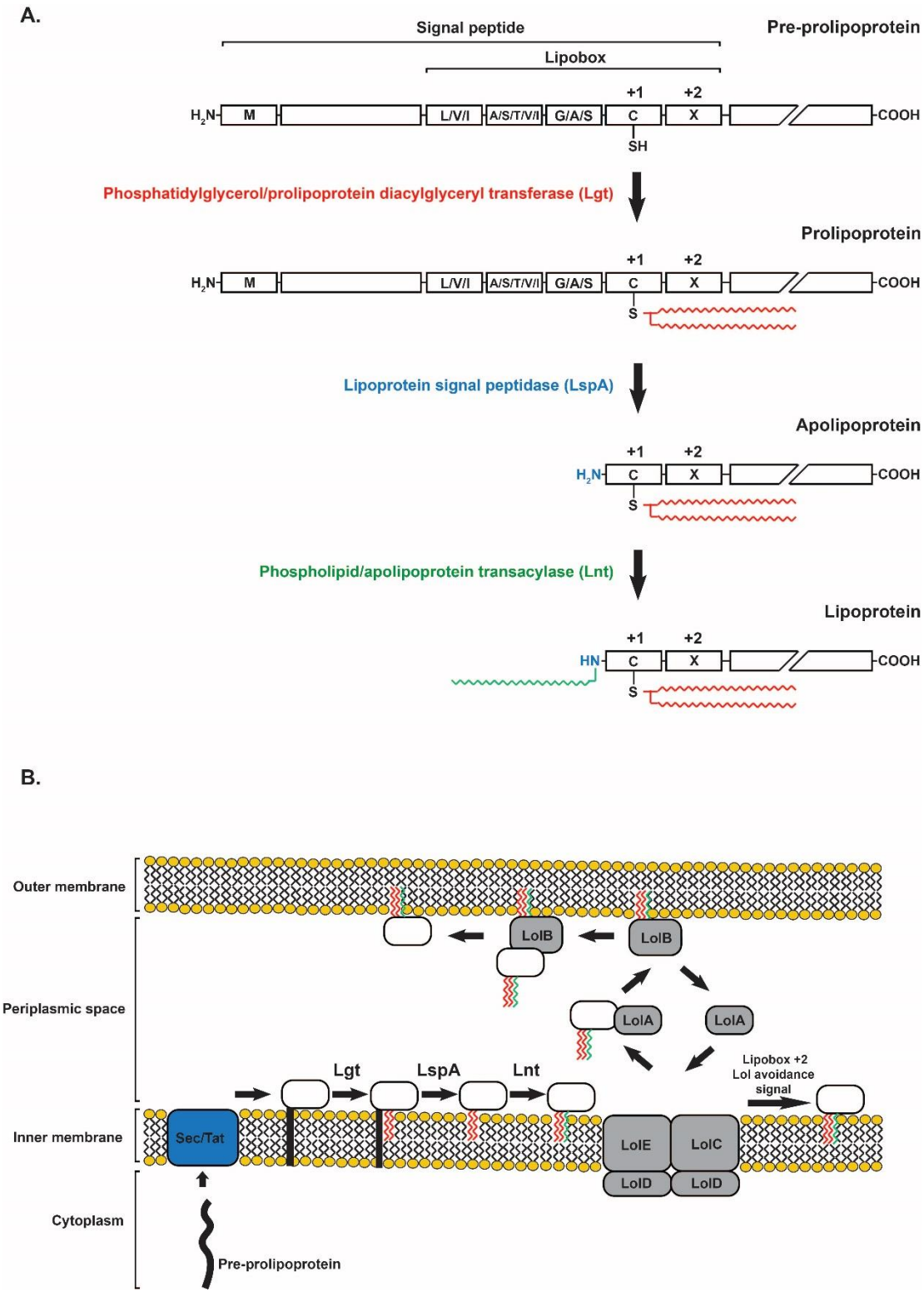
Bacterial strain	Reference
N. gonorrhoeae	
FA1090	Connell *et al*., 1988
FA1090 Δ*ngo1985*	Zielke *et al*., 2014
FA1090 Δ*ngo1985*/P_{lac}::*ngo1985*	Zielke *et al*., 2014
FA1090 Δ*ngo1985*/P_{lac}::*ngo1985* F24D	This study
FA1090 Δ*ngo1985*/P_{lac}::*ngo1985* F24V	This study
FA1090 Δ*ngo1985*/P_{lac}::*ngo1985* F24Y	This study
FA1090 Δ*ngo1985*/P_{lac}::*ngo1985* ΔBON1	This study
FA1090 Δ*ngo1985*/P_{lac}::*ngo1985* ΔBON2	This study
FA1090 Δ*ngo1985*/P_{lac}::*ngo1985* BON2↔1	This study
FA1090 Δ*ngo1985*/P_{lac}::*ngo1985* G91A	This study
FA1090 Δ*ngo1985*/P_{lac}::*ngo1985* G169A	This study
E. coli	
MC1061	Casadaban and Cohen, 1980

Table 4.2. Plasmids and oligonucleotides used in this study.

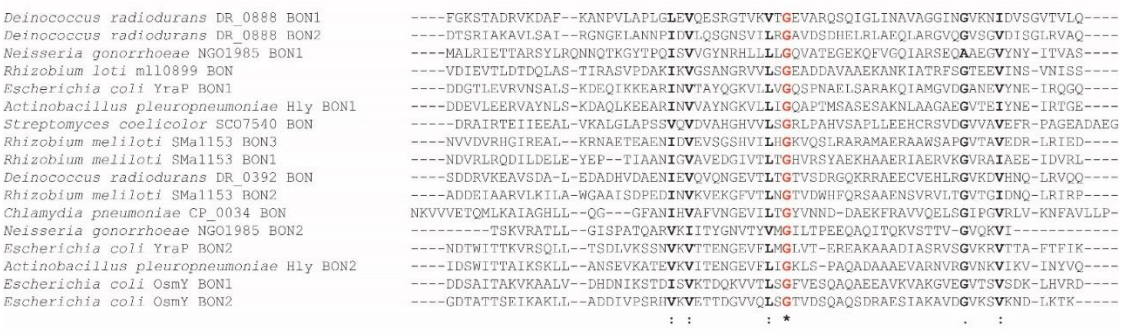
Plasmid Name	**Reference**
pGCC4	Skaar *et al.*, 2002
pUC18-NGO1985	Chapter 3
pUC18-NGO1985 F24D	This study
pUC18-NGO1985 F24V	This study
pUC18-NGO1985 F24Y	This study
pUC18-NGO1985 G91A	This study
pUC18-NGO1985 G169A	This study
pGCC4-NGO1985 F24D	This study
pGCC4-NGO1985 F24V	This study
pGCC4-NGO1985 F24Y	This study
pGCC4-NGO1985 ΔBON1	This study
pGCC4-NGO1985 ΔBON2	This study
pGCC4-NGO1985 BON2↔1	This study
pGCC4-NGO1985 G91A	This study
pGCC4-NGO1985 G169A	This study
Oligonucleotide	**Sequence[1]**
Primers used for site directed mutagenesis:	
F24D-F	CCTCGGCGGCTGCGACAGCGCAGTCGTC
F24D-R	GACGACTGCGCTGTCGCAGCCGCCGAGG
F24V-F	CGGCGGCTGCGTCAGCGCAGT
F24V-R	AGGGCAAGGCTGAGGACGG
F24Y-F	GGCGGCTGCTACAGCGCAGTC
F24Y-R	GAGGGCAAGGCTGAGGACG
G91A-F	CTGCTGCTCGCACAAGTCGCC
G91A-R	CAGGTGGCGGTTGTAGCC
G169A-F	TACGTTATGGCCATCCTCACCC
G169A-R	GGTTACATTGCCGTAGGTAATG
Primers used for deletion and exchanging of BON domains:	
BON1-F1	CCCAAGAAAGGAAGTCCC
BON1-R1	CGCGGCAGTTGGGGCGTGTAGCCTTT
BON1-F2	CGCCCCAACTGCCGCGCACTGCGGGC
BON1-R2	TCAGCGTTGGACGTAGTTTTGGTAGAGGGTAATG
BON2-F1	CCCAAGAAAGGAAGTCCCC
BON2-R1	TAGAGGGTGTTCCAAGTGTCGCCGGC

BON2-F2	CTTGGAACACCCTCTACCAAAACTACGTCCAAC
BON2-R2	GGGCAGTGAGCGCAACGC
BON21-F1	CCCAAGAAAGGAAGTCCC
BON21-R1	CTTTGGACGTTTGGGGCGTGTAGCCTTT
BON21-F2	CACGCCCCAAACGTCCAAAGTCCGCGCC
BON21-R2	GCAGTGCGCGGCAGAATGACTTTTTGTACGCCGACGG
BON21-F3	AAAAGTCATTCTGCCGCGCACTGCGGGCGACATCGCCGG CGACACTTGGAACATCTCCGTCGTCGGCTAC
BON21-R3	GGTAGAGGGTGGAGGCGACGGTAATGTAG
BON21-F4	CGTCGCCTCCACCCTCTACCAAAACTACGTCCAAC
BON21-R4	GGGCAGTGAGCGCAACGC
Primers used for complementation:	
NGO1985-RBS-F	CCCAAGAAAGGAAGTCCC
NGO1985-RBS-R	AAGCTTGGCCGGCCTCAGCGTTGGACGTAGTT
pGCC4-Ver-F	AAATCGCCCTTGATACCG
pGCC4-Ver-R	ACACTTTATGCTTCCGGCTC

[1] Sequences recognized by restriction enzymes are underlined.


A.
Signal peptide
Lipobox
Pre-prolipoprotein
+1
+2
H2N
M
L/V/I
A/S/T/V/I
G/A/S
C
X
COOH
SH
Phosphatidylglycerol/prolipoprotein diacylglyceryl transferase (Lgt)
Prolipoprotein
S
Lipoprotein signal peptidase (LspA)
Apolipoprotein
HN
Phospholipid/apolipoprotein transacylase (Lnt)
Lipoprotein
B.
Outer membrane
Periplasmic space
Inner membrane
Cytoplasm
LolB
LolA
Lgt
LspA
Lnt
Lipobox +2
Lol avoidance
signal
Sec/Tat
LolE
LolC
LolD
Pre-prolipoprotein

Supplementary Figure 4.1. *E. coli* lipoprotein maturation and sorting to the outer- or inner-membrane. (A) The precursor of lipoprotein, pre-prolipoprotein, contains an *N*-terminal signal peptide with a lipobox consensus sequence [LVI][ASTVI][GAS]C. The first enzyme in the lipoprotein maturation pathway, phosphatidylglycerol/ prolipoprotein diacylglyceryl transferase (Lgt) attaches the diacylglyceryl moiety to the thiol group of the invariant cysteine in the lipobox. Following lipidation, lipoprotein signal peptidase (LspA) cleaves the signal peptide preceding the lipobox cysteine of the prolipoprotein. Finally, the phospholipid/apolipoprotein transacylase (Lnt) adds a third acyl chain to the free amino group of the apolipoprotein's lipidated cysteine. (B) Pre-prolipoprotein is synthesized in the cytoplasm and transported across the inner membrane by a Sec or Tat pathway. The immature lipoprotein, anchored to the inner membrane by the signal peptide undergoes processing by Lgt, LspA, and LntA. Unless the aspartate residue is in the position +2 of the lipobox (Lol avoidance signal), the mature lipoprotein is being recognized by LolCDE complex. Lipoprotein is then released and complexed with periplasmic chaperone LolA. The transported protein is received by the outer membrane acceptor LolB which inserts it into the outer membrane. Cargo-free LolA is recycled to the LolCDE.



Supplementary Figure S4.2. Identification of NGO1985 BON domains conserved glycine residues. Individual BON domains of selected proteins belonging to different bacterial species were aligned using online multiple sequence alignment software, Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). BON domain numbering is in order of their appearance in the protein sequence, starting from the N-terminus of the proteins. Conserved glycine residues of individual BON domains are indicated as bold text in red color. Amino acids with similar characteristics are indicated in bold text.

Chapter 5

Discussion and Conclusions

Igor H. Wierzbicki

Overview

The sexually transmitted disease gonorrhea, caused by the Gram-negative obligate human pathogen *N. gonorrhoeae*, has a long recorded history and to this day remains a health and economic burden worldwide. In the United States gonorrhea is the second most commonly reported disease, and it is estimated that annually there are over 78 million new cases of gonococcal infections worldwide (Newman *et al*., 2015). The symptoms of the disease are usually mild or absent (≥50% of women; ≤10% of men); however, untreated gonorrhea can lead to devastating and permanent health consequences among adolescents and neonates. Prolonged *N. gonorrhoeae* infections cause reproductive system damage ultimately leading to infertility and sterility. Newborns delivered by diseased mothers are at high risk of conjunctivitis which can result in corneal scarring and blindness. Moreover, patients with gonorrhea tend to have an increased risk of acquisition and transmission of HIV (Workowski and Levine, 2002).

Among major obstacles in the eradication of this human-specific disease are the constant emerging of antibiotic resistant strains and absence of a protective vaccine. Available treatment options have become limited to dual therapy with extended spectrum cephalosporins, ceftriaxone or cefixime, and the macrolide antibiotic azithromycin (World Health Organization, 2016). However, given the speed of antibiotic resistance determinant acquisition by gonococcus as well as the high cost of treatment, it is believed that these antibiotic regiments will not be long lasting solutions (Unemo *et al*., 2016).

Development of a gonorrhea vaccine has been challenged in the past by phase and antigenic variation of the studied cell surface constituents and the lack of an adequate animal model for screening of potential vaccine antigens (relieved by Jerse *et al*., 2014). The recent development of murine models for gonococcal infections allow study of the physiological function and testing of new vaccine candidates.

Due to the realistic possibility of untreatable gonorrhea in the near future, the World Health Organization and Centers for Disease Control and Prevention stressed the need for new pharmacological interventions including development of low cost antimicrobials with novel mode(s) of action as well as a protective vaccine(s) (World

Health Organization, 2012; Centers for Disease Control and Prevention, 2012). The work conducted and presented in this dissertation emphasizes the characterization and validation of two *N. gonorrhoeae* proteins, $GmhA_{GC}$ and NGO1985, as a molecular target for novel antimicrobials and a potential vaccine antigen, respectively.

$GmhA_{GC}$ involvement in physiology of *Neisseria* species

Over the last few decades, *N. gonorrhoeae* has successfully challenged the arsenal of known and available antimicrobial agents (reviewed by Unemo *et al*., 2016). New combinatorial therapies and derivatives of available antibiotics are being developed to combat the threat of untreatable gonorrhea. However, since resistance to these compounds has already been established, it is just a matter of time until the bacteria will breach these new lines of defense. Indeed, the macrolide derivative solithromycin, which is undergoing Phase 3 clinical trials, has been shown to be less effective for gonococcal isolates with high-levels of resistance to azithromycin (Golparian *et al*., 2012). Therefore, therapeutics affecting new physiological pathways as well as novel drug targets are of demand.

Nucleotide-activated-*glycero*-*manno*-heptoses are ubiquitously present in bacteria and are absent in eukaryotic organisms. Although heptose residues are mainly recognized as a component of the core oligosaccharide of lipopolysaccharides (LPS) and lipooligosaccharides (LOS), they are also components of bacterial capsules, O-antigens, flagella, S-layer glycoproteins, and autotransporters (Valvano *et al*., 2002; Lu *et al*., 2015). In *N. gonorrhoeae*, free heptoses present in the extracellular milieu participate in induction of HIV-1 expression, therefore facilitating viral transmission (Malott *et al*., 2013). The first enzyme in the nucleotide-activated heptose pathway, sedoheptulose-7-phosphate isomerase, GmhA; appears as a promising target for development of new broad spectrum antibiotics (Brooke and Valvano, 1996). GmhA forms a homo-tetrameric complex catalyzing isomerization of D-sedoheptulose 7-phosphate into D-*glycero*-α,β-D-*manno*-heptose-7-phosphate (Eidels and Osborn, 1974; Taylor *et al*., 2008). Abolished GmhA function in some of the studied bacteria was related to a pleiotropic phenotypes including production of shorter LPS/LOS composed only of lipid A and KDO residues, increased susceptibility to antibiotics,

defects in biofilm formation, and decreased virulence (Bauer *et al*., 1998; Brooke and Valvano, 1996; Darby *et al*., 2005; Aballay *et al*., 2003). GmhA function was not essential for viability of the studied bacterial species, and therefore potential inhibitors could function as anti-virulence drugs, which are believed to undergo reduced selection for resistance (Allen *et al*., 2014).

In Chapter 2, we characterized the role and the importance of GmhA for physiology of *N. gonorrhoeae* to validate its potential as a target for new gonorrhea therapeutics. Our experiments showed that $GmhA_{GC}$, encoded by the gene *ngo1986*, is indispensable for synthesis of full length LOS molecules (Fig. 2.1 B and C). However, in contrast to findings from other microorganisms $GmhA_{GC}$ was pivotal for gonococcal viability as bacteria depleted in this protein grew scarcely on solid media and their proliferation was halted in the liquid medium (Fig. 2.1 A, D, and E).

Site directed mutagenesis of the $GmhA_{GC}$ predicted catalytic residues (glutamic acid in position 65 and histidine in position 183) abolished LOS production, yet it did not affect bacterial growth (Fig. 2.4 A and B). These results provided further support for direct involvement of this protein in isomerization of D-sedoheptulose 7-phosphate. At the same time, we have determined that that full length LOS is not indispensable for proper gonococcal growth, and thus the role of GmhA in cell viability is disconnected from heptose synthesis. The reduced viability of bacteria depleted of GmhA was not limited to *N. gonorrhoeae* as a similar decrease in growth was observed for *N. meningitidis* (Fig. 2.5 B). Moreover, GmhA homologues of the two *Neisseria* species functioned interchangeably, rescuing proper LOS production and bacterial growth (Fig 2.5 A and B). In comparison, GmhA of *E. coli* was not able to functionally compensate for the lack of $GmhA_{GC}$, as we could not generate viable *N. gonorrhoeae* expressing only the *E. coli* homologue. These results suggested a functional or structural difference(s) between GmhA from *Neisseria* species and other studied organisms.

This led us to the determination of the $GmhA_{GC}$ three dimensional structure. Based on our initial purification of the recombinant protein utilizing size exclusion chromatography, we have deduced that $GmhA_{GC}$ forms tetramers in solution (Fig. S2.1 A). Subsequent crystallization studies have verified our initial observation that indeed, similarly to known homologues, $GmhA_{GC}$ is a homo-tetramer (Fig. 2.6 A). Crystal

structure indicated that $GmhA_{GC}$ has the so-called "closed" conformation and contains four zinc ions in the active site of the enzyme that are immobilized by the predicted catalytically relevant amino acid residues, including glutamic acid 65 and histidine 183 (Fig. 2.6 C). Our results are in agreement with the solved *Burkholderia pseudomallei* GmhA crystal structure, where zinc ions were found to be embedded within the structure of the protein (Harmer, 2010). Thus, our work has provided additional evidence for GmhA proteins being metalloenzymes. Nevertheless, comparison of our crystal structure with determined structures of GmhA homologues from other bacteria didn't indicate differences in either overall structure of the protein or within the enzymes' active site. Therefore, the extended spectrum of GmhA function in gonococcus and meningococcus is most likely not driven by the difference in protein organization.

In *E. coli*, GmhA shares a significant similarity in structure to the DnaA initiator associating protein A, DiaA. It was shown that DiaA plays a crucial role in initiation and timing of chromosomal replication during the bacterial cell cycle (Ishida *et al*., 2004; Keyamura *et al*., 2007). Study of *Colwellia psychrerythraea* noted that GmhA and DiaA from this bacterium are virtually identical, differing by only 3 amino acids (Do *et al*., 2015). It was also previously reported that many bacterial species encode a DiaA homologue but not a GmhA homologue (Keyamura *et al*., 2007).

Analysis of *N. gonorrhoeae* and *N. meningitidis* genomes indicated that the closest homologues of *E. coli* DiaA are $GmhA_{GC}$ and $GmhA_{NM}$, respectively (Table 2.3). The structural similarity of DnaA and GmhA proteins and the fact that some bacteria encode only one homologue of the two proteins, suggest that $GmhA_{GC}$ could be involved in heptose synthesis and the DNA replication. We found that $GmhA_{GC}$ expression was highest during the mid-exponential phase of bacterial growth (Fig. 2.2 B and C). During that time cells are dividing most actively, and therefore must adequately replicate their genomic DNA. This observation, as well as the fact that the level of $GmhA_{GC}$ is not directly correlated with LOS abundance, supports a potential role of this protein in DNA replication (Fig. 2.2 D and E). Further work involving determination of the potential interaction of $GmhA_{GC}$ with the DnaA homologue as

well as analysis of global changes in metabolism is required to elucidate and understand the whole scope of $GmhA_{GC}$ physiological functions in *N. gonorrhoeae*.

NGO1985 is a surface-exposed lipoprotein of *N. gonorrhoeae*

Due to the constant acquisition of antimicrobial resistance by *N. gonorrhoeae*, development of a protective vaccine(s) would be the most effective way to decrease its prevalence (Craig *et al*., 2015). Moreover, since the human host is the only reservoir for *N. gonorrhoeae*, introduction of a vaccine could plausibly lead to eradication of this pathogen.

One of the main reasons behind the failure of previous vaccine development attempts is the phase and antigenic variability of many of the targeted surface components of *Neisseria*. An excellent example of this obstacle was the experimental vaccine composed of the purified pili protein which failed in clinical trials (Brinton *et al*., 1982; Tramont and Boslego, 1985). Therefore, it was believed that development of a protective vaccine against gonorrhea would not be possible (reviewed by Jerse *et al*., 2014). Although many of the known gonococcal cell envelope constituents are not suitable vaccine antigens, a large body of the membrane proteins remains to be characterized with the regard to their phase/antigenic variability, function, and surface localization.

Recent development of the vaccine(s) against *N. meningitidis* serogroup B, which is based on the constituents of the meningococcal outer membrane, has become a source of encouragement for the gonorrhea vaccine field (reviewed by Jerse *et al*., 2016; Zielke *et al*., 2016). It is important to mention that the search for a suitable meningococcal vaccine began with a set of over 600 protein candidates based on the computer prediction of their localization (Seib *et al*., 2012). After a thorough and complex selection process, five proteins along with MVs were finally formulated into the successful 4CMenB vaccine. In contrast, currently there are only about 12 reported gonococcal proteins that are actively pursued as vaccine candidates, with an additional 5 recently proposed (Jerse *et al*., 2014; Zielke *et al*., 2016) (Table 1.1).

Two things we can learn from the 4CMenB vaccine is that the development of a vaccine for gonorrhea is possible, and that to do so we need to identify and validate more antigens than our currently available set.

Our laboratory has performed two proteomic mining dedicated to the identification of new therapeutic targets against gonorrhea (Zielke *et al*., 2014; Zielke *et al*., 2016). The first work compared the cell envelopes and naturally released membrane vesicles of four well characterized and established gonococcal isolates (MS11, FA1090, F62, and 1291) for identification of proteins expressed with similar abundance among these isolates (Zielke *et al*., 2014). The more recent work focused on analysis of cell envelopes for identification of ubiquitously and differentially expressed proteins of *N. gonorrhoeae* FA1090 strain cultured under conditions mimicking host micro-niches (Zielke *et al*., 2016). A set of 92 proteins were identified as common between these two independent studies. One of these mutual hits was the putative outer membrane protein NGO1985.

Analysis of the predicted amino acid architecture of NGO1985 indicated the presence of a lipoprotein-characteristic lipobox motif and two BON (Bacterial OsmY and Nodulation) domains. Our initial characterizations have shown that bacteria lacking NGO1985 are susceptible to various chemical probes including detergents (bile salts, SDS) and different antibiotics (polymyxin B, chloramphenicol) (Zielke *et al*., 2014). Based on these findings, NGO1985 was hypothesized to participate in maintaining cell envelope integrity and thus became a promising target for pharmaceutical interventions against gonorrhea.

It is generally considered that a good vaccine target should be exposed on the surface of the pathogen. Therefore, the first part of Chapter 3 focuses on determination of NGO1985 localization in the gonococcal cell envelope. As introduced in the paragraph above, NGO1985 contained a putative lipobox, which is a hallmark of lipoproteins. This amino acid motif contains an invariant cysteine residue, crucial for protein maturation and lipidation, which allows for association with either the inner or outer membrane. Up until recently, there was a conviction that outer membrane lipoproteins are in most cases localized on the periplasmic site while surface exposed ones are a rare occurrence (reviewed by Buddelmeijer, 2015). However, with the ever

increasing body of literature, as well as revisiting some of the initial findings, the view on cell surface lipoproteins has undergone a significant change (reviewed by Konovalova and Silhavy, 2015). Increasing number of lipoproteins have been identified to be associated with the surface of many different bacterial species. Our results have indicated that NGO1985 is surface accessible to antisera binding as well as to processing by proteolytic enzymes (Fig. 3.1 E, F, and G). Mutagenic analysis of the predicted conserved cysteine of NGO1985 putative lipobox has revealed that this residue is crucial for protein surface exposure as well as for proper maturation (Fig. 3.1 I). Based on our findings, gonococcal NGO1985 was added to the pantheon of the surface exposed lipoproteins.

Although *N. gonorrhoeae* and *N. meningitidis* differ in their occupied niches within the human body and in the disease symptoms they cause, these two species share a significantly high level of homology between their counterpart proteins. NGO1985 of the FA1090 strain and its meningococcal homologue GNA2091 from the MC58 isolate, differ in only 9 of 202 amino acids. Because of this level of conservation, we did not find it surprising that antibodies raised against NGO1985 readily cross-reacted with GNA2091 (Fig. 3.2 A). GNA2091 is one of the five proteins that were included in the 4CMenB vaccine; however, it was suggested to be localized on the periplasmic site of the outer membrane (Bos *et al*., 2014). It is important to note that there is conflict in the literature regarding GNA2091 orientation within the cell envelope. Later studies performed by another group reported that a component of innate immunity, long pentraxin 3 (PTX3), was able to recognize and bound GNA2091 on the intact meningococcal cells (Bottazzi *et al*., 2015). Moreover, authors of this work refer to GNA2091 as a "surface molecule" without discussing the work of Bos *et al*, 2014. Despite the controversial orientation of GNA2091 in the outer membrane, there are several examples of gonococcal and meningococcal proteins displaying high levels of amino acid conservation but differing in their localization. This includes two of the other 4CMenB vaccine components, NspA and fHbb, which are surface-exposed solely in *N. meningitidis* (Moe *et al*., 1999; Jongerius *et al*., 2013).

Our studies demonstrated that NGO1985 and GNA2091 function interchangeably in both *Neisseria* species (Fig. 3.2 B). This raises the question: why

can proteins with different localizations be functionally similar in these two different organisms? Due to the contradictory reports regarding GNA2091 orientation, localization studies of this protein should be carefully revisited. Furthermore, the topology of NGO1985 and GNA2091 remains unknown. Based on experimental results we were not able to determine if the entirety of NGO1985 is exposed to the extracellular space or if certain regions of the protein are embedded in the lipid bilayer of the bacterial cell membrane. Therefore, the potential difference between the gonococcal NGO1985 and meningococcal GNA2091 may not be entirely due to their localization to either the inner or outer leaflet of the outer membrane, but rather to their topology and to the protein regions that are exposed to the environment.

NGO1985 lipobox +2 residue implication in protein localization and function

As discussed above, our experimental results from Chapter 3 indicate that NGO1985 is an outer membrane lipoprotein exposed on the cell surface. Biogenesis and mechanisms involved in lipoprotein localization to either the inner or outer membrane have been well studied in *E. coli* (reviewed in Konovalova and Silhavy, 2015). It has been shown that residues following the lipid-modified cysteine (+1) play an important role in determining the lipoprotein destination. According to the +2 rule, otherwise called the Lol-avoidance signal, aspartic acid in the position +2 of the lipobox motif causes a retention of the proteins to the inner membrane. However, the dogma of lipoprotein sorting does not apply to bacteria from the *Borrelia* genus or to *Pseudomonas aeruginosa*. The +2 rule has not been investigated for *Neisseria*. The first part of Chapter 4 expands the analysis of NGO1985 lipobox motif residue +2 with respect to protein localization and function. Based on our site directed mutagenesis results, substitution of a phenylalanine residue following the invariant cysteine of NGO1985 lipobox into aspartic acid did not affect protein localization to the outer membrane (Fig. 4.2 C). Moreover, when expressed in a heterologous host, both native and F24D variant of NGO1985 were detected on the surface of *E. coli* cells (Fig. 4.2 E). The fact that NGO1985 underwent maturation and was transported to the *E. coli* outer membrane was not unforeseen. It was shown that SpaI lipoprotein of the Gram-positive bacterium *Bacillus subtilis* was also partially processed when expressed in *E.*

coli (Halami *et al*., 2010). This suggests that due to conservation of machinery involved in lipoprotein maturation and transport, protein substrates even from distinct species of bacteria can be recognized and processed by these pathways. Nevertheless, we found it startling that NGO1985 F24D expressed in *E. coli* was not confined to the inner membrane. These results suggest that lipoprotein sorting might be much more complex and could depend on additional properties of the protein amino acid sequence.

Although the introduction of aspartic acid in the position +2 of NGO1985 did not result in protein localization to the inner membrane, the mutated variant of the protein was not able to fully rescue the antibiotic-sensitive phenotype of Δ*ngo1985* (Fig. 4.2 A). Additionally, substitution of phenyl alanine 24 into the tyrosine, but not the valine residue, also caused a partial abolishment of protein function. These results show that the amino acid following the lipidated cysteine plays an important role in proper NGO1985 function. However, the mechanism for how these mutations have adverse effects on NGO1985 remains unclear. Due to lack of determined NGO1985 topology and structure it is difficult to predict how introduced mutations effect the protein conformation. Also, it is important to remember that macromolecules of the living organism do not exist on their own in the suspension, but are part of a broad network of interactions with other cellular components. An excess of aspartic acid or tyrosine side chains in place of phenylalanine could introduce electrostatic and/or physical interference with other cell envelope components, including negatively charged LOS and proteins interacting with NGO1985.

NGO1985 functions in maintaining cell envelope homeostasis

While the beginning of Chapter 3 focused on determination of the localization and conservation of NGO1985, subsequent work analyzed the role and importance of NGO1985 in physiology of *N. gonorrhoeae*. The initial characterization of NGO1985 performed in our laboratory indicated that deletion of this protein in laboratory strain FA1090 results in increased bacterial susceptibility to different chemical probes, including detergents and antibiotics targeting the cell envelope (polymyxin B) and cytoplasmic molecules (chloramphenicol) (Zielke *et al*., 2014). Based on this

experimental data, NGO1985 was hypothesized to be involved in maintaining cell envelope homeostasis/integrity.

In this work we have expanded the initial study and showed that the absence of NGO1985 in recent clinical isolates of *N. gonorrhoeae* increases their susceptibility to various classes of antibiotics including the last resort treatment options cefixime, ceftriaxone, and azithromycin (Fig. 3.3 A and B). There was none specific pattern in antibiotic susceptibility to tested classes of compounds among analyzed mutants. Therefore, this observation further supported the hypothesis of NGO1985 being involved in cell envelope integrity, rather than functioning specifically in antibiotic resistance. During our studies we observed that elevated levels of NGO1985 provided better fitness to bacteria exposed to ceftriaxone (Fig. 3.3 C). This suggested potential involvement of NGO1985 in sensing and protecting bacterial cells from environmental stress factors. However, we have not observed upregulation of NGO1985 expression during exposure of gonococci to different compounds, growth on solid or in liquid media (Fig. 3.4 D). It is possible that expression of NGO1985 is regulated by certain specific host factors.

Additionally, we determined that NGO1985 provides a growth advantage for gonococci cultured *in vitro* under conditions mimicking host micro-niches: in the presence of normal human serum and during anoxia; as well during experimental *in vivo* murine infection (Fig. 3.4 C and F). Analysis of NGO1985 expression under laboratory host-relevant conditions did not indicate changes in protein abundance (Fig. 3.4 D), which is in agreement with our recent proteomic study (Zielke *et al*., 2016). Lack of NGO1985 upregulation during exposure to human serum and in the absence of oxygen suggests that this protein is not directly involved in survival during non-permissive conditions. Based on immunoblotting analysis we know that NGO1985 is expressed by gonococci during infection in the mice (Fig. 3.4 E). Due to lack of an available loading control for our immunoblotting samples, we cannot deduce if NGO1985 expression varies throughout infection. During mice coinfection, the NGO1985 mutant was 100- to 1000-fold outcompeted by the wild type strain. We speculate that NGO1985 most likely is not associated with adhesion, as we would expect the fitness to decrease steadily over time. Instead, we saw a stable decreased

fitness over the course of the experiment, further supporting the hypothesis of NGO1985 playing general role in maintaining membrane homeostasis.

Bacterial membrane defects are often accompanied by leakage of periplasmic contents into the extracellular milieu and increased membrane vesicle release (Lopes *et al*., 1972; McBroom and Kuehn, 2007). Culture supernatants of NGO1985 mutants contained increased levels of periplasmic and cytoplasmic proteins and produced approximately 3-fold more membrane vesicles than wild type *N. gonorrhoeae* (Fig. 3.5 A, B, and D). The presence of cytoplasmic proteins suggests that besides increased leakage of periplasmic contents, NGO1985 mutants undergo frequent lysis. We have also identified that cells of Δ*ngo1985* contain decreased amounts of periplasmic chaperones SurA and DsbA, and an elevated abundance of cytoplasmic protein Zwf (Fig. 3.5 D and E). This decrease in periplasmic chaperones could potentially lead to incorrect folding of outer membrane proteins (Sklar *et al*., 2007; Heras *et al*., 2009). Upregulation of Zwf has been linked to cell envelope stress and to the presence of reactive oxygen species (Bernsetin *et al*., 1999; Merritt and Donaldson, 2009; Sandoval *et al*., 2011). Collectively, this indicates that the cell envelope of NGO1985 mutant could be under constant stress due to an accumulation of misfolded proteins. Analysis of NGO1985 mutant cell envelopes did not show a dramatic change in their overall protein profile, with the exception of a decreased abundance of TamA components of the TAM complex involved in autotransporter assembly (Fig. 3.5 F and G) (Selkrig *et al*., 2012). In contrast, abundance of many proteins was elevated in the membrane vesicles of Δ*ngo1985* (Fig. 3.5 F and G). Membrane vesicles are described in the literature as a means for bacteria to dispose of misfolded and toxic proteins (McBroom and Kuehn, 2007). Therefore, based on our combined results, we conclude that absence of NGO1985 leads to a general cell envelope defect responsible for the pleiotropic phenotype of Δ*ngo1985*. Leakage of periplasmic chaperones potentially results in misfolding and aggregation of the outer membrane proteins which ultimately promotes increased release of membrane vesicles.

NGO1985 association with β-barrel Assembly Machinery (BAM) Complex

Our results described in the above section suggest NGO1985 involvement in maintaining cell envelope integrity. To gain a more in-depth understanding of protein function, we have investigated the NGO1985 interactome by applying tandem affinity purification (TAP) coupled with mass spectrometry (MS) analysis. The TAP method involves fusion of the protein of interest with a tag composed of Protein A and Calmodulin Binding Protein allowing for two step affinity purification of the protein complexes (Puig *et al*., 2001). Our analysis of proteins co-purified with TAP-tagged NGO1985 revealed a broad network of interaction (Fig. 3.6 A, B, C). The 246 proteins identified in complexes with NGO1985 belong to different functional classes including transport of various molecules, metabolism, energy production, and cell envelope biogenesis (Fig. 3.6 E, Table S4.4). The methods involving combination of affinity purification of protein complexes and MS analyses are known for their sensitivity and very often large quantities of proteins are identified during these studies (Kaiser *et al*., 2008; Chang *et al*., 2009; Willmann *et al*., 2016; Boyarchuk *et al*., 2016). Most of the proteins come into interaction with multiple alternative complexes during their lifetime, e.g. involved in their synthesis, maturation, transport, and finally their physiological function(s). Therefore, experiments utilizing MS analysis of protein complexes represent an average of binding partners. Moreover, it is important to be aware that some of the identified proteins can be indirectly associated with the true target through a secondary binding to other components of the complexes. Some authors consider these as non-specific and exclude DNA repair proteins, translation/initiation factors, ribosomal proteins, and chaperones from their analysis due to their identification in various data sets utilizing TAP-MS approach (Boyarchuk *et al*., 2016).

Among the proteins identified in complexes with TAP-tagged NGO1985 were the BAM complex components: BamA (NGO1801), BamC (NGO0948), BamD (NGO0277), and BamE (NGO1780) (Table S3.4, Fig. 3.6 D). The BAM complex is involved in folding and assembly of outer membrane β-barrel proteins into the outer membrane and therefore it is indispensable for maintaining integrity of the cell envelope and bacterial physiology (Ricci and Silhavy, 2012). The function of

NGO1985 as a part of the BAM complex could explain its involvement in cell envelope homoeostasis. Our subsequent experiments have shown that proteins co-purified with TAP-tagged BamE contained all four BAM components and NGO1985, further supporting the idea of these proteins functioning together (Fig. 3.6 G). The two known homologues of NGO1985, *E. coli* YraP and *N. meningitidis* GNA2091, have been suggested previously as potential components of the BAM complex; however, experimental data supporting this interaction have never been shown (Onufryk *et al*., 2005; Bos *et al*., 2014).

During our trans-complementation studies, we observed, that the phenotype of *N. gonorrhoeae* Δ*ngo1985* is rescued by GNA2091 but not by YraP protein (Fig. 3.2 B). Studies regarding BamA have shown that meningococcal and *E. coli* homologues did not cross-complement their function in these two species (Volokhina *et al*., 2013). Authors of this work speculated that lack of functional redundancy could be related to the evolutionary distance between *E. coli* and *N. meningitidis,* belonging to the class of γ- and β-proteobacteria, respectively; leading to inability to interact with other components of the complex. Therefore, lack of functional complementation of NGO1985 absence by *E. coli* YraP could be due to slight, progressive evolutionary changes in their interacting partners between these species.

BamA is the main component of the BAM complex, spanning through the entire bacterial outer membrane (Ricci and Silhavy, 2012). The N-terminal part of BamA is localized in the periplasm and is composed of five Polypeptide Translocation Associated (POTRA) domains which serve as the site of interaction with other known components of the BAM complex (Kim *et al*., 2007). Although interactions of BAM components appear to be limited to the periplasmic space, studies regarding BamC indicated that this lipoprotein is at least partially exposed on the surface of the outer membrane (Webb *et al*., 2013). Authors speculated that the surface exposed portion of BamC could potentially come into interaction with surface loops of BamA. Based on our results NGO1985 is associated with the surface of gonococcal cells, and since this protein possibly comes into interaction with the BAM complex one could speculate that it contacts the surface exposed portion of BamA. However, as described in the previous sections, due to the lack of determined topology of NGO1985 in the outer

membrane, the potential interaction with periplasmic localized BAM components should not be excluded at this stage.

Although the BAM complex is conserved among bacteria, species specific differences exist in the form of absence, duplication, or presence of additional elements of the complex (Anwari *et al*., 2012; Volokhina *et al*., 2009). An example of such a difference is the lack of a typical BamB homologue in *Neisseria* species. In *E. coli*, BamB has recently been shown to be involved in biogenesis of BamA (Misra *et al*., 2015). During our analyses of MVs derived from Δ*ngo1985*, we have observed elevated levels of BAM components, with the biggest increase in abundance of BamA (Fig. 3.5 G). As discussed before, it is hypothesized that membrane vesicles are a means for bacterial cells to dispose of misfolded, aggregated, or toxic proteins. This suggests that the absence of NGO1985 effects the assembly and/or stability of BamA leading to its release in the membrane vesicles. Therefore, NGO1985 could have evolved in *N. gonorrhoeae* to have a similar function(s) as BamB in *E. coli*. Future studies will be required to validate these hypotheses as well as to further determine the potential interaction of NGO1985 with BAM complex.

BON domains of NGO1985

To gain an understanding of NGO1985 function, we have analyzed individual amino acid components of this protein. As discussed in Chapter 3 and the beginning of Chapter 4, we have investigated the importance of lipobox residues +1 and +2 with respect to the protein localization and function. The remaining work discussed in Chapter 4 focused on analysis of the BON domains of NGO1985. Available knowledge regarding BON domain function and critical residues is limited and based mainly on predictions. It can be summarized that BON domains are on average 60 amino acids in length, contain several hydrophobic regions and predicted conserved glycine residues, and that they bind phospholipids in the periplasmic space. The phospholipid binding function was concluded based solely on the deduced function of the *E. coli* periplasmic OsmY protein participating in protection against osmotic shock (Liechty *et al*., 2000; Oh *et al*., 2000). We found that NGO1985 is not associated with osmoregulation, as the Δ*ngo1985* mutant did not display an increased susceptibility to osmotic shock

inducing compounds (Fig. S3.1). Based on our findings, NGO1985 is associated on the cell surface of gonococcal cells (Fig. 2.1 E, F, and G). In Gram-negative bacteria, the inner leaflet of the outer membrane is composed of phospholipids, while its proximal site is composed mainly of LPS/LOS (Kamio and Nikaido, 1976). Therefore, BON domains of NGO1985 could not be involved in the binding of phospholipids. Nevertheless, as was discussed in the section regarding NGO1985 localization, topology of NGO1985 in the outer membrane is not known. We should not rule out the possibility that BON domains could be entirely or partially embedded in the lipid bilayer and potentially interact with the phospholipids of the outer membrane. Based on our results, both of the NGO1985 BON domains are required in their native order for the proper protein function (Fig. 4.3 A). This suggests that these individual motifs could be selective towards different molecules they potentially bind. Therefore, re-arrangement of BON domains, with respect to the protein orientation in the membrane, would inhibit their association with the appropriate targets. As discussed above, we hypothesize that NGO1985 is part of the BAM complex. One could propose that both or one of the BON domains come in contact with proteins composing the BAM complex or with the substrate β-barrel protein. Binding studies with individual BON domains should be performed to determine potential binding to phospholipids, LOS, and components of the BAM complex to finally elucidate role of these protein motifs.

Function of the BON domains remains enigmatic and the potential implication(s) of their conserved glycine residues was never assessed. The final part of Chapter 4 analyzed the importance of glycine residues G91 and G169 of BON domains 1 and 2, respectively, in the physiological role of NGO1985. Introduction of alanine substitutions in these individual amino acid residues (G91A, G169A) has led to decreased protein abundance in comparison to the wild type NGO1985 when expressed in the native as well as heterologous host (Fig. 4.4 B, C, D). As expected, decreased protein levels were correlated with abolished NGO1985 function in *N. gonorrhoeae* (Fig. 4.4 A). Therefore, we conclude that the invariant glycine is most likely an important structural element of BON domains, necessary for proper folding and stability.

Conclusions and future directions

Due to the constant development of antibiotic resistance determinants, infections caused by *N. gonorrhoeae* are believed to become untreatable in the near future. Because of this threat it is critical to formulate new strategies to combat gonorrhea. Development of therapeutic interventions, in the form of new antibiotics or vaccines, is a multistep process requiring combined years of work from academic and private-sector scientists. Work presented in this dissertation focuses on characterization and validation of two previously unstudied gonococcal proteins: $GmhA_{GC}$ and NGO1985; as molecular targets for new antimicrobial agents and a vaccine antigen, respectively.

Chapter 2 explored the physiological implications and structure of the predicted gonococcal heptose biosynthetic pathway enzyme, sedoheptulose 7-phosphate isomerase, $GmhA_{GC}$. We have found that this protein, similarly to homologues described in other bacteria, is essential for synthesis of full length LOS molecules. However, in contrast to other species, $GmhA_{GC}$ is pivotal for gonococci viability. Our experimental results indicate that catalytic activity of $GmhA_{GC}$ involved in heptose biosynthesis is independent from this protein involvement in *N. gonorrhoeae* proliferation. Importance of $GmhA_{GC}$ in LOS synthesis as well as bacterial viability, underscores the potential of this protein as a target for the development of novel therapeutics. The crystal structure of $GmhA_{GC}$ should guide future identification of compounds inhibiting heptose synthesis as well as gonococcal growth.

Only scarce information and predictions regarding the potential functions of NGO1985 or its BON domains were available in the literature. Therefore, Chapters 3 and 4 were dedicated to the characterization of this protein. Throughout our studies we have shown that NGO1985 is conserved among diverse gonococci isolate lipoproteins exposed on the surface of the cell. In contrast to lipoproteins characterized in *E. coli*, NGO1985 does not undergo sorting to the inner and outer membrane, as dictated by the position +2 of the lipobox sequence. Based on experimental evidence, NGO1985 participates in maintaining cell envelope integrity, potentially as a novel component of the β-barrel assembly (BAM) complex. Dysregulation of bacterial homeostasis as a result of NGO1985 mutations results in overall cell envelope defects leading to an

increase in susceptibility to antibiotics, decreased viability during *in vitro* host environment related growth conditions, and attenuated virulence in the in *vivo* model of infection. We have found that both BON domains are required for proper function of NGO1985 and that their conserved glycine residues play an important role in maintaining protein stability. Based on conservation, surface exposure, and importance in physiology, NGO1985 appears to be a promising candidate for vaccine development.

This work has provided insights into $GmhA_{GC}$ and NGO1985 function and their validation as therapeutic targets against gonorrhea. Despite the body of results and information obtained from this dissertation, much more work is required in order to fully understand and utilize these two proteins for development of new pharmacological interventions. Studies of $GmhA_{GC}$ interacting protein partners should help to elucidate the involvement of this protein in gonococcal viability. Furthermore, our laboratory is actively working on development of an assay allowing for screening of inhibitors of sedoheptulose 7-phosphate isomerase activity of $GmhA_{GC}$. NGO1985 is currently undergoing an experimental trial where mice subjected to immunization with this protein will be analyzed for development of protection to gonococcal colonization. Studies determining the three dimensional structure of NGO1985 are in progress to facilitate our understanding of protein function as a whole and of its individual BON domains. Further work regarding association of NGO1985 with BAM will be required to determine how this protein fits into the whole structure of the complex and what roles it plays.

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Zielke, R. A., Wierzbicki, I. H., Baarda, B. I., Gafken, P. R., Soge, O. O., Holmes, K. K., Jerse, A. E., Unemo, M., Sikora, A. E. (2016). "Proteomics-driven Antigen Discovery for Development of Vaccines Against Gonorrhea". Mol Cell Proteomics. **15**(7): 2338-55.

Zielke, R. A., Wierzbicki, I. H., Baarda, B. I., Sikora, A. E. (2015). "The *Neisseria gonorrhoeae* Obg protein is an essential ribosome-associated GTPase and a potential drug target". BMC Microbiol **15**: 129.

Zielke, R. A., Wierzbicki, I. H., Weber, J. V., Gafken, P. R., Sikora, A. E. (2014). "Quantitative proteomics of the Neisseria gonorrhoeae cell envelope and membrane vesicles for the discovery of potential therapeutic targets". Mol Cell Proteomics. **13**(5): 1299-317.

Appendices: Abstracts of Additional Manuscripts

Appendix 1

Quantitative proteomics of the *Neisseria gonorrhoeae* cell envelope and membrane vesicles for the discovery of potential therapeutic targets

Authors: Zielke, R. A., Wierzbicki, I.H., Weber, J. V., Gafken, P. R., Sikora, A. E.

Journal: Molecular & Cellular Proteomics. 2014. **13**(5):1299-317.

Abstract:

Neisseria gonorrhoeae (GC) is a human-specific pathogen, and the agent of a sexually transmitted disease, gonorrhea. There is a critical need for new approaches to study and treat GC infections because of the growing threat of multidrug-resistant isolates and the lack of a vaccine. Despite the implied role of the GC cell envelope and membrane vesicles in colonization and infection of human tissues and cell lines, comprehensive studies have not been undertaken to elucidate their constituents. Accordingly, in pursuit of novel molecular therapeutic targets, we have applied isobaric tagging for absolute quantification coupled with liquid chromatography and mass spectrometry for proteome quantitative analyses. Mining the proteome of cell envelopes and native membrane vesicles revealed 533 and 168 common proteins, respectively, in analyzed GC strains FA1090, F62, MS11, and 1291. A total of 22 differentially abundant proteins were discovered including previously unknown proteins. Among those proteins that displayed similar abundance in four GC strains, 34 were found in both cell envelopes and membrane vesicles fractions. Focusing on one of them, a homolog of an outer membrane protein LptD, we demonstrated that its depletion caused loss of GC viability. In addition, we selected for initial characterization six predicted outer membrane proteins with unknown function, which were identified as ubiquitous in the cell envelopes derived from examined GC isolates. These studies entitled a construction of deletion mutants and analyses of their resistance to different chemical probes. Loss of NGO1985, in particular, resulted in dramatically decreased GC viability upon treatment with detergents, polymyxin B, and chloramphenicol, suggesting that this protein functions in the maintenance of the cell envelope permeability barrier. Together, these findings underscore the concept that the cell envelope and membrane vesicles contain crucial, yet under-explored determinants of

GC physiology, which may represent promising targets for designing new therapeutic interventions.

Appendix 2

In situ gelling polyvalerolactone-based thermosensitive hydrogel for sustained drug delivery

Authors: Mishra, G. P., Kinser, R., Wierzbicki, I. H., Alany, R. G., Alani, A. W.

Journal: European Journal of Pharmaceutics and Biopharmaceutics. 2014. **88**(2): 397-405.

Abstract:
Biodegradable poly(ethyleneglycol)-poly(valerolactone)-poly(ethyleneglycol) [PEG-PVL-PEG] copolymers were synthesized through ring opening polymerization of δ-valerolactone (VL) followed by the coupling of monomethoxy poly(ethyleneglycol-poly(valerolactone) (mPEG-PVL) with hexamethylene diisocyanate (HDI). The copolymers were characterized by (1)H NMR, FT-IR, and GPC. Block copolymers of PEG and PVL with different VL/PEG molar ratios were successfully synthesized. One of the copolymers (Copolymer 2, PEG550-PVL6768-PEG550) displayed a sol-gel transition at a physiological temperature based on the test tube inverting method and rheological studies. The thermogelling copolymer demonstrated a characteristic crystalline peak for PVL block as determined by DSC and XRD analysis. In vitro release from the copolymer hydrogel matrix indicated that dexamethasone (DEX), a hydrophobic model drug, released comparatively slower than 5-fluoruracil (5-FU), a hydrophilic model drug, due to the potential partitioning of DEX into the PVL core. 5-FU in vitro release from copolymer 2 was 86% in 22 h, whereas only 14% of DEX was released in 24h. Cell viability studies confirmed that hydrogels composed of block copolymers are biocompatible. Copolymer 2 showed more than 80% relative cell viability at all concentrations, including concentrations greater than 200 fold CMC. In vivo gel formation studies indicate that gel integrity was maintained for 7 days upon subcutaneous injection into mice. These results indicate that PEG-PVL-PEG copolymers are suitable for drug delivery applications.

Appendix 3

A metalloprotease secreted by the type II secretion system links *Vibrio cholerae* with collagen

Authors: Park, B. R., Zielke, R. A., Wierzbicki, I. H., Mitchell, K. C., Withey, J. H., Sikora, A. E.

Journal: Journal of Bacteriology. 2015. **197**(6):1051-64.

Abstract:
Vibrio cholerae is autochthonous to various aquatic niches and is the etiological agent of the life-threatening diarrheal disease cholera. The persistence of *V. cholerae* in natural habitats is a crucial factor in the epidemiology of cholera. In contrast to the well-studied *V. cholerae*-chitin connection, scarce information is available about the factors employed by the bacteria for the interaction with collagens. Collagens might serve as biologically relevant substrates, because they are the most abundant protein constituents of metazoan tissues and *V. cholerae* has been identified in association with invertebrate and vertebrate marine animals, as well as in a benthic zone of the ocean where organic matter, including collagens, accumulates. Here, we describe the characterization of the *V. cholerae* putative collagenase, VchC, encoded by open reading frame VC1650 and belonging to the subfamily M9A peptidases. Our studies demonstrate that VchC is an extracellular collagenase degrading native type I collagen of fish and mammalian origin. Alteration of the predicted catalytic residues coordinating zinc ions completely abolished the protein enzymatic activity but did not affect the translocation of the protease by the type II secretion pathway into the extracellular milieu. We also show that the protease undergoes a maturation process with the aid of a secreted factor(s). Finally, we propose that *V. cholerae* is a collagenovorous bacterium, as it is able to utilize collagen as a sole nutrient source. This study initiates new lines of investigations aiming to uncover the structural and functional components of the *V. cholerae* collagen utilization program.

Appendix 4

The *Neisseria gonorrhoeae* Obg protein is an essential ribosome-associated GTPase and a potential drug target

Authors: Zielke, R. A., Wierzbicki, I. H., Baarda, B. I., Sikora, A. E.

Journal: BMC Microbiology. 2015. **15**: 129.

Abstract:
BACKGROUND: *Neisseria gonorrhoeae* (GC) is a Gram-negative pathogen that most commonly infects mucosal surfaces, causing sexually transmitted urethritis in men and endocervicitis in women. Serious complications associated with these infections are frequent and include pelvic inflammatory disease, ectopic pregnancy, and infertility. The incidence of gonorrhea cases remains high globally while antibiotic treatment options, the sole counter measures against gonorrhea, are declining due to the remarkable ability of GC to acquire resistance. Evaluating of potential drug targets is essential to provide opportunities for developing antimicrobials with new mechanisms of action. We propose the GC Obg protein, belonging to the Obg/CgtA GTPase subfamily, as a potential target for the development of therapeutic interventions against gonorrhea, and in this study perform its initial functional and biochemical characterization.
RESULTS: We report that NGO1990 encodes Obg protein, which is an essential factor for GC viability, associates predominantly with the large 50S ribosomal subunit, and is stably expressed under conditions relevant to infection of the human host. The anti-Obg antisera cross-reacts with a panel of contemporary GC clinical isolates, demonstrating the ubiquitous nature of Obg. The cellular levels of Obg reach a maximum in the early logarithmic phase and remain constant throughout bacterial growth. The in vitro binding and hydrolysis of the fluorescent guanine nucleotide analogs mant-GTP and mant-GDP by recombinant wild type and T192AT193A mutated variants of Obg are also assessed.
CONCLUSIONS: Characterization of the GC Obg at the molecular and functional levels presented herein may facilitate the future targeting of this protein with small

molecule inhibitors and the evaluation of identified lead compounds for bactericidal activity against GC and other drug-resistant bacteria.

Appendix 5

Polymeric Micelles as Carriers for Nerve-Highlighting Fluorescent Probe Delivery

Authors: Hackman, K. M., Doddapaneni, B. S., Barth, C. W., Wierzbicki, I. H., Alani, A. W., Gibbs, S. L.

Journal: Molecular Pharmaceutics. 2015. **12**(12): 4386-94.

Abstract:
Nerve damage during surgery is a common morbidity experienced by patients that leaves them with chronic pain and/or loss of function. Currently, no clinically approved imaging technique exists to enhance nerve visualization in the operating room. Fluorescence image-guided surgery has gained in popularity and clinical acceptance over the past decade with a handful of imaging systems approved for clinical use. However, contrast agent development to complement these fluorescence-imaging systems has lagged behind with all currently approved fluorescent agents providing untargeted blood pool information. Nerve-specific fluorophores are known, however translations of these agents to the clinic has been complicated by their lipophilic nature, which necessitates specialized formulation strategies for successful systemic administration. To date the known nerve-specific fluorophores have only been demonstrated preclinically due to the necessity of a dimethyl sulfoxide containing formulation for solubilization. In the current study, a polymeric micellar (PM) formulation strategy was developed for a representative nerve-specific fluorophore from the distyrylbenzene family, BMB. The PM formulation strategy was able to solubilize BMB and demonstrated improved nerve-specific accumulation and fluorescence intensity when the same fluorophore dose was administered to mice utilizing the previous formulation strategy. The success of the PM formulation strategy will be important for moving toward clinical translation of these novel nerve-specific probes as it is nontoxic and biodegradable and has the potential to decrease the necessary dose for imaging while also improving the safety profile.

Appendix 6

Proteomics-driven Antigen Discovery for Development of Vaccines Against Gonorrhea

Authors: Zielke, R. A., Wierzbicki, I. H., Baarda, B. I., Gafken, P. R., Soge, O. O., Holmes, K. K., Jerse, A. E., Unemo, M., Sikora, A. E.

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Abstract:
Expanding efforts to develop preventive gonorrhea vaccines is critical because of the dire possibility of untreatable gonococcal infections. Reverse vaccinology, which includes genome and proteome mining, has proven very successful in the discovery of vaccine candidates against many pathogenic bacteria. However, progress with this approach for a gonorrhea vaccine remains in its infancy. Accordingly, we applied a comprehensive proteomic platform-isobaric tagging for absolute quantification coupled with two-dimensional liquid chromatography and mass spectrometry-to identify potential gonococcal vaccine antigens. Our previous analyses focused on cell envelopes and naturally released membrane vesicles derived from four different *Neisseria gonorrhoeae* strains. Here, we extended these studies to identify cell envelope proteins of *N. gonorrhoeae* that are ubiquitously expressed and specifically induced by physiologically relevant environmental stimuli: oxygen availability, iron deprivation, and the presence of human serum. Together, these studies enabled the identification of numerous potential gonorrhea vaccine targets. Initial characterization of five novel vaccine candidate antigens that were ubiquitously expressed under these different growth conditions demonstrated that homologs of BamA (NGO1801), LptD (NGO1715), and TamA (NGO1956), and two uncharacterized proteins, NGO2054 and NGO2139, were surface exposed, secreted via naturally released membrane vesicles, and elicited bactericidal antibodies that cross-reacted with a panel of temporally and geographically diverse isolates. In addition, analysis of polymorphisms at the nucleotide and amino acid levels showed that these vaccine candidates are highly conserved among N. gonorrhoeae strains. Finally, depletion of BamA caused a loss of

N. gonorrhoeae viability, suggesting it may be an essential target. Together, our data strongly support the use of proteomics-driven discovery of potential vaccine targets as a sound approach for identifying promising gonococcal antigens.

Appendix 7

Combinatorial Polymeric Conjugated Micelles with Dual Cytotoxic and Antiangiogenic Effects for the Treatment of Ovarian Cancer

Authors: Rao, D. A., Mishra, G., Doddapaneni, B. S., Kyryachenko, S., Wierzbicki, I. H., Ngyuen, D. X., Shah, V., Al Fatease, A. M., Alany, R. G., Alani, A. W.

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Abstract:
Emerging treatment paradigms like targeting the tumor microenvironment and/or dosing as part of a metronomic regimen are anticipated to produce better outcomes in ovarian cancer, but current drug delivery systems are lacking. We have designed and evaluated paclitaxel (PTX) and rapamycin (RAP) micellar systems that can be tailored for various dosing regimens and target tumor microenvironment. Individual and mixed PTX/RAP (MIX-M) micelles are prepared by conjugating drugs to a poly(ethylene glycol)-block-poly(β-benzyl L-aspartate) using a pH sensitive linker. The micelles release the drug(s) at pH 5.5 indicating preferential release in the acidic endosomal/lysosomal environment. Micelles exhibit antiproliferative effects in ovarian cell cancer lines (SKOV-3 (human caucasian ovarian adenocarcinoma) and ES2 (human ovarian clear cell carcinoma)) and an endothelial cell line (HUVEC; human umbilical vein endothelial cells) with the MIX-M being synergistic. The micelles also inhibited endothelial migration and tube formation. In healthy mice, micelles at 60 mg/kg/drug demonstrated no acute toxicity over 21 days. ES2 xenograft model efficacy studies at 20 mg/kg/drug dosed every 4 days and evaluated at 21 days indicate that the individual micelles exhibit antiangiogenic effects, while the MIX-M exhibited both antiangiogenic and apoptotic induction that results in significant tumor volume reduction. On the basis of our results, MIX-M micelles can be utilized to achieve synergistic apoptotic and antiangiogenic effects when treated at frequent low doses.