SYSTEMS OF THIAMINE PYROPHOSPHATE BIOSYNTHESIS AND TRANSPORT IN *NEISSERIA GONORRHOEAE*

by

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Dissertation submitted to the Faculty of the
Emerging Infectious Diseases Graduate Program
Uniformed Services University of the Health Sciences
In partial fulfillment of the requirements for the degree of
Doctor of Philosophy 2018

UNIFORMED SERVICES UNIVERSITY, SCHOOL OF MEDICINE GRADUATE PROGRAMS
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FINAL EXAMINATION/PRIVATE DEFENSE FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
IN THE EMERGING INFECTIOUS DISEASES GRADUATE PROGRAM

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Date of Examination: Tuesday, March 20, 2018
Time: 1:00 PM
Place: C2095 Conference room

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Title of Dissertation: "Systems of Thiamine Pyrophosphate Biosynthesis and Transport in *Neisseria gonorrhoeae*"

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ACKNOWLEDGMENTS

I would like to thank everyone who played a role in the fulfillment of this dissertation. I would like to thank Dr. Ann Jerse for inviting me to her laboratory and subsequently providing me with great freedom to pursue this project. She has been incredibly generous with her resources, encouragement, and kindness over the years, and I am greatly appreciative of her support. I also owe the members of my committee a debt of gratitude for all of their insights and guidance, particularly my committee chair Dr. Frank Shewmaker, who offered me tireless support and who often seemed to have more faith in my ability to complete this task than I did. I would like to acknowledge all members of the Jerse lab, past and present, for their assistance in the lab. Dr. Adriana LeVan worked with me on several elements of this project that were critical to its completion- without her expertise, patience, and friendship I most certainly would not be here today. Finally, thank you to my family, who provided me with the love and encouragement that has made all of this possible. To my parents, who understood better than anyone the long road to earning a degree, and to my siblings, who never once asked me when I was going to be done. To my wife, Sarah, who will always be the best thing that happened to me- both in graduate school and in life. Her love and support is the foundation of any and all successes I have had, and has given me the confidence to move through the world in ways I once only dreamed of being possible.

DEDICATION

This dissertation is dedicated to the memory of my grandmother, Marjorie Brodkin. The depth of her love, and what was lost with her, is still difficult to comprehend.

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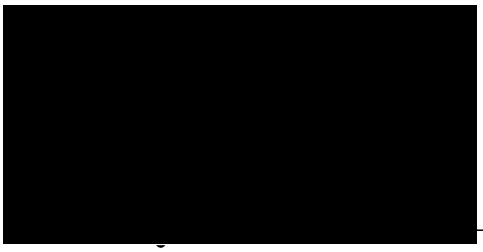

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ABSTRACT

Systems of Thiamine Pyrophosphate Biosynthesis and Transport in *Neisseria gonorrhoeae*

Riley T. Sennett, Doctor of Philosophy, 2018

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Gonorrhea is the second-most commonly reported notifiable infection in the United States and a serious threat to public health. The rapid evolution of antibiotic resistance in *Neisseria gonorrhoeae* (Gc) has decreased the number of options available for treatment, and there has not been a successful vaccine developed for Gc due to the ability of the pathogen to evade detection and killing by the host immune system. Gc is an extremely successful human-specific pathogen that has tailored its genome to allow it to adapt to and survive in nutrient-restrictive environments.

Here we examined the impact of thiamine pyrophosphate (TPP) biosynthesis and transport on Gc growth *in vitro*, its fitness *in vivo* using a genital tract infection model, and resistance to innate immune effectors. We identified the genetic components of a potential TPP biosynthetic pathway in commonly used laboratory strains of Gc. We hypothesized that this pathway was functional, and that the capacity of Gc to produce TPP through this *de novo* pathway was important for survival in the host. We found that

loss of a key enzyme, glycine oxidase (ThiO), in the pathway inhibited growth *in vitro* in the absence of exogenously provided TPP. Complementation of the mutant fully restored growth on TPP-deficient agar, and partially restored growth in liquid cultures under TPP-depleted conditions. In addition, the *thiO* mutant was attenuated relative to the WT strain in female BALB/c mice, but not in C57BL6 mice, which do not experience a vaginal polymorphonuclear leukocyte (PMN) response. The complemented mutant was not attenuated in either model of lower genital tract infection.

TPP has some described functions as a scavenger of reactive oxygen species (ROS), which can be produced by invading PMNs, a hallmark of Gc genital tract infections. Therefore we also hypothesized that the production of TPP through this biosynthetic pathway could facilitate bacterial resistance to PMN-derived ROS during infection. We found that the *thiO* mutant was more sensitive to two sources of ROS, H_2O_2 and paraquat, as well as to killing by isolated murine PMNs after the mutant was depleted of internal reserves of TPP. However, the *thiO* mutant was not more resistant to challenge from PMNs that lack the capacity to generate ROS through the phagocytic oxidative burst, suggesting that PMN-derived ROS are not responsible for our observed *in vivo* phenotype. Preliminary results indicate that the *thiO* mutant may be more susceptible to polymyxin B when starved for TPP, suggesting that TPP may be important for resistance to antimicrobial peptides. We conclude that the pathway of TPP biosynthesis is important for meeting the basic nutritional needs of Gc under nutrient-poor conditions, such as those in the lower genital tract, and may have an additional role in resistance to non-oxidative mechanisms of killing by the innate immune response.

We noted that the TPP biosynthesis-deficient *thiO* mutant could replicate in the presence of extremely low concentrations of added TPP. Furthermore, previous work indicated that Gc TPP auxotrophs can be isolated from the urethra and cervix of humans, and thus can survive in the lower genital tract. Therefore, we also hypothesized that a functional system of TPP transport was present in Gc, and could be critical for adaptation to host environments with varying levels of micronutrient availability. We identified an open reading frame (*thiB*) that had homology to the substrate-binding component of a putative TPP transporter of *Salmonella enterica*, and insertionally inactivated the *thiB* gene. We then evaluated the ability of the *thiB* mutant to grow in the absence of provided TPP. The mutant strain did not have a phenotype *in vitro* or during competitive infections with the wild-type parent strain, but the gonococcal *thiB* gene was able to complement a TPP transport-deficient strain of *Salmonella enterica*. However, a gonococcal candidate for the transporter permease ThiP was not able to rescue the *Salmonella* mutant, indicating that our description of the TPP transporter is incomplete.

To assess whether gonococci that lacked both biosynthesis and the ThiB transporter system were able to establish infection, we constructed a *thiB*, *thiO* double mutant. The double mutant required high concentrations of exogenous TPP to grow *in vitro*, and was not able to establish lower genital tract infection in mice. We conclude that a functional TPP transporter is present in Gc, and is necessary for bacterial survival *in vivo* when *de novo* production of TPP is impaired.

This is the first study of the TPP biosynthetic pathway in Gc and in the context of Gc infection. The outcome of this work underscores the importance of metabolic factors and pathways for bacterial pathogenesis. Furthermore, this work indicates that mechanisms for the production and transport of TPP may be viable candidates as targets of novel therapeutic agents.

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CHAPTER 1: INTRODUCTION

NEISSERIA GONORRHOEAE

Neisseria gonorrhoeae (the gonococcus, Gc) is an exclusive human pathogen and the causative agent of the sexually transmitted infection (STI) gonorrhea. The gonococcus is one of two pathogenic species of *Neisseria*, along with *N. meningitidis*, and the majority of the other species in this genus are considered to be commensal organisms. Gc is a Gram-negative, un-encapsulated diplococcus and an extremely successful pathogen, as evidenced by its long history of residing within its human hosts (170). There are no known reservoirs for Gc, and it has therefore evolved adaptation mechanisms specific for humans in order for transmission to be continuous.

In order to survive and persist within the host so effectively, Gc has evolved multiple mechanisms of resistance to the defenses of the human immune system. Gc expresses antigenically and phase variable external structures such as pilin, the opacity (Opa) proteins, and lipooligosaccharide, that facilitate evasion of immune recognition. Gc also suppresses the adaptive immune response, and is a poor inducer of memory effector cells, making re-infections common. In addition, Gc has developed mechanisms for the acquisition and utilization of host nutrients, and can use human iron-binding glycoproteins as a source of iron to support bacterial replication in nutrient-limited environments. Furthermore, Gc is a naturally competent pathogen and routinely picks up genetic elements from its environment. This capacity for the acquisition and integration of DNA has facilitated the uptake of antibacterial resistance determinants, which has

subsequently complicated treatment options for gonococcal infections. Gc is not limited to incorporating genetic material from other bacteria, however, as it is known that approximately 11% of Gc strains carry fragments of human DNA sequences (4).

EPIDEMIOLOGY AND PUBLIC HEALTH IMPORTANCE

Infections caused by Gc are considered to be a serious public health concern worldwide. The World Health Organization (WHO) global surveillance for sexually transmitted infections estimates that there were approximately 78 million new cases of gonorrhea in 2012 (126). In the United States, gonorrhea is the second-most commonly reported notifiable infection, with 395,216 cases reported in 2015 (123.9 cases per 100,000 population) (28). This represents a 12.8% increase in the rate of reported gonorrhea cases since 2014, and an increase of 19.9% since 2011. In 2016, the most recent year of available surveillance data, 468,514 cases of gonorrhea were reported in the U.S. (146 per 100,000 population) as rates of STIs continue to rise (160). Gonorrhea disproportionately affects the Southern regions of the United States, and rates are higher in racial and ethnic minority groups compared to whites (28). In addition, the burden of disease is carried mostly by sexually active young people, with approximately 50% of the estimated new cases occurring in those 15-24 years in age. The recent increases in the rates of gonorrhea and other STIs are worrisome enough, however, the severity of the situation is compounded by the development of antibiotic resistance by Gc.

Since the introduction of antimicrobial agents for the treatment of gonorrhea, Gc has steadily evolved resistance to each new class of drugs such that we are now facing the

prospect of untreatable gonococcal infections in the near future. This trend began as early as the late 1930s, when sulfonamides (sulfa drugs) were prescribed as the first effective treatment for gonorrhea (82, 170). Just as the sulfonamides were losing effectiveness in the early 1940s, penicillin was introduced with extremely high rates of success. The penicillin era saw great reductions in the burden of gonococcal disease, however, resistance determinants began to emerge in Gc with the acquisition of beta-lactamase encoding plasmids (91, 170). By the mid-1980s, penicillin resistance had become so prevalent among circulating strains, which was now also due to chromosomal mutations, that penicillin could no longer be recommended for treatment. Penicillin resistance is no longer so prevalent in the U.S., but it continues to be widespread in developing countries where antibiotic usage is poorly regulated. In 1993, only the fluoroquinolone class of antibiotics, along with third-generation cephalosporins ceftriaxone and cefixime, were recommended as the primary treatment for gonorrhea (42, 170). At the time this recommendation was made, however, signs of fluoroquinolone resistance had already been reported nationally in Hawaii and overseas in Asia. By 2007, fluoroquinolone resistance was widespread and these drugs were contraindicated for treatment in the United States leaving only one class of antibiotics, the cephalosporins, as the primary treatment for gonorrhea. In the years following this development, increasing resistance to cefixime have resulted in dual therapy consisting of injectable ceftriaxone and azithromycin as the only regimen currently recommended for the empirical treatment of gonorrhea (28, 169).

PATHOGENESIS

Gonococcal infections are typically localized to the mucosal surface of the genital epithelium. In women, the endocervix is the primary site of infection; for men, the urethra, although gonococcal urethritis often also occurs in women (112, 174). Gc can establish infection at other body sites as well, including the pharynx and the rectal mucosae, and neonates can acquire conjunctival infections during birth (28). Additionally, in a small percentage of cases, disseminated gonococcal infection (DGI) can occur, leading to infection of the joints and skin, resulting in septic arthritis and dermatitis, respectively.

Infections of the urogenital tract may be symptomatic or asymptomatic. Symptomatic gonococcal infections are marked by a robust polymorphonuclear leukocyte (PMN) response and mucopurulent discharge. Indeed, the observation of a PMN-rich exudate containing intracellular, Gram-negative diplococci is considered a defining feature of infection (99). However, asymptomatic infections are frighteningly common, and may occur in both women and men (29, 181).

In women, gonococcal disease may present in a variety of ways. In the lower genital tract and urinary tract, common sites of infection include the cervix and the urethra, respectively. Clinical presentations may include vaginal bleeding and discharge, dysuria, and menorrhagia. An estimated 45% of infections in women ascend to the tissues of the upper reproductive tract, where more severe pathology can occur (40, 174). Ascendant infections can involve inflammation of the fallopian tubes as well as the uterine lining, resulting in salpingitis and endometritis, respectively, which collectively is called pelvic

inflammatory disease (PID). Complications associated with PID include ectopic pregnancy, infertility, and chronic, life-long pelvic pain. The likelihood of ascendant infection is increased when cervical infections are asymptomatic, resulting in delayed detection and treatment. Unfortunately, asymptomatic disease in women is fairly common, with over half of cervical infections showing no clinical indications of disease (40).

VIRULENCE FACTORS AND EVASION OF INNATE IMMUNE EFFECTORS

The mucosae of the lower genital tract is considered a hostile environment that is replete with immune effectors that pose a challenge to gonococcal colonization. Limited availability of nutrients and low vaginal pH levels, along with complement, antimicrobial peptides, and reactive oxygen species (ROS) produced by host cells are all factors that Gc must successfully evade in order to establish infection. During the long history of Gc co-evolving alongside humans, the gonococcal genome has become tailored to retain a vast number of factors that facilitate colonization and infection (40).

The human body naturally sequesters iron, an essential nutrient, as a host-defense mechanism that limits iron availability to invading pathogens. Gc can overcome this defense through the products of the *tbpA* and *tbpB* genes, which encode a receptor for human transferrin (33, 105). Gc can also acquire iron from human iron-binding lactoferrin through a similar mechanism that is mediated by a receptor encoded by *lbpA* and *lbpB* (3, 104). Capture of the iron-loaded transferrin or lactoferrin by Gc provides a source of iron to support bacterial replication in nutrient-limited environments.

Experimental infections in male volunteers has demonstrated that the presence of either a functional transferrin or lactoferrin receptor is required for gonococcal colonization (33). Gc can also acquire iron from hemoglobin through the receptor HpuAB, which is thought to be important during DGI and possibly menstruation (31, 77, 168).

Additionally, Gc can vary the composition and expression of several surface structures in order to escape detection by the host immune system. The gonococcal type IV pilus mediates initial attachment of the bacterium to host cell surfaces, and promotes bacterial micro-colony formation during infection (47, 50). Although Gc is a non-motile organism, retraction of the type IV pilus results in twitching motility that may be important for bacterial distribution both in localized areas of the epithelium, and for access to the tissues of the upper genital tract during ascendant infection (40). Antigenic variation of the pilus occurs by recombination events between the major pilus subunit, encoded by *pilE*, and one of several copies of *pilS*, which are considered transcriptionally silent loci (1, 60).

Gonococcal lipooligosaccharide (LOS) is a surface structure that is found on the bacterial outer membrane in place of lipopolysaccharide (LPS), which is characteristic of many Gram-negative pathogens. LOS is responsible for a wide range of activities that influence bacterial pathogenesis, such as epithelial cell attachment and PMN recruitment (40). LOS is constructed through the activity of many glycosyl transferases encoded by *lgt* genes in Gc, and the structure of this antigen may vary significantly due to phase variable expression of the transferases (148, 184). In addition, sialylation of gonococcal LOS

using host-derived neuraminic acid enhances resistance to PMN killing and decreases binding of anti-LOS antibodies (182). Gc can also modify LOS through decoration of the lipid A subunit with phosphoethanolamine (PEA) which can result in increased resistance to human complement in serum and antimicrobial peptides (92). PEA modifications that affect serum resistance are placed on the 4' position of lipid A, and the process is catalyzed by the PEA transferase LptA (92). PEA-decorated lipid A also increases Gc resistance to cationic antimicrobial peptides, and *lptA* mutants that cannot make this modification are attenuated during infection of male volunteers and female mice (61, 92, 121).

The Opa (opacity) protein family also has a critical role in mediating interactions between Gc and host cells. Opa proteins facilitate bacterial invasion of host cells by binding to members of the carcinoembryonic antigen cell adhesion molecule (CEACAM) family (133). Opa proteins are phase variable and antigenically diverse, and several different Opa variants may be expressed at one time by a single strain of Gc (69, 71). Opa expression is not required for infection of the genital tract, but can influence the immunological response to the bacterium (62, 71). For example, Opa protein interactions are capable of activation of the innate immune system by stimulating PMN responses via the oxidative burst, but can also result in suppression of adaptive immunity through inhibition of T-lymphocyte activation and proliferation and B-cell antibody production (9, 133). In addition, Opa expression increases resistance to antibodies and complement-mediated killing, and promotes bacterial persistence and dissemination in the genital tract (32, 133).

Gc also has more direct ways of dealing with unwanted immune effector molecules and antimicrobial compounds. Efflux pump systems are common in prokaryotes and can be used to detoxify the cellular environment. In particular, multidrug resistance (MDR) pumps are useful for the expulsion of various antibiotic compounds and antimicrobial peptides to limit their damage to the bacterial cell. The MtrCDE efflux pump is important for survival and pathogenesis in Gc. MtrCDE is active against bile salts, nonionic detergents, and several classes of antibiotics (150). Expression of the *mtrCDE* operon is controlled by an activator, MtrA, and a repressor MtrR (175, 176). Loss of the repressor MtrR results in increased fitness of Gc in a female mouse model of infection, and inactivation of MtrA results in decreased fitness (175). In addition, Gc has other efflux pump systems, including the FarAB, MtrE pump that recognizes and removes long-chain fatty acids and is thought to be important for colonization of the rectal mucosa (70, 132).

Gonococcal infections are frequently characterized by a robust inflammatory response, with the recruitment of large numbers of polymorphonuclear leukocytes (PMNs) to the affected site. PMNs are important for early control of infection, and are capable of killing bacteria through both oxygen-dependent and independent mechanisms such as the production of reactive oxygen species (ROS) and the release of antimicrobial peptides, respectively (19, 48). However, viable gonococci can be recovered from the PMN-rich exudates of infected patients, and it has been demonstrated that a subset of Gc will remain viable after exposure to human PMNs *in vitro* (6, 141, 180). In addition, Gc appears to be able to survive and even replicate within PMN phagosomes (151).

Therefore, it is apparent that Gc is highly adept at evading killing by PMNs, and multiple mechanisms that promote its survival within PMNs have been described.

Gc may escape killing by manipulation of the oxidative burst in several ways. Firstly, exposure to lactate generated by PMNs undergoing glycolysis increases Gc oxygen consumption, reducing the amount available to be used as a substrate for the NADPH oxidase (22). Secondly, bacterial porin and the Opa proteins can suppress the oxidative burst, however, these mechanisms have not been fully elucidated (35, 94). In addition, Gc can detoxify ROS through superoxide dismutase (SOD) and catalase activity (142, 158). Gc can also promote DNA repair mechanisms after exposure to ROS, such as the MsrAB methionine sulfoxide reductases, the recombinase RecA and DNA-binding protein RecN, and the putative metalloprotease NGO1686 (153, 162, 163). Finally, exposure to ROS has been shown to result in the upregulation of expression of various antioxidant genes and their products (161).

Interestingly, despite the many mechanisms Gc has for resisting oxidative killing, multiple studies have found that non-oxidative processes appear to be more important for PMN killing of Gc (23, 127). Antimicrobial peptides and other bactericidal compounds are present in PMN granules, and can be released to create a hostile environment for invading bacteria at the site of infection (20, 48). Gc has evolved several ways to escape these challenges as well. Gonococcal pilin and porin proteins can reduce the fusion of PMN granules with the plasma membrane and phagosomes, subsequently decreasing granule release (17, 94). In addition, modifications to some surface structures discussed

previously, such as LOS and Opa, can decrease the interactions of antimicrobial peptides with the bacterial cell surface (133, 147). Lastly, the efflux pump system MtrCDE can export toxic compounds, and has been shown to be involved in resistance to antimicrobial peptides (149).

EXPERIMENTAL MODELS OF INFECTION

The specificity of gonococcal infections for the human host has presented considerable challenges to studying dynamics of infection *in vivo*. Experimental urethral infection of male volunteers has provided a great deal of information about the early stages of infection, and is still used to evaluate the importance of virulence genes in infection (62). The human urethral infection model has also been used to assess the effectiveness of gonococcal vaccine candidates, such as a pilin vaccine developed in the 1980s that was tested in male service members stationed in Korea (21). Unfortunately, the relevance of this model to other types of Gc infections is limited. For example, experimental urethral infection in males does not accurately represent the infection kinetics seen in women, which are influenced by reproductive hormones.

Animal models of gonococcal infection have been pursued in many different species. Early animal models of Gc infection include mouse peritoneal and rabbit intraocular systems developed in the 1930s, however, the success of penicillin for treatment caused interest in these models to wane (7). The next major development involved the use of non-human primates for modeling pathogenesis and sexual transmission of genital Gc infections. Experimental infection of male chimpanzees mimicked many features of

infection in humans and natural transmission from males to females occurred (7, 8). However, because of the prohibitively high cost of chimpanzees and the inability of many researchers to access this model, non-human primates are no longer considered a usable model.

The use of mice for modeling Gc genital tract infection has been under development for several decades. Studies in the 1980s showed that female mice could be colonized with Gc during the proestrus phase of the murine estrous cycle, when estrogen levels are increasing and levels of Gc-inhibiting progesterone are low. However, bacteria were only recovered for a few days before the cycle transitioned into the post-ovulatory phases, resulting in clearance of infection (74). Later developments showed that treatment of germ-free mice with 17β-estradiol could prolong the period of susceptibility and support sustained recovery of Gc (165). The Jerse laboratory investigated and confirmed these results independently, and refined the model to include the use of antibiotic injections to suppress potentially inhibitory commensal flora (73). In the current protocol, estradiol and antibiotics are administered prior to inoculation, and bacteria can be recovered from vaginal swabs for 7-12 days, depending on the form of estradiol used. The mechanism by which estradiol treatment promotes Gc colonization is unclear, but may involve suppression of the innate immune response and decreases in cytokine production and immune receptor concentrations (73).

NUTRITIONAL REQUIREMENTS

Despite its success as a human pathogen, Gc is considerably more difficult to culture in the laboratory environment. Gc is a fastidious, microaerophilic organism, requiring increased carbon dioxide and the provision of nutrients and chemical supplements in order to be successfully cultured *in vitro*. Specifically, Gc requires 3-5% CO_2 and will only grow between 35-37 degrees Celsius with high humidity. Growth of Gc on agar media was found to be improved with the addition of thiamine pyrophosphate (TPP) and glutamine, however, it is not clear why these specific nutrients are of such high importance for Gc (84, 87, 88). It was also observed that Gc requires glutamic acid, histidine, glucose, starch, glutathione, sodium chloride, iron salts, magnesium, and phosphates for *in vitro* growth (54). Most of these compounds are provided in the GC medium base that is typically used in the preparation of growth media (GC agar) for culture of Gc. The composition of GC agar is peptone digest, corn starch, dipotassium and monopotassium phosphate, and sodium chloride. Kellogg's Supplement I is added to provide glucose, TPP, and glutamine, and ferric nitrate is added as an iron source (84). When Gc is grown aerobically, sodium bicarbonate is added as a source of carbon dioxide. Chocolate agar, a variation of blood agar that has been heated to lyse red blood cells, can also be used to culture Gc (18). In a clinical setting, Thayer-Martin agar, a chocolate agar medium that contains added nutrients and antibiotics to inhibit the growth of commensal flora and contaminating organisms, is used to isolate Gc from patient samples (116).

THIAMINE PYROPHOSPHATE

Thiamine pyrophosphate (TPP, also referred to as thiamine diphosphate and co-carboxylase) is an essential micronutrient for all living organisms. Humans, as well as most other eukaryotic organisms, are dependent on dietary sources to fulfill their TPP intake requirements. Good sources for this nutrient include whole grain and fortified grain products, where it is provided in the form of vitamin B1 (thiamine B1) (46). Thiamine has a relatively short half and is not stored within the body (46, 155). Without proper daily intake of thiamine, deficiency and resulting pathologies will quickly develop. Typically, vitamin B1 deficiency manifests as beriberi, which can involve cardiac and neurological complications (46). Conditions such as Wernicke's encephalopathy and Korsakoff's syndrome, involving the development of lesions of central nervous system and neuronal cell loss, respectively, may also occur. These diseases are relatively rare in the global population, but are known to be exacerbated by chronic alcoholism and may be underdiagnosed (46).

The essential nature of thiamine and TPP is no surprise considering its versatility and wide usage in a variety of cellular processes. TPP serves as a critical cofactor for enzymes that are active in numerous biochemical pathways. Most notably, this includes activity as a cofactor for the pyruvate dehydrogenase and alpha-ketoglutarate dehydrogenase complexes, which are active in the Citric Acid Cycle; as well as transketolase, an essential component of the Pentose-Phosphate Pathway (93). These pathways are important for the generation of ATP and provide energy to power the cell. In addition, TPP serves as one of five cofactors for the branched-chain alpha-keto acid dehydrogenase complex (BCKDC), which is involved in the catabolism of branched-

chain amino acids in animals and the production of long-chain fatty acids in prokaryotes (38). These processes, among others that utilize TPP as a coenzyme, are necessary to produce adequate metabolic capacity, as well as to maintain cellular homeostasis and general cellular well-being.

BIOSYNTHESIS OF THIAMINE PYROPHOSPHATE IN PROKARYOTES

Unlike humans, prokaryotic organisms, as well as some fungi and plants, are not dependent on dietary intake of TPP, and can produce TPP through a unique biosynthetic pathway. Pathways of *de novo* TPP biosynthesis have been described in several model organisms, and the chemistry of these pathways has been is complex. In addition, while the pathways all ultimately result in the production of TPP, enzymatic components and their specific substrates may vary between genera.

The TPP biosynthesis pathway is more accurately described as two pathways combined into one. There are two arms of the pathway, each involved in the production of a separate component of TPP. One side of the pathway involves the production of the thiazole moiety (THZ), and the other side produces the pyrimidine moiety (HMP) (Figure 1). In *Escherichia coli* and *Salmonella*, two of the best studied model organisms for TPP biosynthesis, the thiazole moiety is produced from the substrates of tyrosine, 1-deoxy-D-xylulose, and cysteine (11, 38, 90). This process is mediated by the products of the *thiG* and *thiH* genes, which form a thiazole synthase complex and are assisted by the cysteine desulfurase IscS, the sulfur carrier protein ThiS, and the adenyltransferase ThiF (11). Interestingly, in *Bacillus subtilis*, a Gram-positive model organism, ThiH is replaced by

ThiO, an oxygen-dependent glycine oxidase that utilizes glycine rather than tyrosine as a substrate (122). The remainder of the biosynthetic components are shared between the enteric bacteria and *Bacillus*. For the production of the pyrimidine moiety, the products of the *thiC* and *thiD* genes are required to convert intermediates of the purine biosynthesis pathway to HMP (11, 30, 172, 185). The condensation of the two moieties is mediated by the product of the *thiE* gene to create thiamine monophosphate (TMP). Afterwards, a final phosphorylation step is catalyzed by the product of the *thiL* gene to produce the active cofactor TPP (11, 90, 172).

Secondary to this primary biosynthetic pathway, networks of thiamine salvage enzymes can utilize pathway intermediates produced by the cell or imported from the environment to produce TPP when biosynthesis is impaired. Salvage enzymes include the products of the genes *thiM*, a kinase involved in thiazole salvage; as well as *thiK* and *thiN,* which convert thiamine B1 to TMP in *E. coli* and to TPP in *B. subtilis*, respectively (38, 130, 140). In addition, the ThiD kinase is sometimes classified as a salvage enzyme as it can use imported HMP precursors to produce the pyrimidine moiety when the ThiC HMP synthase is not functional (38).

The distribution of TPP biosynthesis genes throughout the chromosomes of different bacteria is frequently similar, but the organization may differ slightly. Biosynthesis genes are primarily found grouped into two operons with at least one gene independently located. In *Salmonella*, the operons are organized as *thiMD* and *thiCEFSGH* (123, 177). These two operons are transcriptionally regulated by a TPP-responsive riboswitch

element, the THI-box, and expression is repressed in the presence of exogenous TPP (106, 178). The remaining primary biosynthesis gene, *thiL*, is not found in these operons and does not appear to be regulated by TPP.

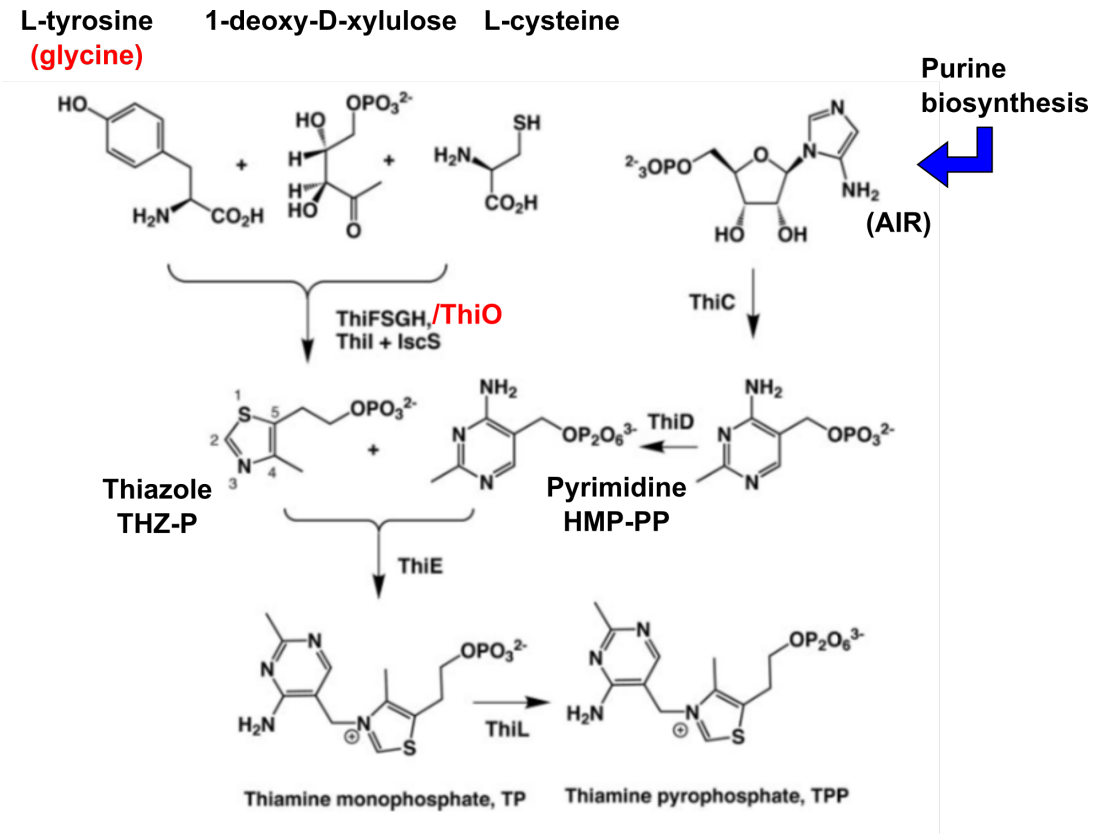

Figure 1. The thiamine pyrophosphate biosynthesis pathway in prokaryotes. The basic structure of the TPP biosynthesis pathway is shown. The formation of the thiazole moiety (THZ), as mediated by ThiFSGH(O), ThiI, and IscS from tyrosine, deoxy-xylulose, and cysteine, is shown on the left. Purine biosynthesis feeds into the production of the pyrimidine moiety (HMP), shown on the right. The moieties are condensed by ThiE to produce thiamine monophosphate (TMP), which is phosphorylated to TPP by ThiL. The substitution of ThiO and glycine for ThiH and tyrosine in *Bacillus subtilis* are shown in red. Figure adapted from Leonardi and Roach, J. Biol. Chem. 2004; 279(17):17054-17062.

PROKARYOTIC SYSTEMS OF THIAMINE PYROPHOSPHATE TRANSPORT

In addition to the pathways of *de novo* biosynthesis and TPP salvage, prokaryotic organisms possess systems to import TPP or other intermediate molecules from the growth environment, as shown in Figure 2. The best characterized of these is the ThiBPQ transporter. The *thiBPQ* locus was identified in *Salmonella enterica* and was found to encode an ABC transporter specific for the translocation of thiamine and thiamine pyrophosphate (177). This locus was previously identified in *E. coli* as *sfuABC* (83, 177). Functional analyses indicated that *thiBPQ* encodes three components- a substrate binding subunit, transmembrane channel protein, and an ATP-binding subunit, respectively. Furthermore, the *thiBPQ* locus in *Salmonella* and *E. coli* is transcriptionally regulated in the same manner as the biosynthesis genes, in which a riboswitch element represses expression in the presence of excess TPP (177).

Other systems of TPP and thiamine transport have been identified; however, the descriptions of these systems are not considered complete, and they are less well understood. The product of the *thiT* gene in *Lactobacillus casei* and *Bacillus subtilis* was shown to be involved in the transport of thiamine B1 (43, 44). ThiT belongs to the family of energy-coupling factor (ECF) transporters, which are dependent on small integral membrane proteins to mediate binding and transport of substrates. ThiT is one such integral membrane protein, and is thought to be a part of a multimeric transporter system (128). However, the other components of this system have not been definitively identified, and there is evidence that the integral membrane proteins alone can mediate

transport. A homolog of ThiT was also identified in *Listeria monocytogenes*, where it was found to be involved in thiamine uptake (139).

Comparative genomics and sequence mining for TPP responsive riboswitches has also identified a host of putative transporters involved in the uptake of thiamine or its precursor molecules. The functionality of the ECF transporters YkoCDEF and ThiW, for importing the HMP and THZ moieties, respectively, has been demonstrated in Gram-positive organisms (128, 130, 140). In addition, the role of the ThiXYZ ABC transporter in HMP salvage was experimentally validated in *B. subtilis* (68). Recently, a predicted thiamine transporter PnuT was shown to use a facilitated diffusion mechanism of transport, which is significantly different from the active transport systems previously identified (52, 65). Finally, the products of the *cytX* and *thiV* genes are predicted to play a role in HMP moiety transport, but the functionality of these systems has not been confirmed (129).

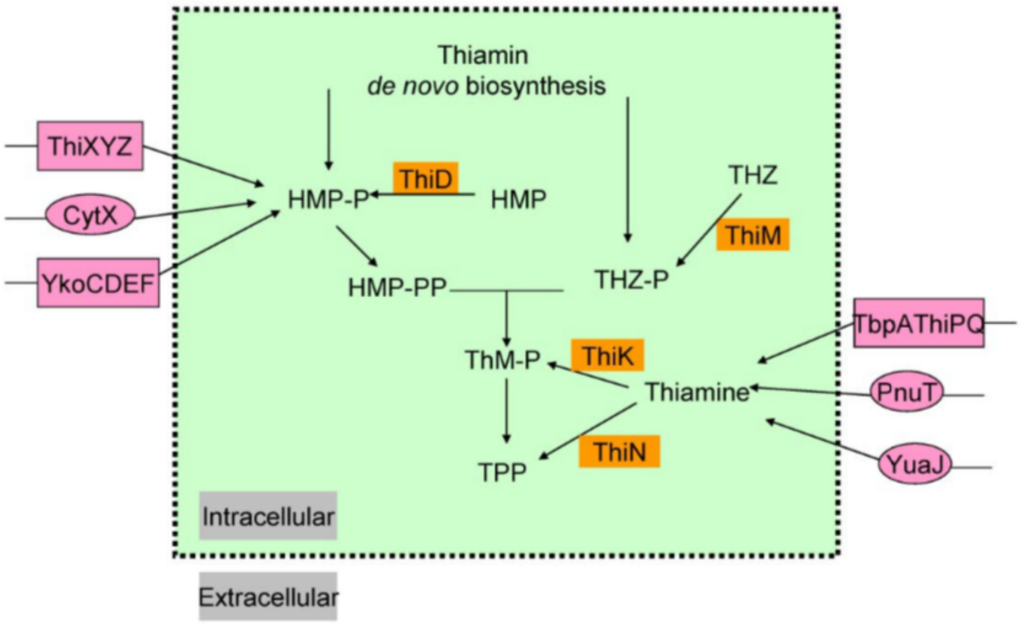

Figure 2. Systems of transport and salvage of thiamine, thiamine pyrophosphate, and related precursor compounds in prokaryotes.

The TPP biosynthesis pathway is represented in the intracellular environment, and salvage enzymes are shown in orange with their substrates and products. Transport systems are shown in pink; those in boxes have confirmed functionality, those in circles are putative. PnuT was recently experimentally validated, and YuaJ is the previously used nomenclature for ThiT, which has been confirmed as a transporter in several organisms, including *Bacillus subtilis*. Figure adapted from: Du, Wang, and Xie, Int. J. Biol. Sci. 2011; 7(1):41-52.

THIAMINE PYROPHOSPHATE AND REACTIVE OXYGEN SPECIES (ROS)

TPP may have additional benefits beyond its contribution to basic processes of cellular metabolism. Many studies have investigated the potential antioxidant activity of TPP, and have found that it is capable of protecting against oxidative damage in a variety of mechanisms. These mechanisms may be direct or indirect. It was observed that TPP may act as a scavenger of reactive oxygen species (ROS), including superoxide anions and hydroxyl radicals (53, 81, 111). Interaction of TPP with reactive substrates results in the opening of the thiazole ring and donation of a hydrogen atom, as well as transfer of two hydrogens from the side group of the pyrimidine ring. Consequently, the toxic free radicals are reduced and thiamine is oxidized to produce thiochrome and thiamine disulfide (95).

Accumulation of thiamine B1 in Arabidopsis plants subjected to environmental stressors helped to alleviate damage caused by production of oxidative compounds (167). In *E. coli*, the addition of TPP to paraquat-treated cultures reversed a decrease in the growth rate, and suppressed the expression of manganese-containing superoxide dismutase (Mn-SOD) and glucose-6-phosphate dehydrogenase (G6PD), which are typically expressed in the presence of oxidative stress (81). In *Salmonella enterica*, provision of TPP rescued growth of paraquat-treated cultures better than other nutrients such as methionine (166). This effect was determined to be due to the oxidative disruption of iron-sulfur cluster containing proteins, some of which are enzymes involved in the production of TPP. Interestingly, TPP is important in preventing lipid peroxidation, which can occur during oxidative-damage induced hepatotoxicity in rats and involves damage to cellular

membranes (59, 143, 171). This phenomenon is thought to be largely indirect, as TPP is a cofactor for enzymes involved in the production of NADPH, which is required for the reduction of glutathione. Reduced glutathione (GSH) is an important endogenous antioxidant, capable of converting free radicals and peroxides to harmless byproducts, and depletion of GSH results in an imbalance of cellular redox status. However, some of this effect is thought to be due to direct interactions between TPP and free radicals as well (95).

Furthermore, TPP may be able to influence the production of ROS. Studies have found that both thiamine B1 and TPP could suppress the production of hydroperoxide from the auto-oxidation of linoleic acid (119). Also, the presence of thiamine or TPP was capable of suppressing the generation of oxygen radicals from stimulated human neutrophils. In both the cell-free and isolated neutrophil assays, TPP showed considerably stronger suppressive effects than thiamine B1, although both displayed impressive antioxidant activity (119). Collectively, these intriguing results indicate that TPP in important for cellular resistance to oxidative damage, and may even be able to help modulate the damage caused by PMN- generated ROS.

STATEMENT OF PROBLEM

Gonococcal disease occurs worldwide and is capable of causing severe pathologies with long-term health effects. Increasing antibiotic resistance and the lack of a gonorrhea vaccine has resulted in limited options for both empirical and preventive treatment of gonococcal infections. Gc is well-adapted to promote its own survival in the human host,

and has many mechanisms for evading detection and killing by the host immune system. In particular, Gc is extremely adept at resisting pathogen-control strategies mounted by the innate immune system. Gonorrhea infections are frequently marked by a robust PMN response and high levels of inflammation. However, Gc persists during inflammation, and can survive and replicate even within the harsh environment of PMN phagosomes. This unique capacity to resist immune cell effectors so successfully makes Gc an even more formidable threat to public health.

Mechanisms of bacterial resistance to PMNs and PMN-derived effectors have long been investigated in efforts to understand the relationship between Gc and the host immune system. These efforts have identified many different pathways and resources that Gc employs to protect itself from PMN killing. However, less is known about the contributions of bacterial metabolism to pathogenesis in the host. Here we sought to investigate those connections by specifically examining the metabolic pathway of TPP biosynthesis as it relates to gonococcal fitness and survival during infection.

HYPOTHESES AND SPECIFIC AIMS

The goal of this work was to expand our knowledge of metabolic pathways in Gc, and to explore the contributions of metabolism to bacterial pathogenesis. To this end, we outlined two specific aims to guide the work of this dissertation.

Specific Aim 1: To investigate the functionality of a potential pathway of *de novo* thiamine pyrophosphate biosynthesis in Gc.

Subaim 1.1: To describe the *in vitro* growth phenotypes and nutritional requirements of a TPP biosynthesis-deficient mutant of Gc.

Subaim 1.2: To investigate the importance of TPP biosynthesis *in vivo* in a murine model of lower genital tract infection.

Subaim 1.3: To determine the contributions of TPP biosynthesis to bacterial resistance to reactive oxygen species and other effectors produced by innate immune cells.

Hypothesis: We hypothesized that the *de novo* production of TPP is critical to sustain Gc during growth in nutrient-poor environments, and contributes to resistance to damage from oxidative stress.

Specific Aim 2: To explore potential systems of transport for TPP or other thiamine derivatives in Gc.

Subaim 2.1: To examine the *in vitro* growth requirements of a TPP transport-deficient mutant of Gc.

Subaim 2.2: To assess the importance of TPP transport for bacterial growth and survival *in vivo* in the presence or absence of the ability to synthesize TPP.

Hypothesis: We hypothesized that a transporter for thiamine and its derivatives is active in Gc, and is important for adaptation to the lower genital tract.

CHAPTER 2: MATERIALS AND METHODS

CULTURE CONDITIONS

All strains of bacteria, including *N. gonorrhoeae* and *E. coli*, were grown for 18-20 hours in an incubator maintained at 37°C with 7% CO_2. *N. gonorrhoeae* was grown on GC agar containing Kellogg's supplements I (glucose, L-glutamine, and thiamine pyrophosphate) and 12 uM ferric nitrate (84). For liquid culture, bacteria were grown with aeration in GC broth supplemented with 5 mM sodium bicarbonate (107). For TPP deprivation conditions, TPP was omitted from Kellogg's supplement I, which was then used in the preparation of TPP-agar and broth. For the *thiB*, *thiO* double mutant strain RS9, GC media was prepared with a high final concentration (1 mM) of TPP to allow growth (GC+ agar). Luria-Bertani (LB) agar and broth was used for the growth of *E. coli* TOP10. Antibiotics used for selection or propagation of mutants were kanamycin (50 ug/mL), ampicillin (100 ug/mL), streptomycin (100 ug/mL), and erythromycin (1 ug/mL), and chloramphenicol (1 ug/mL).

BACTERIAL STRAINS AND GENETIC MANIPULATIONS

The bacterial strains used in this study are described in Table 1. A description of the oligonucleotide primers used for the polymerase chain reaction (PCR) is found in Table 2 (10 base-pair DUS sequence and restriction enzyme sites are underlined). To create an insertionally inactivated *thiO* gene, we designed a modified wild-type *thiO* sequence from strain FA1090 in which an internal 303 bp sequence (nucleotides 400 to 702) was omitted to allow for the insertion of a non-polar kanamycin resistance-encoding cassette

(*thiO*::*aphA3*) (103). This altered sequence was submitted to Thermo Fisher Scientific GeneArt service, where it was synthesized and cloned into vector pMA (AmpR) to create pRS8. The *thiO*::*aphA3* sequence was PCR-amplified from this vector using primers ThiO-F UP and ThiO-R UP, purified, and spot transformed into FA1090 (55). Successful transformants were selected on GC agar with Km, and one Km^R isolate was passaged further and confirmed as containing the inactivated *thiO* gene by PCR amplification and nucleotide sequencing. This mutant was designated strain RS8 (Table 1). For complementation, the WT *thiO* sequence was PCR-amplified from FA1090 using primers ThiO-F PAC and ThiO-R PME. The amplicon was digested with PacI and PmeI, and then cloned into Pac/Pme- digested neisserial complementation vector (NICS) pGCC3 (I.J. Mehr and H.S. Seifert, unpublished data) to create pRS8.2. The resultant plasmid was then digested with ClaI to release a large (~8 kb) fragment containing the *thiO* sequence flanked by the neisserial *aspC* and *lctP* genes as well as an erythromycin (Erm) resistance marker. The 8 kb fragment was spot-transformed in RS8 and complemented mutants were isolated on GC agar with Erm. An Erm^R, Km^R isolate, strain RS8.2, was chosen for further characterization and confirmed by PCR analysis and sequencing.

To create an insertionally inactivated *thiB* gene, we designed a modified wild-type *thiB* sequence from strain FA1090 in which an internal 204 bp sequence (nucleotides 400 to 603) was omitted to allow for the insertion of a chloramphenicol resistance-encoding cassette (*cat*). This altered sequence was submitted to Thermo Fisher Scientific GeneArt service as before, where it was synthesized and cloned into vector pMA (AmpR) to create pRS6. The *thiB*::*cat* sequence was amplified by PCR from this vector using primers

ThiB-F UP and ThiB-R UP, purified, and spot transformed into FA1090 (55). Successful transformants were selected on GC agar with Cm, and one isolate was passaged further and confirmed as containing the inactivated *thiB* gene by PCR amplification and nucleotide sequencing. This mutant was designated strain RS6 (Table 1). For complementation, the WT *thiB* sequence was PCR-amplified from FA1090 using primers ThiB-F PAC and ThiB-R PME. The amplicon was cloned into the neisserial complementation vector (NICS) pGCC3 as before to create pRS6.2. The resultant plasmid was then digested with ClaI to release a large (~8 kb) fragment containing the *thiB* sequence flanked by the neisserial *aspC* and *lctP* genes as well as an erythromycin (Erm) resistance marker. The 8 kb fragment was spot-transformed in RS6 and complemented mutants were isolated on GC agar with Erm. An Erm^R, Cm^R isolate, strain RS6.2, was chosen for further characterization and confirmed by PCR analysis and sequencing. A *thiB*, *thiO* double mutant was created by PCR-amplifying the inactivated *thiB* sequence from RS6 and spot transforming the fragment into the *thiO* mutant RS8. The resultant strain was selected on GC+ agar containing chloramphenicol.

Table 1. Genotype and TPP- phenotype of strains used in this study

Strain	Genotype	TPP- Phenotype	Reference
FA1090	WT	Normal growth	(98)
RS8	*thiO*::*aphA3*	Reduced colony diameter	This study
RS8.2	RS8 complemented with FA1090 *thiO*	Normal growth	This study
RS6	*thiB*::*cat*	Normal growth	This study
RS6.2	RS6 complemented with WT *thiB*	Normal growth	This study
RS7	*thiP*::*cat*	Normal growth	This study
RS7.2	RS7 complemented with WT *thiP*	Normal growth	This study
RS9	*thiB*::*cat*, *thiO*::*aphA3*	No growth	This study
RS1	*thiC*::*cat*	Normal growth	This study
RS12	*cytX*::*aphA3*	Normal growth	This study
RS13	*thiC*::*cat*, *cytX*::*aphA3*	Normal growth	This study

Table 2. Oligonucleotide primers used in this study

Primer	Nucleotide Sequence
ThiO-F UP	5’ GCCGTCTGAACGGTACGCCTTTTC
ThiO-R Up	5’ GCCGTCTGAAAGGGTGTCCTTTCGG
ThiO-F PAC	5’ GGGGTTAATTAACGGTACGCCTTTTC
ThiB-R PME	5’ GGGGTTTAAACAGGGTGTCCTTTCGG
ThiB-F UP	5’ GCCGTCTGAAGTTAGAATGTTATTTTACAGCG
ThiB-R Up	5’ GCCGTCTGAAGGCGGTATGTTTGTTTTATTTC
ThiB-F PAC	5’ GGGTTAATTAACGGTCGGGTTTTCTTGC
ThiB-R PME	5’ AAAGTTTAAACCTCTGCGGGGCGGTATG
ThiP-F UP	5’ GCCGTCTGAAACGGCGGGAGGAAG
ThiP-R Up	5’ GCCGTCTGAACTGAAAAAGGGTTCGC
ThiP-F PAC	5’ GGGTTAATTAATAAGGCGCTGAAGCACCGG
ThiP-R PME	5’ AAAGTTTAAACTTTGCCGTCCTCTGAAAAAGGG
CytX-F UP	5’ GCCGTCTGAAAGGACGTCATATGTCG
CytX-R UP	5’ GCCGTCTGAACAATGCGGTCAGCCTTC

COLONY SIZING AND QUANTIFICATION

Wild-type FA1090, mutant, and complemented mutant bacteria were grown on GC agar plates (+TPP) overnight, suspended in PBS, and plated on both GC and TPP- agar. After 24 hours incubation, plates were imaged on a Leica AF6000 microscope and over 100 colonies were photographed and their diameters recorded and averaged. A Mann-Whitney test was used to detect significant differences between the GC and TPP- agar groups for each strain.

GROWTH KINETICS AND INDEPENDENT GROWTH CURVES

Bacteria were subcultured from -80 stocks and passed on GC agar. Strains were harvested after 18 hours of incubation, suspended in GC broth (GCB), and used to inoculate flasks containing supplemented GC broth with or without TPP to an initial OD_{600} of ~0.07. To starve the bacteria of TPP, strains were grown in TPP-broth (10 mL) to mid-to-late log phase before harvesting. A 4 mL aliquot was removed, centrifuged, and re-suspended in an equal volume of fresh GCB without supplements. The suspension was adjusted to an OD_{600} of ~0.7 before being used to inoculate flasks containing 35 ml of GCB with or without TPP. Cultures were incubated at 37°C with shaking at 220 rpm, and the OD was taken every hour over the course of eight hours. Every other hour a sample was plated on GC agar to determine the number of recovered CFUs. The mean and SEM of three independent experiments is shown. A two-way ANOVA with repeated measures was used to detect significance.

THIAMINE PYROPHOSPHATE TITRATION

RS8 bacteria were grown under TPP starvation conditions as described above except that increasing concentrations of TPP were added to the culture broth as follows. A 100 mM stock solution of TPP was serially diluted to achieve the target concentrations ranging from 10 nM to 10 pM before addition of 350 μL to 35 mL cultures of GCB for a final 1:100 dilution. Cultures were then incubated for 6 hours at 37°C, 220 rpm, and OD_{600} measurements were taken every hour. For the 0 TPP condition, an equal volume (350 uL) of water was added to the flask instead of TPP. For the TPP titration of the double mutant RS9, bacteria were harvested from GC+ agar and used to inoculate cultures containing a range of TPP concentrations from 1 mM to 1 uM in ten-fold dilutions. Cultures were incubated and assessed for the OD_{600} and recovered CFUs as above.

SUBSTRATE UTILIZATION ANALYSIS

RS8 bacteria were grown under the following TPP starvation conditions: bacteria were grown in TPP-broth (10 mL) to mid-to-late log phase before harvesting. Four milliliter aliquots were removed, centrifuged, and re-suspended in an equal volume of fresh GCB without supplements. The suspension was adjusted to an OD_{600} of ~0.7 before being used to inoculate large cultures with varying concentrations of TPP, TMP, or B1 (Sigma-Aldrich). A 100 mM stock solution of TPP, TMP, or B1 was serially diluted before the addition of 350 μL of each dilution into the 35 mL cultures of GCB for a final 1:100 dilution to achieve the target concentrations. Cultures were then incubated for 6 hours at 37°C, 220 rpm, and OD_{600} measurements were taken every hour. For the 0 TPP, TMP, or B1 condition, an equal volume (350 uL) of water was added to the flask.

COMPLEMENTATION OF *SALMONELLA ENTERICA*

Plasmids were constructed that placed wild-type *S. enterica thiB* or the gonococcal *thiB* under the control of an inducible promoter. These plasmids were transformed into a thiazole (THZ) auxotrophic strain of *Salmonella* in which a point mutation in ThiB abolishes the transport of TPP. The transformed strains were then tested on minimal media containing thiamine B1, TPP, or the THZ moiety. THZ or thiamine B1 in the media could rescue growth, but TPP could be used only if ThiBPQ was functional. As a negative control, an empty vector was transformed into *S. enterica*. As a positive control an HMP auxotrophic strain (will grow on TPP and B1, but not THZ) was also tested for its ability to grow on the different media types.

IDENTIFICATION OF THIAMINE DERIVATIVES IN MEDIA BASE

A *thiC* mutant strain of *Salmonella enterica* was grown in nutrient broth (NB), washed, and used to inoculate cultures of minimal medium containing varying concentrations of GC Medium Base (BD Difco), thiamine B1, or glucose alone. Concentrations of GC Medium that were tested were 0.2% and 0.02%. For B1 cultures, 100 nM of B1 was present in the culture medium. For glucose only conditions the final concentration used was 11 mM. Cultures were incubated for 20 hours, with OD_{650} reading taken every 5 hours. For further analysis, growth of *Salmonella thiH*, *thiE*, and *thiL* mutants was assessed on agar media containing glucose only (minimal media), thiazole (THZ), TPP, thiamine B1, nutrient agar (rich media), or 0.2% GC Medium Base.

IN VITRO COMPETITIVE CULTURE ASSAYS

Mutant RS8 or RS8.2 and wild-type bacteria were grown in 10 ml of GCB TPP- to mid-log phase, then aliquots were combined to create a mixture containing similar numbers of each strain. The 1:1 mixture was used to inoculate 35 ml cultures of GCB that were either fully supplemented (full TPP), contained no TPP (0), or contained a minimal concentration (10 nM) of TPP. Cultures were incubated for 6 hours as above, and OD_{600} measurements taken every hour. At 0 (input), 1, 3 and 5 hours post inoculation, aliquots were serially diluted and inoculated on GC agar with and without Km selection to determine total (GC agar) and mutant or complement (GC + Km) bacterial counts. Numbers of wild-type bacteria were calculated by subtracting the number of mutant bacteria from the total number. The competitive index was calculated as: [(mutant CFUs/parent CFUs)output/(mutant CFUs/parent CFUs)input]. All competitive culture experiments were conducted three times.

H_2O_2 AND PARAQUAT SENSITIVITY DISC DIFFUSION ASSAYS

Wild-type FA1090, RS8, and RS8.2 bacteria grown on GC agar or passaged on TPP- agar for 24 hours were suspended in PBS to an initial OD_{600} of 0.1 (~10^8 CFU/mL), and then plated as a lawn on GC agar. Filter discs soaked in 1, 5, 10 and 20 mM solutions of H_2O_2 (a source of exogenous ROS) or paraquat (an inducer of intracellular ROS) were placed on top of the bacterial lawn. Plates were incubated overnight and the zone of inhibition was calculated by measuring the area where no bacteria grew and subtracting the diameter of the paper disc (6 mm). A one-way ANOVA was used to detect significant differences between RS8 and WT at each dose of H_2O_2 or paraquat administered.

PMN ISOLATION AND KILLING ASSAYS

Groups of 5-6 female BALB/c mice (Charles River Laboratories) were injected intraperitoneally with 2.5 mL of 4% thioglycollate broth to induce peritonitis. After 5 hours, mice were sacrificed and peritoneal lavage with 10 mL cold HBSS buffer was performed to recover the infiltrating PMNs. The PMN suspension was centrifuged at a low-speed spin and the pellet re-suspended in HBSS II (HBSS with 0.1% gelatin and 10 mM glucose). Trypan blue staining was performed to determine cell number and viability. Bacteria were opsonized by incubating in 10% pooled normal mouse serum (NMS) or heat-inactivated normal mouse serum (HI-NMS). PMNs (approximately 10^6) were combined with NMS- or HI-NMS-opsonized wild-type, *thiO* mutant, and complemented mutant bacteria in a 1:1 ratio in HBSS. The mixtures were placed in a rotary shaker and incubated at 37°C. After 45 minutes of incubation, aliquots were removed, serially diluted, and plated on GC agar with streptomycin to recover viable bacteria. The degree of killing was measured by comparing the number of bacteria recovered from the experimental condition to those recovered from the HI-NMS control. For testing the involvement of the phagocytic respiratory burst, C57BL/6J and B6.129S6-Cybbtm1Din/J (gp91phox-, in the C57BL6/J background) mice (Jackson Labs) were used instead, and only bacteria harvested from TPP-plates were assessed in the PMN killing assay.

MINIMUM INHIBITORY CONCENTRATION (MIC) OF POLYMYXIN B

Supplemented GC agar was prepared and added to 50 mL conical tubes containing polymyxin B diluted to the appropriate concentration in GC broth. The tubes were

inverted 5-6 times to mix, then the contents were poured into the wells of a six-well plate. We assessed bacterial susceptibility to a range of concentrations from 300-100 ug/mL, in 25 ug/mL increments. Wells containing agar with no polymyxin B were included as growth controls. The agar was allowed to cure for two days before use. On the day of the assay, suspensions of WT, RS8, and RS8.2 bacteria harvested from TPP-starved and un-starved conditions were prepared at an OD_{600} of 0.05-0.055 in sterile PBS. Samples were serially diluted and plated on GC agar to quantify the number of viable bacteria per milliliter of bacterial suspension. Ten microliters (~$2x10^5$ CFUs) of each strain were spotted onto the agar plugs in the six-well plate containing polymyxin B, and the spots were allowed to dry at room temperature. Plates were then inverted and placed in the incubator. After 24 and 48 hours, plates were removed and the growth of the spots were scored. Inhibition of growth was considered to be accomplished when fewer than 5 colonies were counted in a spot, and the MIC is reported as the lowest concentration at which inhibition occurred.

IN VIVO COMPETITIVE MURINE INFECTIONS

For *in vivo* competitions, female BALB/c or C57BL/6J (Charles River Laboratories) mice were treated with 0.5 mg of 17β-estradiol in sesame oil on days -2, 0, and +2 of the infection timeline. Antibiotics were also administered to suppress commensal flora as described (73). Bacteria to be tested were cultured overnight on GC agar (TPP-replete) and mice were inoculated intra-vaginally with equal numbers (total dose ~10^6 CFU/mL) of wild-type (WT) combined with RS8, RS8.2, RS6, or RS9 bacteria. The double mutant RS9 was cultured overnight on GC+ agar prior to inoculation of mice. On days 1, 3, 5,

and 7 post-inoculation, vaginal swabs were quantitatively cultured on selective and non-selective GC agar to calculate the competitive index. Selective agar for the competition of WT with RS8 or RS8.2 contained streptomycin (Sm) and kanamycin (Km). For the inoculum containing WT and RS6, Sm and chloramphenicol (Cm) medium was used. Non-selective medium consisted of GC agar containing Sm alone. The mixture prepared with the double mutant RS9 was cultured on GC+ agar containing Sm and Sm+Cm, as described above. The competitive index was calculated as: [(mutant CFUs/parent CFUs)output/(mutant CFUs/parent CFUs)input]. Vaginal swabs were also inoculated onto HIA agar to monitor commensal flora, and streaked on a glass slide to monitor PMN influx. Slides were stained with a modified Wright stain, and the presence of vaginal PMNs was quantified as the number of PMNs among 100 cells as determined by light microscopy. For noncompetitive infections, female BALB/c mice were treated as described above before inoculation with 10^6 CFUs of RS9 bacteria. Mice were cultured on GC+ agar with Sm and Cm every day for 5 days, with monitoring of commensal flora and vaginal PMNs.

EPITHELIAL CELL ADHERENCE AND INVASION ASSAYS

Human cervical epithelial carcinoma cells, ME-180, were maintained in McCoy's 5A Medium supplemented with 10% heat inactivated FBS. For adhesion and invasion assays, epithelial cells were seeded in 24 well plates (1 X 10^5 cells/well) and incubated overnight at 37°C in 5% CO_2. Bacterial strains were grown for 18 hours on GCR or GC+ agar. For starvation conditions, WT and RS6 bacteria were grown on TPP- agar prior to addition to ME-180 cells. Bacteria were suspended in McCoy's 5A Medium supplemented with 10%

heat inactivated FBS and containing 0.5% Kellogg's supplement, then added to the epithelial cells at a MOI of 10:1. For adherence assays, cells and bacteria were incubated for 4 hours, washed with PBS, then treated with 1% saponin for 15 minutes. Lysates were collected and aliquots plated on GCR and GC+ agar plates. For invasion assays, cells were incubated for an additional 2 hours, then treated with 100 μg/ml of gentamicin for another 2 hours. After washing the cells extensively, the number of invasive GC was quantified by lysing the cells with 1% saponin for 15 minutes. Aliquots of the lysates were plated onto GCR or GC+ agar plates.

STATISTICAL METHODS

A Mann-Whitney test was used to detect significant differences between WT, mutant, and complemented mutant colony diameters when grown on GC or TPP- agar. For the *in vitro* growth curves, a one-way ANOVA with repeated measures was used to detect differences between growth under TPP+ or TPP- conditions. CI values from the *in vivo* competitions of WT vs. *thiO* mutant and WT vs. complemented mutant were log-transformed and compared by the Mann-Whitney test to determine if significant differences in the average CI values were present over the course of infection. For the H_2O_2 and paraquat disc diffusion assay, a one-way ANOVA test with multiple comparisons was used to detect significant differences between RS8 and WT at each dose tested. For the PMN killing assays, the internal replicates from the NMS condition were averaged and normalized against the average of the HI-NMS internal replicates for each individual experiment. The normalized value was recorded as the percent bacteria recovered from the NMS condition compared to the HI-NMS controls for the individual

experiments. A two-way ANOVA with multiple comparisons was used to detect significance. Statistical analyses were performed by GraphPad Prism software package.

ANIMAL USE ASSURANCES

Animal experiments were conducted in the laboratory animal facility at Uniformed Services University, which is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, under a protocol approved by the University's Institutional Animal Care and Use Committee (MIC-16-488).

CHAPTER 3: Biosynthesis of Thiamine Pyrophosphate is Important for Growth and Survival of *Neisseria gonorrhoeae in vitro* and *in vivo* and May Contribute to Protection from Effectors of Innate Immunity

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ABSTRACT

Thiamine pyrophosphate (TPP) is an essential cofactor for many metabolic processes and can act as a potent scavenger of reactive oxygen species (ROS). *Neisseria gonorrhoeae* (Gc) is an obligate human pathogen that is adept at surviving within its host by co-opting resources and evading immune responses. Historically, TPP has been provided as a supplement to culture media to improve the recovery of Gc from infected patients, but the importance of TPP for Gc in the context of infection is unknown. Here we identified the components of a *de novo* TPP biosynthetic pathway in Gc by *in silico* analysis, and constructed a glycine oxidase (*thiO*) mutant to assess the importance of thiamine biosynthesis in Gc. We found that the ability of Gc to produce TPP *de novo* is important for growth *in vitro* under nutrient-poor conditions, and increases Gc resistance to exogenous H_2O_2 and paraquat. The *thiO* mutant also exhibited decreased resistance to opsonophagocytic killing by murine polymorphonuclear leukocytes (PMNs), but studies

with PMNs from *gp91phox-* mice showed the increased susceptibility of the mutant was not due to PMN-derived ROS. Interestingly, however, the *thiO* mutant was attenuated in experimentally infected BALB/c but not C57/BL6 mice, which, unlike BALB/c mice, did not produce a PMN response to infection. The *thiO* mutant exhibited consistently lower MICs to polymyxin B when deprived of TPP, suggesting thiamine confers resistance to oxygen-independent PMN defenses. We conclude that the capacity of Gc to produce TPP is important for adaptation to growth in nutrient-poor environments, and may also contribute to protection from damage caused by PMN-derived effectors during infection.

Note: The creation of all mutant and complemented strains and the experiments reported in this study were performed by R. Sennett.

INTRODUCTION

Neisseria gonorrhoeae (the gonococcus, Gc) is a human-specific pathogen and the causative agent of the sexually transmitted infection gonorrhea. Gonorrhea is a disease that has ancient roots, with cases being described in humans as early as biblical times (170). In the long process of evolving alongside humankind, Gc has become remarkably adept at tailoring its genome to allow it to establish and maintain infection. For example, Gc sialylates the N-lactoneotetraose variant of its lipooligosaccharide (LOS) using host-derived neuraminic acid to increase resistance to complement-mediated killing (182), and serum-resistant strains bind human factor H and C4BP-binding proteins, which are soluble regulators of the complement cascade, to down-regulate complement activation

(101, 113, 114). In addition, Gc expresses specific receptors that enable the bacterium to acquire iron from human lactoferrin and transferrin (105).

Symptomatic gonococcal infections characteristically produce a strong innate immune response in the form of a robust polymorphonuclear leukocyte (PMN) influx at the site of infection. However, Gc can evade this host defense by surviving within PMNs and proliferating within the PMN phagosomes (75, 141, 151). It was previously shown that production of catalase or cytochrome c peroxidase by Gc, as well as the accumulation of intracellular manganese through the MntABC transporter, were not sufficient to impact the susceptibility of Gc to killing by isolated PMNs. (159, 183). However, a gonococcal zinc metalloprotease was found to be required for resistance to reactive oxygen species (ROS) and PMN-mediated killing by aiding in the physical expression of the pilus (162). Furthermore, Gc can affect the recruitment of the components of the NADPH oxidase to cellular membranes by modulating expression of the opacity protein (Opa) (154). Gc is also adept at evading non-oxidative mechanisms of killing by PMNs. Expression of the MtrCDE efflux pump is crucial for bacterial resistance to antimicrobial peptides (149). Also, decoration of gonococcal lipid A with phosphoethanolamine (PEA) protects Gc from antimicrobial peptides, complement-mediated killing, and confers a survival advantage *in vivo* (57, 92, 121). Other unique mechanisms of PMN resistance have been investigated. Notably, production of a secreted gonococcal thermonuclease can degrade neutrophil extracellular traps (NETs), and TonB-dependent transporters assist Gc in escaping nutritional depletion by binding PMN-produced calprotectin and preventing the sequestration of zinc (67, 80). This bacterial resilience to host innate defenses has proven

to be an additional obstacle to overcome in the treatment of gonorrhea infections, especially as antibiotic resistance in Gc continues to spread rapidly (28).

Metabolic pathways, in particular mechanisms of nutrient production and acquisition, are increasingly considered a new class of virulence factors for many human pathogens (38). Several studies in recent years have described the contributions of metabolic processes to maintaining or enhancing bacterial virulence. In *Francisella tularensis*, a biotin biosynthesis protein was identified as a virulence factor with essential activity for the escape of the pathogen from macrophage phagosomes and subsequent intracellular replication (76, 110). In addition, the ability to synthesize tryptophan *de novo* is essential for maintaining *Mycobacterium tuberculosis* in activated macrophages that attempt to control the infection by depletion of their internal tryptophan stores (186). In Gc the ability to acquire and utilize lactate increases bacterial growth rate and resistance to complement-mediated killing by enhancing sialylation of LPS or LOS (100, 108). Additionally, a gonococcal mutant that was unable to obtain lactate from the growth environment was more susceptible to human complement-mediated killing and attenuated in experimentally infected mice, demonstrating the importance of lactate metabolism for Gc colonization of the lower genital tract (45).

Thiamine pyrophosphate (TPP) is an essential metabolite for all living organisms. TPP serves as a cofactor for enzymes in the Citric Acid Cycle and Pentose-Phosphate Pathway, and is therefore important for the generation of energy in the form of ATP and NADPH, as well as the production of carbohydrates and branched-chain amino acids (14,

38). In addition, TPP has activity as an antioxidant and a scavenger of ROS, and the presence of thiamine and its phosphorylated esters may increase cellular resistance to oxidative stress (119, 166, 167). Humans are dependent on dietary sources of TPP, but many bacteria, plants, and fungi are capable of producing TPP through a *de novo* biosynthetic pathway. Recently, the TPP pathway has been considered a source of novel targets for drug treatment in *Plasmodium falciparum* and *Mycobacterium tuberculosis*, which are two pathogens that display increasing resistance to traditionally used therapeutics (5, 109).

Improvements in culturing techniques for *N. gonorrhoeae* have included the use of TPP to optimize growth of the pathogen *in vitro* (88). TPP was first described as a growth factor of Gc in 1946, and since then it has been routinely added to culture media in Kellogg's Supplement I (84). Despite the practice of supplementing media with TPP, little is known about the ability of Gc to produce its own TPP, or what the importance of this metabolite is for replication and colonization in the host. We hypothesized that the *de novo* production of TPP is critical to sustain *Neisseria gonorrhoeae* during growth in nutrient-poor environments and contributes to resistance to damage from oxidative stress. To test this hypothesis, we examined the genome of a commonly used laboratory strain of Gc, and determined that a complete pathway of TPP biosynthesis appeared to be present. As the functionality of this pathway has never been addressed, we disrupted the *thiO* gene, which encodes an essential glycine oxidase, and tested the resultant strains for their ability to grow *in vitro* in the absence of supplemental TPP, and *in vivo* in the female mouse model of Gc lower genital tract infection. Furthermore, we examined the capacity

of Gc-produced TPP to protect the bacterium against oxidative and non-oxidative stress and killing by murine PMNs. Our data indicate that TPP production in Gc may have function as a previously unrecognized form of resistance to host innate effectors.

RESULTS

Commonly studied gonococcal strains possess the components of a potentially functional thiamine pyrophosphate biosynthesis pathway. Prokaryotic pathways of TPP biosynthesis have been most comprehensively studied in the model organisms of *B. subtilis* and enteric bacteria such as *E. coli* and *S. enterica*. Results of these studies have shown that TPP is produced by the creation of two separate moieties, a thiazole and a pyrimidine unit, which are then condensed to form thiamine monophosphate (TMP) (11). A final phosphorylation step produces the active cofactor TPP. In most model organisms, such as *B. subtilis*, the *thi* biosynthetic genes are organized into two operons and one to two single gene loci (130).

We examined the genomes of common lab gonococcal strains FA1090 and MS11 to determine whether the components of a TPP biosynthetic pathway could be identified. FA1090 was originally isolated from a case of disseminated gonococcal infection and is serum resistant, whereas MS11 is serum sensitive and was recovered from the cervix of a patient with an uncomplicated lower genital tract infection (77). Therefore, it is possible that the metabolic requirements of these strains may differ based on their different infectious niches. However, in both strains we identified all of the major genetic components of the TPP biosynthetic pathway (Table 3). Four of these *thi* genes are

grouped into a single putative operon (Figure 3), and the other four, as well as two genes predicted to encode accessory enzymes involved in TPP biosynthesis, occur as single genes throughout the genome. The putative operon that we identified in Gc was previously identified in *N. meningitidis* (130). We conclude that it is likely that most gonococcal strains possess the capacity to produce TPP through a functional biosynthetic pathway. In addition, we hypothesized that the *de novo* production of TPP is critical to sustain *N. gonorrhoeae* during growth in nutrient-poor environments.

Table 3. TPP biosynthesis genes identified in FA1090

thi gene product	FA1090 putative *thi* gene	Putative gene product function
Dxs	NGO_0036	DXP synthase
IscS	NGO_0636	NifS-like cysteine desulfurase
ThiD	NGO_1160	HMP-P kinase
ThiL	NGO_1575	Thiamine monophosphate kinase
ThiG	NGO_2005	Thiazole synthase
ThiS	NGO_2006	Sulfur carrier protein
ThiE	NGO_2007	Thiamine phosphate pyrophosphorylase (thiamine monophosphate synthase)
ThiO	NGO_2008	Glycine oxidase
ThiF	NGO_2019	Adenyltransferase
ThiC	NGO_2041	HMP-P synthase

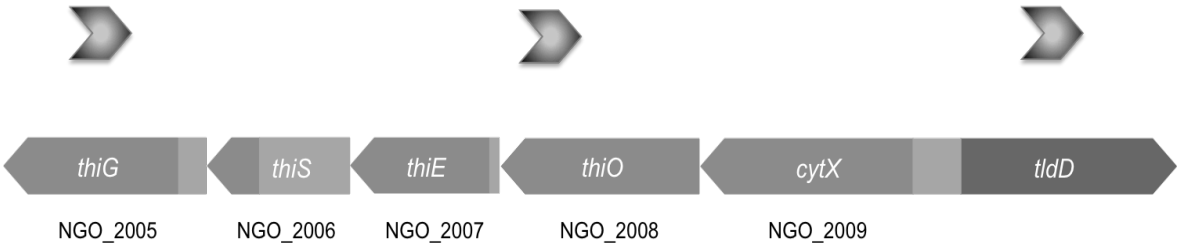

Figure 3. *thiO* is located in a potential operon of TPP biosynthesis genes.
The putative *thi* operon that was identified in FA1090 is shown above. *cytX*, which has homology to the cytosine permease *codB* in *E. coli*, is the first gene in the operon, and is preceded by a potential TPP-responsive riboswitch element. *thiO* (NGO_2008) is immediately downstream of *cytX*. The *thi* genes not found in this operon are distributed throughout the chromosome and are involved in the production of the thiazole moiety, the pyrimidine moiety, and the final pathway steps that result in the formation of the active cofactor.

Interruption of *thiO* severely reduced the average colony diameter in the absence of TPP. In order to examine whether a functional pathway of TPP biosynthesis was necessary to support growth of Gc under nutrient-restricted conditions, we insertionally inactivated the *thiO* gene in strain FA1090 to create mutant RS8 as described in Materials and Methods (Table 1). In *B. subtilis*, *thiO* encodes a glycine oxidase that has essential activity in the production of the thiazole moiety (145, 146). When grown on agar media lacking TPP supplementation (TPP-), RS8 exhibited a drastic reduction in the average colony diameter compared to growth on GC agar (Table 1, Figure 4). WT FA1090 did not display a growth defect on TPP- media, and complementation of the *thiO* mutation in strain RS8.2 restored the colonies to normal size on TPP- plates. Despite the apparent growth defect, colonies of RS8 taken from TPP- plates could be successfully passaged further on the same media, albeit the colonies remained very small. Passage of RS8 colonies from TPP- agar to GC agar resulted in restoration of normal colony growth (data not shown).

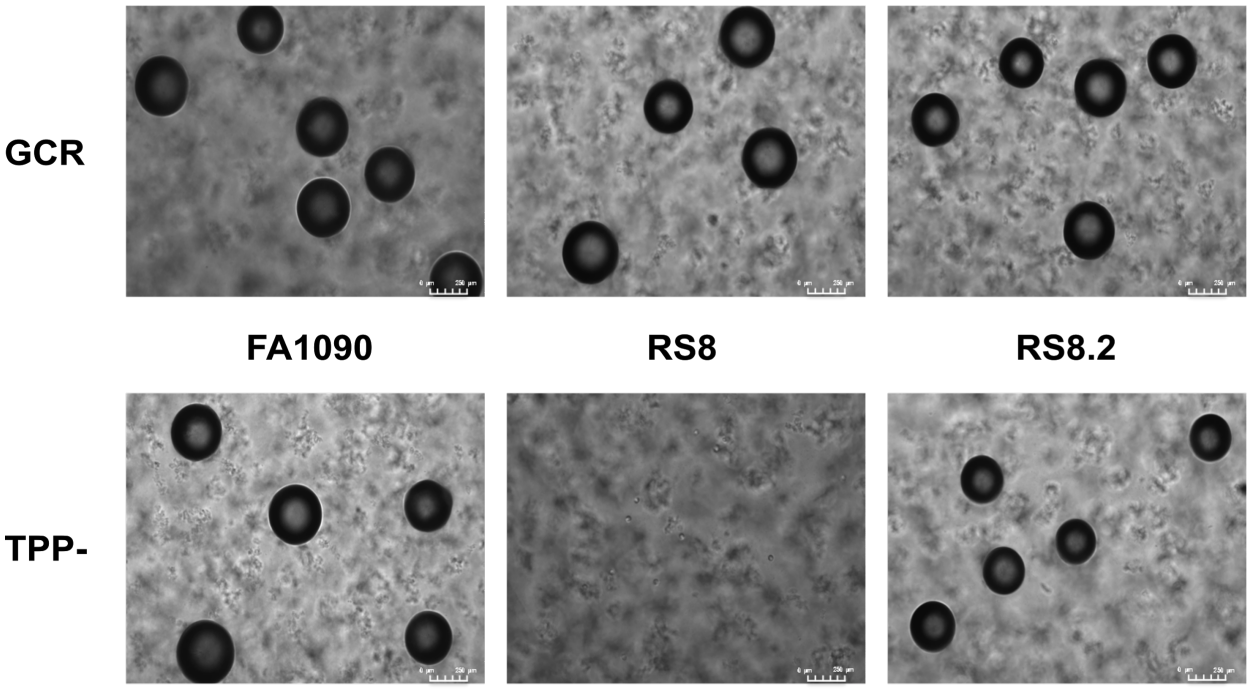

Figure 4. Growth of the *thiO* mutant RS8, complemented mutant RS8.2, and WT FA1090 on agar media lacking TPP.

Microscope images are shown for each strain grown for 24 hours on both fully supplemented media (GC agar, top), and media in which TPP has been excluded from supplement I (bottom). RS8 displayed a drastic reduction in colony diameter in the absence of TPP, but complementation with *thiO* reversed this phenotype. Scale bars represent 0-250 micrometers.

Starvation for TPP resulted in failure of the *thiO* mutant to grow in broth without TPP, and a decline in the number of viable bacteria recovered. We then investigated the effects of the *thiO* mutation on growth kinetics in broth culture. When bacteria were harvested from TPP-replete conditions, inoculation of cultures lacking exogenous TPP did not result in a growth defect for WT, RS8, or RS8.2 (Figure 5A, B). We hypothesized that this might be due to the ability of RS8 bacteria to build internal stores of TPP during growth on supplemented GC agar prior to culture inoculation. In order to deplete the internal TPP reserves, bacteria were grown in GC broth lacking TPP before being inoculated into GC broth containing or lacking added TPP. With the additional TPP starvation step, RS8 displayed a growth defect when grown in cultures where TPP was absent (Figure 5C, D). The optical density of the RS8 culture failed to double after two hours of incubation (Figure 5C), and the number of viable bacteria recovered decreased sharply (Figure 5D). Interestingly, complementation of *thiO* in strain RS8.2 did not fully restore growth in the absence of TPP, however, absorbance readings and recovered CFUs were significantly increased from those of RS8 under the same conditions. This result may be due to insufficient expression of the WT gene from the site of insertion, as the putative *thi* operon appears to be under the control of a potential TPP-responsive riboswitch element (106). A riboswitch is located 170 nucleotides upstream of the transcriptional start site of NGO_2009, the first open reading frame in the *thiO* operon, and was not preserved in the complementation construct.

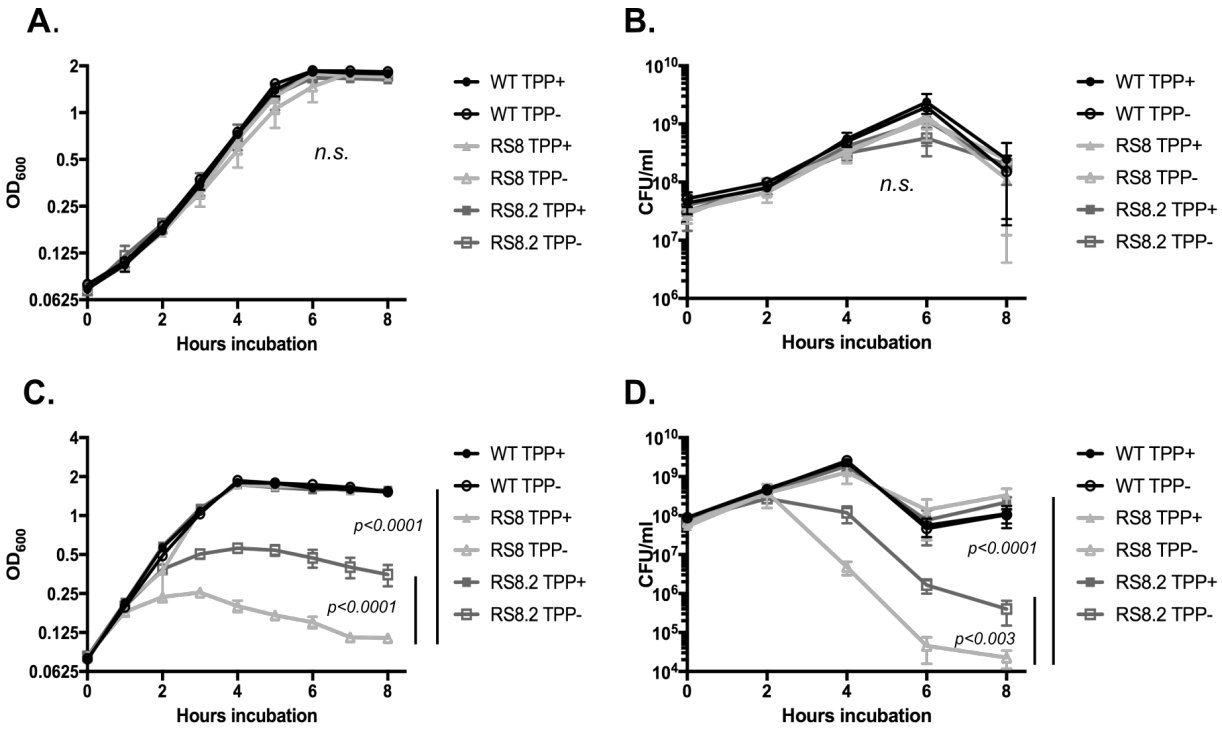

Figure 5. Growth kinetics of RS8, RS8.2, and WT in TPP-supplemented or un-supplemented broth.

Bacteria were harvested from GC agar plates (A, B), or were harvested from GC agar and grown to mid-log phase in mini-cultures without TPP before inoculating larger broth cultures in which TPP was either present or excluded (C, D). Cultures in which TPP was present are indicated by filled symbols, and cultures lacking TPP are indicated by open symbols. The results show the averages of 3 independent experiments, ± SEM.

Picomolar concentrations of TPP were unable to fully support growth of the TPP-starved *thiO* mutant in a dose-dependent manner. We then sought to identify the lowest concentration of TPP that could rescue starved RS8 bacteria. Starved RS8 bacteria were used to inoculate a series of cultures containing dilutions of exogenously provided TPP. Fully supplemented GC media contains a final concentration of approximately 4.3 uM TPP, and we began by testing a range of TPP concentrations starting from 1% (43 nM) to 0.2% (8.6 nM). There were no differences in growth of RS8 under these conditions compared to fully supplemented GC broth (data not shown). Further reduction in the TPP concentrations showed that a minimum concentration of 10 nM TPP would fully support bacterial growth, but further dilution of TPP into the picomolar range resulted in a dose-dependent inhibition of growth (Figure 6). It is unclear whether these results have biological significance, as the availability of TPP in the lower genital tract has not been well described. However, results of studies examining TPP concentrations within tissues of the reproductive tract have indicated that the levels could be in the picomolar range, which is comparable to our results (51).

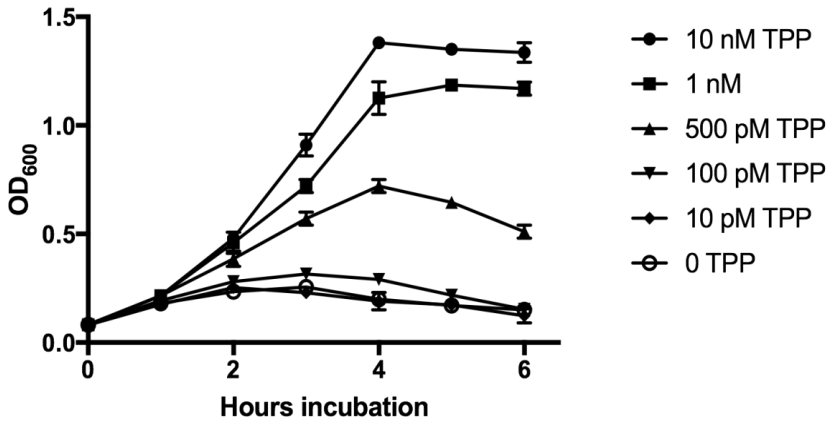

Figure 6. Titration of TPP into cultures of TPP-starved RS8.
RS8 bacteria cultured in TPP-deficient broth were used to inoculate GC broth with no TPP or TPP concentrations ranging from 10 pM to 10 nM. No differences in growth were observed when concentrations of added TPP were in the nM range, but a dose-dependent inhibition of growth was seen when TPP was provided in pM concentrations.

WT bacteria could not rescue the growth disadvantage of the *thiO* mutant when co-cultured in the absence of TPP. We next examined the *in vitro* fitness of RS8 and RS8.2 compared to FA1090 under co-culture conditions to determine whether the mutant bacteria were capable of utilizing TPP produced by the WT strain. In three independent experiments, similar numbers of WT and either RS8 or RS82 bacteria were inoculated into GC broth containing 0, 10 nM or 4.3 uM GC broth, and the competitive index was determined at 1, 3 and 5 hours post-inoculation. In the absence of TPP, a 100- to 1000-fold fitness cost was observed for RS8 at 3 and 5 hrs post-inoculation as expected, based on the noncompetitive growth experiments (Figure 7A, squares). The two strains exhibited equal fitness in broth cultures containing 4.3 uM and 10 nM TPP, the latter of which was also expected based on the results of the TPP titration experiment (Figure 6). No attenuation was observed for the complemented strain RS8.2 compared to WT under the same conditions at any time point (Figure 7B). We conclude that TPP production in the WT strain was not able to rescue the mutant at the time points analyzed.

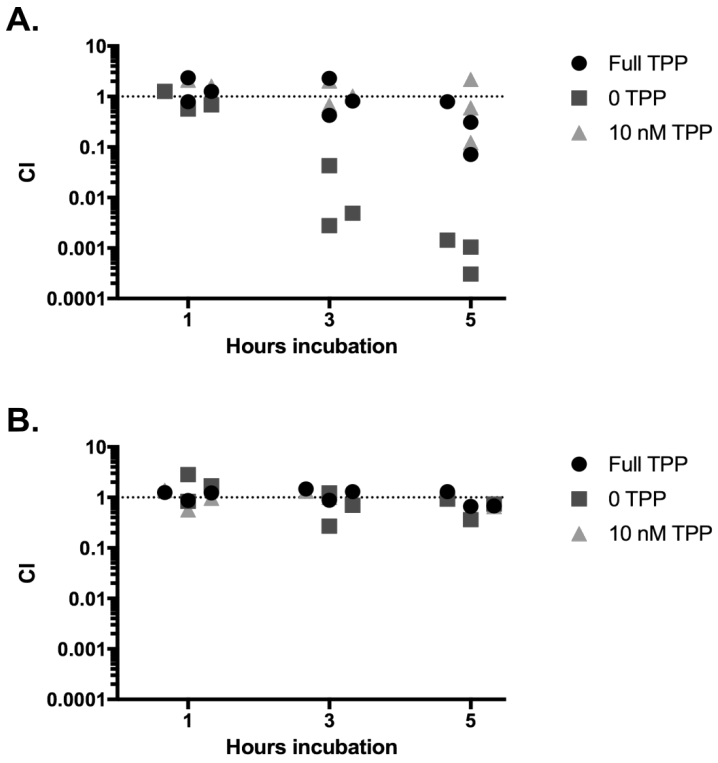

Figure 7. Competitive co-cultures of WT with RS8 or RS8.2 under conditions of varying TPP availability.

Bacteria were grown under TPP-starved conditions, combined in equal numbers, and used to inoculate cultures containing a range of TPP concentrations. Samples taken at 0, 1, 3, and 5 hours were used to calculate the competitive index. Competitive cultures were performed 3 times, and the results of each experiment are shown above. (A) WT vs. RS8, (B) WT vs. RS8.2.

WT bacteria significantly outcompeted the *thiO* mutant in a symptomatic mouse model of genital tract infection. In order to determine whether TPP biosynthesis is important for bacterial growth or survival *in vivo*, we experimentally infected groups of BALB/c mice vaginally with similar numbers of WT and RS8 or RS8.2 bacteria and determined the ratio of each strain in vaginal cultures over time as previously described (73). Combined data from two independent experiments showed that the mutant RS8 had an approximately 10-fold reduction in the competitive index on days 5 and 7 of infection (Figure 8A); this decrease was significantly different than the competitive index observed in mice inoculated with WT and RS8.2 bacteria (Figure 8B). The observed attenuation of RS8 coincided with a rise in vaginal PMNs on day 5 of infection (Figure 8E), suggesting that the fitness decrease may be due to pressure from the inflammatory response.

To further examine this hypothesis, we conducted competitive infection experiments in C57/BL6 mice, which provide a model of asymptomatic infection because they do not produce pro-inflammatory cytokines or a vaginal PMN influx in response to Gc (120). In the C57/BL6 mice, RS8 did not exhibit a decrease in the competitive index at any point during infection (Figure 8C). When RS8.2 and WT were competed in the asymptomatic model of infection, there was a slight increase in fitness of the complemented mutant strain on days 3 and 5 of infection (Figure 8D). As shown previously (44), no PMN influx was observed in infected C57/BL6 mice (Figure 8F).

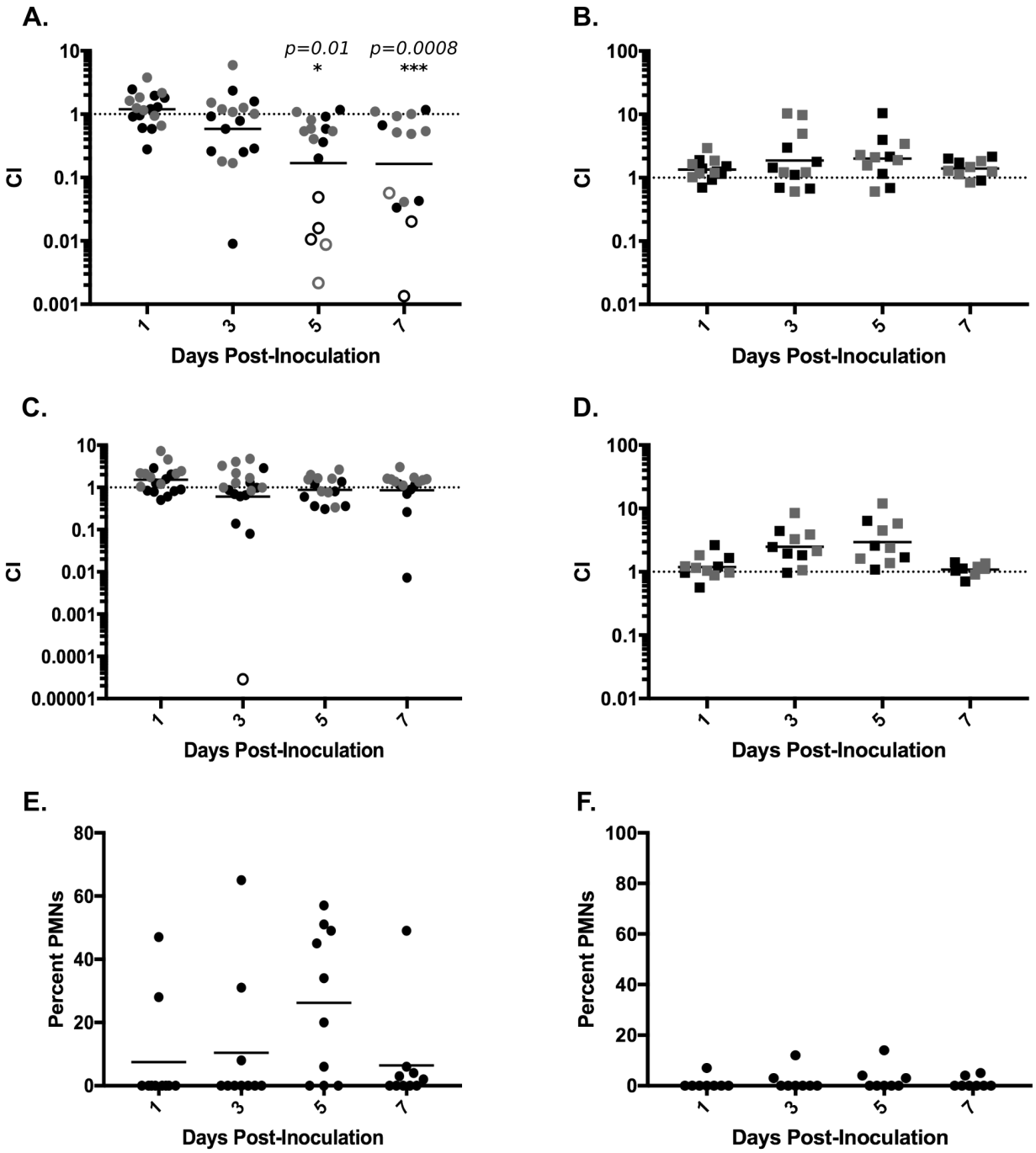

Figure 8. *In vivo* competitions of WT and RS8 or RS8.2 bacteria in BALB/c or C57BL/6 mice.

Similar numbers of WT and RS8 (A, B) or WT and RS8.2 (C, D) were inoculated vaginally in estradiol-treated BALB/c (A, C) or C57BL/6 mice (B, D). The relative recovery of either mutant or complemented mutant bacteria over the 7 day course of infection was determined by culturing equal volumes of vaginal swab suspensions on GC

agar with streptomycin (Sm) (total CFUs) and GC agar with Sm and kanamycin (Km) (mutant and complemented mutant CFUs). Results show the CI for each mouse on the respective day of infection. Horizontal bars indicate the geometric mean. The dotted line at CI=1 indicates equal fitness. A CI value <1 indicates decreased fitness relative to WT, whereas a value > 1 indicates increased fitness. Open symbols represent mice from which only WT bacteria were recovered; the limit of detection (1 CFU/ 100 uL of swab suspension) was used to calculate the number of mutant bacteria in these cases. Competitive infections were performed twice; the results of first experiment are shown in black, the second experiment is shown in gray. The percent of PMNs among 100 vaginal cells are shown for one WT vs. RS8 competitive experiment in BALB/c (E) and C57BL6 mice (F) and are representative of what was observed in all other experiments. For statistical analysis, the CI values for each group, WT vs. RS8 or WT vs. RS8.2, were log transformed and compared on each day of infection. A Mann-Whitney test was used to determine if significant differences between the CI values for each group were present.

The *thiO* mutant was more sensitive to ROS *in vitro* when starved for TPP. In order to explore the potential link between TPP biosynthesis and resistance to PMN-derived antimicrobial effectors, we used a disc diffusion assay to examine the effect of the *thiO* mutation on bacterial susceptibility to ROS. WT, RS8 and RS8.2 bacteria harvested from TPP-replete conditions showed no difference in susceptibility to H_2O_2 or paraquat (PQ) (Figure 9A, C). However, when bacteria were starved for TPP prior to exposure to PQ, RS8 exhibited a significant increase in susceptibility to paraquat in a dose-dependent manner compared to WT and RS8.2 (Figure 9D). A similar trend was observed with H_2O_2, although the results were not as striking (Figure 9B). Importantly, when the assay was performed using penicillin-G as a control, there were no differences in ROS susceptibility for bacteria grown under TPP-replete or starved conditions (Figure 9E, F).

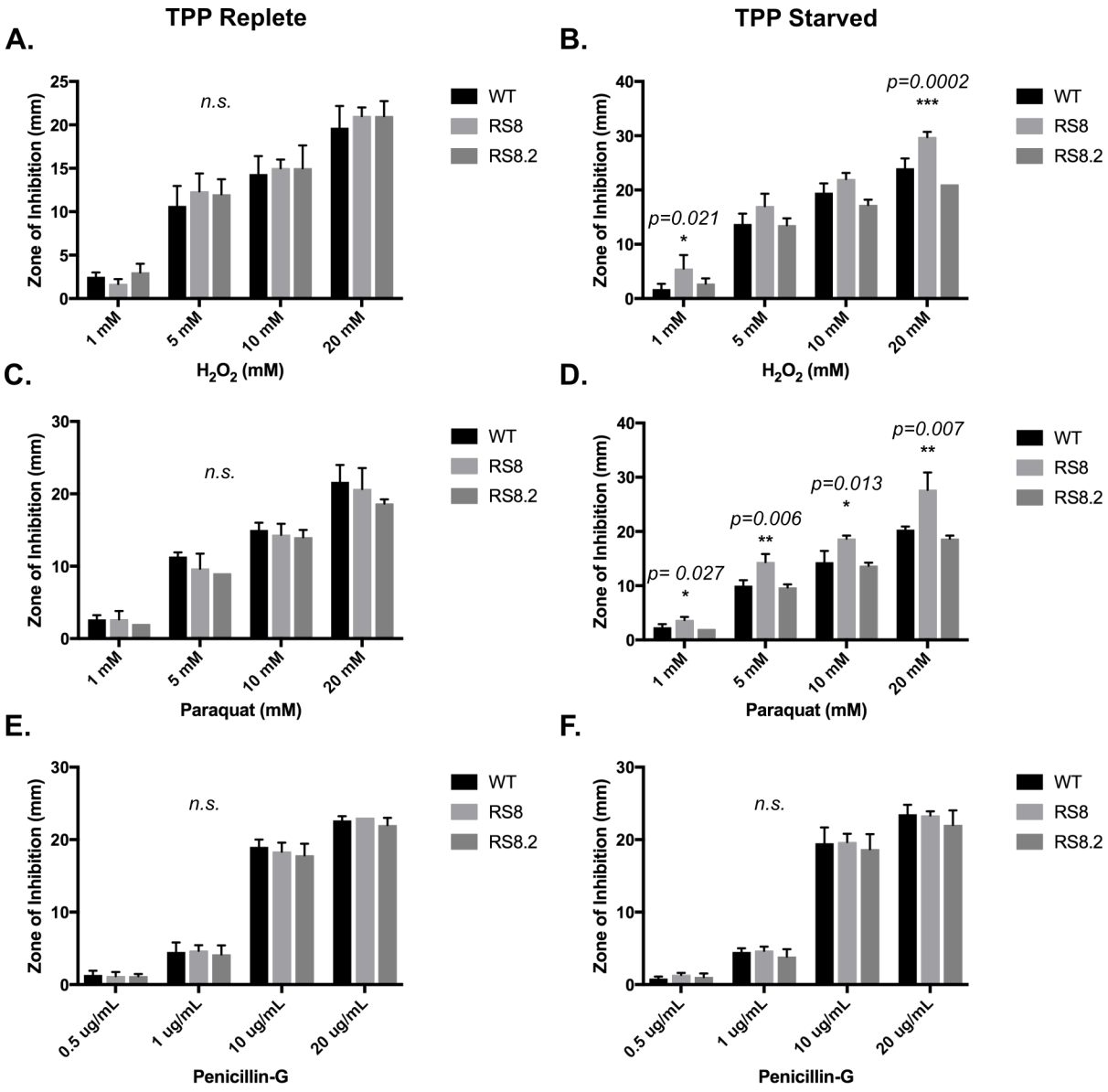

Figure 9. *In vitro* susceptibility of WT, RS8, and RS8.2 to hydrogen peroxide and paraquat.

Bacteria were grown under TPP-replete (A, C, E) or TPP-starved conditions (B, D, F), suspended in PBS to an OD_{600} of 0.1, and 100 uL were plated as a lawn onto GC agar. Filter discs soaked in increasing concentrations of H_2O_2 (A, B), paraquat (C, D), or penicillin-G (E, F) were placed on top of the lawn, and plates were incubated for 24 hours. Results show the average zone of inhibition from 3 independent experiments.

TPP-starved RS8 bacteria were more susceptible to PMN killing than WT or RS8.2 bacteria. To test the hypothesis that the inability to produce TPP increases susceptibility to the host inflammatory response, we performed PMN killing assays using WT, RS8 or RS8.2 bacteria harvested from TPP-replete or starved conditions. Bacteria were opsonized with normal mouse serum (NMS) prior to adding to the PMNs. There was no appreciable decrease in the number of bacteria recovered compared to the bacteria pre-incubated in heat-inactivated NMS after 45 min of incubation when the bacteria were grown under TPP-replete conditions. After TPP starvation, however, a significant decrease in the number of RS8 bacteria recovered was observed compared to WT and RS8.2 (Figure 10). To test whether the greater susceptibility of the RS8 mutant was due to PMN-derived ROS, we used PMNs from *gp91phox-* mice, which are unable to produce a phagocytic respiratory burst due to a mutation in the NADPH oxidase gene. We found that TPP-starved RS8 bacteria were equally vulnerable to Phox-deficient PMNs as to PMNs from C57BL6 mice, indicating that oxidative mechanisms of killing are not responsible for the observed phenotype.

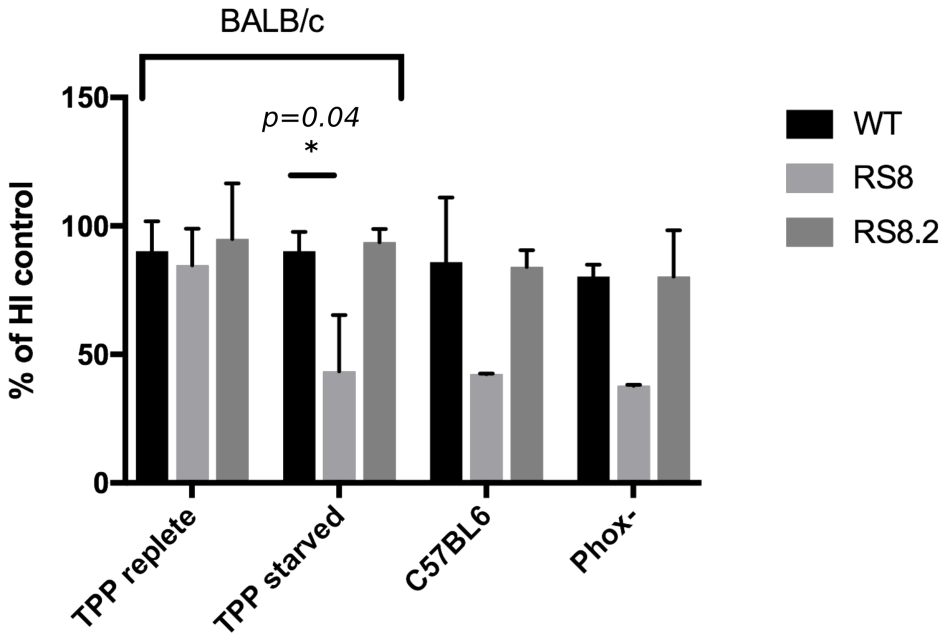

Figure 10. Susceptibility of WT, RS8, and RS8.2 to killing by murine-derived neutrophils.

Bacteria were harvested from GC or TPP- agar, incubated with NMS or HI-NMS, and incubated with PMNs. Experiments with BALB/c PMNs were performed a total of 3 times. For experiments in C57BL/J and Phox- mice, only bacteria harvested from TPP- agar were tested and the results of two independent experiments are shown. The mixture was incubated, with rotation, at 37°C. Samples were taken in triplicate after 45 minutes of incubation, serially diluted, and plated on GC + Sm agar to recover viable CFUs. Results shown represent the average CFUs recovered from NMS- treated samples as a percentage of the average CFUs recovered from HI-NMS treated control samples. A two-way ANOVA was used to detect significance. The difference between WT and RS8 under starved conditions was statistically significant at 45 minutes when tested with PMNs from BALB/c mice, but not C47BL6 or Phox- mice, although the trend observed was similar.

The *thiO* mutant RS8 was more sensitive to the antimicrobial peptide polymyxin B when starved for TPP. Following these results, we sought to examine whether non-oxidative mechanisms of killing could be involved in the increased sensitivity of RS8 to challenge by PMNs. We tested WT, RS8, and RS8.2 bacteria grown under TPP replete and starved conditions on a range of polymyxin B concentrations in GC agar. After 24 hours of incubation, TPP-depleted RS8 displayed increased susceptibility to polymyxin B compared to WT, RS8.2 and RS8 grown under TPP-replete conditions (Figure 11A). This trend continued after 48 hours of growth, with growth of TPP-starved RS8 routinely inhibited at lower concentrations of polymyxin B than the other strains under starved and un-starved conditions (Figure 11B).

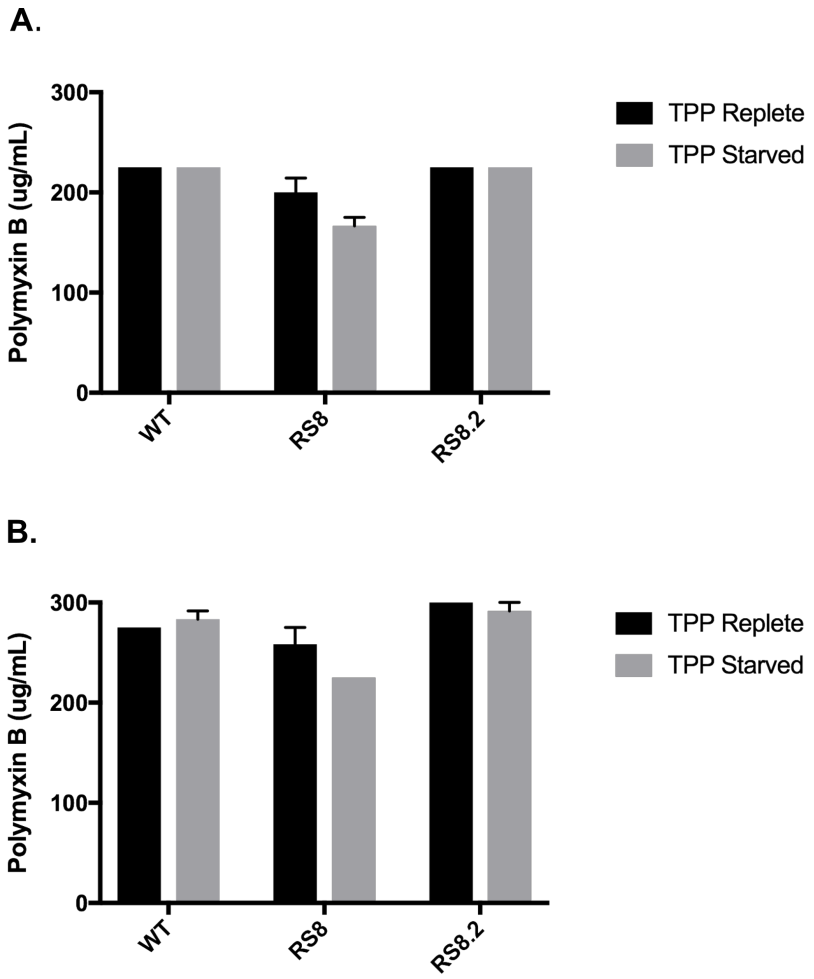

Figure 11. MIC values for WT, RS8, and RS8.2 grown under TPP-replete and depleted conditions.

Approximately $2x10^5$ CFUs of bacteria were spotted onto agar containing a range of polymyxin B concentrations. Plates were incubated at 37°C and the spots scored for growth at 24 (A) and 48 (B) hours to determine the MIC. The assay was performed 3 times, and results show the average MIC value recorded + SEM.

CONCLUSIONS

We conclude that *Neisseria gonorrhoeae* possesses the components of a *de novo* biosynthetic pathway required to produce thiamine pyrophosphate, and that this metabolite may have protective properties for Gc when challenged with a PMN influx and resulting oxidative pressure during infection.

DISCUSSION

N. gonorrhoeae is a highly successful pathogen that is capable of utilizing many resources in order to adapt to human mucosal surfaces. In the laboratory environment, Gc is a fastidious organism that requires special conditions such as increased CO_2 and a set of defined nutritional supplements in order to achieve optimal growth. Thiamine pyrophosphate is one component that has historically been added to solid and broth media for culturing Gc (88). This metabolite has important functions as a cofactor for many enzymatic processes that result in the production of ATP and NADPH, among other critical molecules. Most notably, TPP serves as a cofactor for the pyruvate dehydrogenase and alpha-ketoglutarate complexes in the Citric Acid Cycle, as well as for transketolase in the Pentose-Phosphate Pathway (11). Interestingly, it was previously determined that Gc relies primarily upon the Entnerr-Doudoroff and Pentose-Phosphate pathways in order to metabolize glucose (108). Therefore, we can reasonably deduce that TPP is important for the production of energy in dividing bacterial cells.

In this report, we show that Gc possess the components of a putative pathway of TPP biosynthesis. Functionality of the pathway was demonstrated by interruption of a key

glycine oxidase, *thiO*, which resulted in diminished colony diameter when grown in the absence of TPP. In broth cultures, prior starvation for TPP was necessary in order to observe inhibited growth kinetics of a *thiO* mutant in the absence of TPP. This finding suggests that Gc is capable of accumulating TPP from its growth environment, possibly through the process of active transport. In support of this hypothesis, we found that the *thiO* mutant could survive and replicate in the presence of picomolar levels of TPP. Our *in silico* analysis of the genome of strain FA1090 suggested that the components of the TPP transporter may be present in Gc.

For this study, we utilized the female mouse model of lower genital tract infection to test the capacity of the WT, mutant, and complemented mutant bacteria to establish infection. We found that the mutant strain was attenuated relative to WT on days 5 and 7 of infection, which corresponded to an increase in vaginal PMNs. TPP availability in the female lower genital tract has not been well described but is likely to be low given that TPP is primarily intracellular where it is bound as a cofactor. In addition, mammals do not store TPP in large quantities, and a constant supply is needed to support biological functions. It is possible that the attenuation of the mutant strain is due to nutrient starvation rather than selective pressure from the responding PMNs, however it is important to note that gonococcal TPP auxotrophs have previously been recovered from clinical samples, suggesting that enough TPP is available in the genital tract to support bacterial survival in the absence of TPP biosynthesis (25–27). Furthermore, the results of the competitive infections in C57BL6 mice displayed no difference in the fitness of the *thiO* mutant relative to the WT strain, in contrast to infections performed in BALB/c

mice. The C57BL6 mice did not experience an influx of vaginal PMNs during the course of infection, and therefore the implications of this result are striking.

From the results of the *in vivo* studies, we sought to examine the connection between PMN influx and the attenuation of the *thiO* mutant in greater detail. We found that prior TPP starvation was required to increase susceptibility of the *thiO* mutant to ROS and PMNs *in vitro* and *ex vivo*, respectively. Gc is capable of surviving the oxidative burst produced by infiltrating PMNs during infection, and the antioxidant defenses of Gc are multifactorial. The implication of TPP biosynthesis in resistance to oxidative damage in Gc represents an interesting addition to the many factors that are considered to be involved in resistance to oxidative stress. In fact, a previous study identified a gene encoding a putative thiamine binding and transport protein as upregulated in a screen assessing the transcriptomic response of Gc to hydrogen peroxide (161). In a TPP-starved *thiO* mutant, many cellular functions are likely to be negatively impacted. Exposure of *S. enterica* to paraquat results in a nutritional requirement for thiamine (166). This requirement was linked to the destabilization of Fe-S clusters in several thiamine biosynthesis proteins by the exposure to superoxide. In addition, we speculate that decreased intracellular TPP may interfere with the ability of the cell to produce NADPH via transketolase and the Pentose-Phosphate Pathway. NADPH in turn is required for the reduction of glutathione (GSH), an important endogenous antioxidant. However, given the complexity of these metabolic networks, it is unlikely that there is a specific mechanism by which TPP biosynthesis influences susceptibility to ROS.

In addition, we note that because PMN-derived reactive oxygen species (ROS) do not appear to challenge Gc, it is considered more likely that killing of Gc occurs by the non-oxidative mechanisms of PMN killing (75, 141). The results of the *in vivo* competitions may be due to the effects of these mechanisms, and the PMN challenge assays with Phox- cells support this conclusion. When we assessed susceptibility to the antimicrobial peptide polymyxin B we found that the *thiO* mutant displayed a lower MIC when starved for TPP, indicating that TPP biosynthesis may be more important for resistance to non-oxidative mechanisms of PMN killing. The Jerse laboratory has previously shown that the influx of PMNs to the genital tract of Gc-infected female BALB/c mice resulted in increased levels of several pro-inflammatory cytokines and chemokines, including MIP-2, which help to promote further inflammatory responses (120). The arrival of PMNs to the site of infections results in phagocytosis of bacteria, followed by the release of granules into the phagolysosome that contain many antimicrobial compounds (75). We hypothesize that the lack of attenuation of the *thiO* mutant in C57BL6 mice is therefore due to the failure of PMNs to arrive, phagocytose the bacteria, and deploy effector molecules, sparing the mutant from the selective pressure of the granule contents.

Finally, it is worth noting that the strain background in this study, FA1090, already has multiple auxotrophies (AHU, proline), and was originally isolated from a disseminated gonococcal infection (37, 77). Therefore, it is not necessarily representative of strains that are typically isolated from the urogenital tract. However, when the *thiO* mutation was moved into strain MS11 (originally from an uncomplicated lower genital infection), the same phenotype as strain RS8 was observed when grown on agar media lacking TPP

(data not shown). It seems possible then, that the importance of this pathway is conserved among gonococcal strains that may inhabit different niches in the human body.

In summary, we have demonstrated that Gc requires TPP in order to achieve optimal growth under *in vitro* conditions, and interruption of a *de novo* pathway of TPP biosynthesis results in attenuated fitness in a symptomatic model of infection, as well as increased susceptibility to sources of ROS and challenge by PMNs. However, we acknowledge that gonococcal resistance to oxidative mechanisms of killing are complicated and are likely to be influenced by several redundant factors. Furthermore, we found evidence that Gc may possess a system for the active transport of TPP from the growth environment, which would represent a system that has not previously been defined.

Acknowledgments

We gratefully acknowledge Dr. Cara Olsen for assistance with statistical analyses, Dr. Diana Downs for her helpful consultation on mechanisms of TPP biosynthesis, and Dr. Adriana LeVan for her help with the PMN killing assays. This work was supported by the National Institutes of Health grant ROI AI42053, Uniformed Services University of the Health Sciences intramural grant USU MIC-73 2493, and USU Student Award T073377116.

CHAPTER 4: A Transporter for Thiamine Pyrophosphate or Related Derivatives is Active in *Neisseria gonorrhoeae*, and is Critical for Experimental Genital Tract Infection in the Absence of a Functional Thiamine Biosynthesis Pathway

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ABSTRACT

Thiamine pyrophosphate (TPP) is an essential cofactor for several critical cellular processes. In prokaryotes, biosynthetic pathways for TPP have been described. Complementary to these pathways are TPP transportation systems that can be important for adaptation to different environmental conditions. *Neisseria gonorrhoeae* (Gc) is a strict human pathogen that is capable of colonizing diverse niches. We recently identified the TPP biosynthesis pathway in Gc. In this study, we identified the components of a potential TPP transporter and created a TPP-binding subunit (ThiB) mutant to assess functionality in Gc. Interruption of this single component failed to produce an *in vitro* phenotype, however, the gonococcal *thiB* gene complemented a TPP-transport deficient *Salmonella* mutant. A putative transporter permease component (*thiP*) failed to complement *Salmonella*, and identification of the other transporter components is incomplete. When the *thiB* mutation was combined with a mutation in *thiO*, an essential

biosynthesis enzyme, the resultant strain required high amounts of TPP in order to survive and was not infectious in mice.

Interestingly, the *thiO* mutant could use TPP and thiamine monophosphate (TMP), but not thiamine B1 for growth, suggesting deficiencies in the network of TPP salvage genes. In addition, we investigated a possible pyrimidine moiety (HMP) transporter that appeared to be linked to an operon containing TPP biosynthesis genes, but were unable to confirm functionality of this transporter in Gc. We conclude that a TPP transporter is present and active in Gc, and is important for survival in biological niches that may have low levels of nutrient availability.

Note: Experimentation involving the creation of and *in vitro* growth analyses of the gonococcal transporter mutants was conducted by R. Sennett. Complementation of *Salmonella* and analysis of GC medium base with *Salmonella* TPP biosynthesis mutants was performed by J.L. Irons and D.M. Downs. Competitive genital tract infections of female mice were performed by A. LeVan.

INTRODUCTION

Neisseria gonorrhoeae (the gonococcus, or Gc) is the causative agent of the sexually transmitted infection gonorrhea and an extremely successful pathogen. Gc is restricted to humans and has no outside animal or environmental reservoir, although it is capable of colonizing multiple different niches within the host (170). Gc preferentially colonizes mucous membranes, such as those of the lower urogenital tract. However, if an infection

is allowed to progress untreated, ascending infections can occur that affect the organs of the upper reproductive tract (70). Additionally, Gc can colonize the pharynx and the rectum, and can also be the cause of neonatal conjunctivitis. Gc also has intracellular niches, including epithelial cells and neutrophils (75). In a low percentage of cases, infections can disseminate to the bloodstream, skin and joints (70).

Gc shares mucosal niches with many other bacterial species, and can survive in a number of diverse environments in the human body (173). Therefore, it is possible that the capacity to obtain nutrients from the host environment through active transport is key for facilitating Gc survival during infection. Transporters of essential nutrients are diverse and are widespread in pathogenic and non-pathogenic microorganisms (130), (128). These systems of are often important for assisting bacteria survival under difficult or unique growth conditions. For example, the intracellular bacterium *Chlamydia trachomatis* can obtain biotin through a complex system involving host and bacterial transporters that enables bacterial survival within inclusion vacuoles in the host cell (49).

Thiamine pyrophosphate (TPP) is an essential micronutrient that plays a critical role as a cofactor for several enzymes that are involved in important cellular processes (11) (39). There is also evidence that TPP is a scavenger of reactive oxygen species (119), (167). In prokaryotes, TPP can be produced *de novo* through a complex, multistep pathway of biosynthesis. This pathway involves the formation of two separate moieties, a pyrimidine and a thiazole, which are condensed to produce thiamine monophosphate (TMP), which is then phosphorylated to produce the active cofactor TPP (11). In addition to this

pathway, bacterial mechanisms for the active transport of TPP from the growth environment have been described. These transporters can be equally as important as *de novo* biosynthesis for survival and adaptation to the host. For example, *Listeria monocytogenes* requires a functional TPP transport system to successfully replicate in host cell cytoplasm (97) (139).

The ABC transporter ThiBPQ, originally described in enteric bacteria, is a well-described system of TPP uptake. This transporter consists of three discrete subunits: the TPP-binding subunit, ThiB; the permease or transmembrane channel, ThiP; and the ATP-binding subunit, ThiQ (177). In *Salmonella*, ThiBPQ transports thiamine (B1), TPP, and possibly TMP, making it an extremely versatile system (177). Other mechanisms for the importation of TPP and its esters have been described. These include energy-coupled modular transporters (energy-coupled factors, ECFs) in *Bacillus subtilis* and *Lactobacillus casei*, which feature a conserved transmembrane component and bind specific substrates with a small integral membrane protein (128). A homolog of the thiamine-binding protein of these systems, ThiT, is present in *Listeria monocytogenes* and *Lactococcus lactis* (139) (43).

We recently reported that Gc appeared to have the components of a functional TPP biosynthesis pathway, and showed that interruption of this pathway through inactivation of the essential glycine oxidase gene *thiO* resulted in inhibited growth under TPP-depleted conditions (Chapter 3). Growth of the *thiO* mutant responded to exogenously provided TPP in a dose-dependent manner, and was capable of surviving at

concentrations of TPP as low as 100 pM. The ability of the *thiO* mutant to replicate under such TPP-restricted conditions suggests the presence of an active transport system. The isolation of thiamine and TPP auxotrophs from genital tract infections also suggests the presence of transport and salvage systems of TPP or precursor molecules (26) (25). However, whether Gc possesses a system capable of transporting TPP from its growth environment has not yet been examined.

Here we hypothesized that a thiamine transporter is active in Gc, and is important for adaptation to and colonization of the genital tract. Using *in silico* analysis, we identified the gene encoding a likely substrate-binding component, ThiB, of a putative TPP transporter, and confirmed its role in TPP transport by heterogenous complementation of *Salmonella* with the Gc *thiB* gene. We also evaluated the ability of a *thiB* mutant and a *thiB*, *thiO* double mutant to grow in the absence of exogenous TPP, and to establish lower genital tract infection in female mice. Finally, we defined whether Gc could utilize different substrates to salvage growth under TPP-depleted conditions, and analyzed GC medium base for TPP precursor molecules that may help to support growth of TPP-auxotrophic strains. Our data indicate that a TPP transporter is present and assembled in Gc, and in the absence of thiamine biosynthesis, this transporter is critical for establishing gonococcal genital tract infection.

RESULTS

Several *Neisseria* species possess the substrate-binding component of a putative TPP transporter. The ThiBPQ transporter was identified in *S. typhimurium*, where it is

responsible for the transport of thiamine pyrophosphate (177). A similar three-gene operon is present in *E. coli*, previously designated *sfuABC*, that corresponds to the ThiBPQ locus in *Salmonella* (177). Expression of this operon is controlled by a TPP-responsive riboswitch that suppresses transcription in the presence of exogenous TPP (178) (106).

The putative TPP-binding component gene *thiB* (NGO_2056) was identified in the genomic sequence of the laboratory Gc strain FA1090. We also identified this gene in strain MS11, and comparative genomic analysis had previously identified the same *thiB* homolog in *Neisseria meningitidis* (130). NGO_2056 displays 32% amino acid identity with 87% coverage compared to the ThiB subunit of *E. coli*. Unlike the ThiBPQ systems identified in *E. coli* and *Salmonella*, the gonococcal *thiB* candidate was identified as a single gene, not in any apparent operon, and with no preceding Thi-element riboswitch (125). Attempts to locate candidates for the other transporter components, ThiP (permease subunit) and ThiQ (ATP-binding subunit) were less successful. NGO_2078 was identified as a possible *thiP* homolog, but was not as comparable to the *E. coli* subunit (33% amino acid similarity with 36% coverage) as the putative *thiB*. A strong candidate for the ThiQ subunit was not identified in Gc.

Inactivation of *thiB* did not attenuate Gc growth in the absence of TPP supplementation. In order to test the functionality of the putative transporter components, we interrupted the sequence of NGO_2056 with a chloramphenicol (Cm) resistance-encoding cassette to create the *thiB* mutant strain RS6 as described in

Materials and Methods. The same procedure was used to insertionally inactivate NGO_2078 to create the putative *thiP* mutant RS7. We then tested these mutants for the ability to grow on GC agar media with TPP excluded from Kellogg's supplement I (TPP- media) compared to the wild-type (WT) FA1090 parent strain and the corresponding complemented mutant strains RS6.2 and RS7.2. Neither RS6 nor RS7 displayed a difference in colony diameters compared to WT bacteria or the complemented strains when grown on TPP- agar (Figure 12A and RS7 data not shown). In broth cultures, RS6 grew similarly to WT bacteria in the presence or absence of added TPP (Figure 12B). The numbers of CFUs for WT, RS6, and RS6.2 recovered from broth with and without TPP were also similar (Figure 12C). In addition, bacteria that were harvested from TPP- agar prior to the inoculation of broth cultures lacking TPP did not display inhibited growth (data not shown). The lack of growth defects in RS6 and RS7 when cultured in the absence of TPP is likely due to the presence of a functional system of *de novo* TPP biosynthesis, which we previously investigated (Chapter 3).

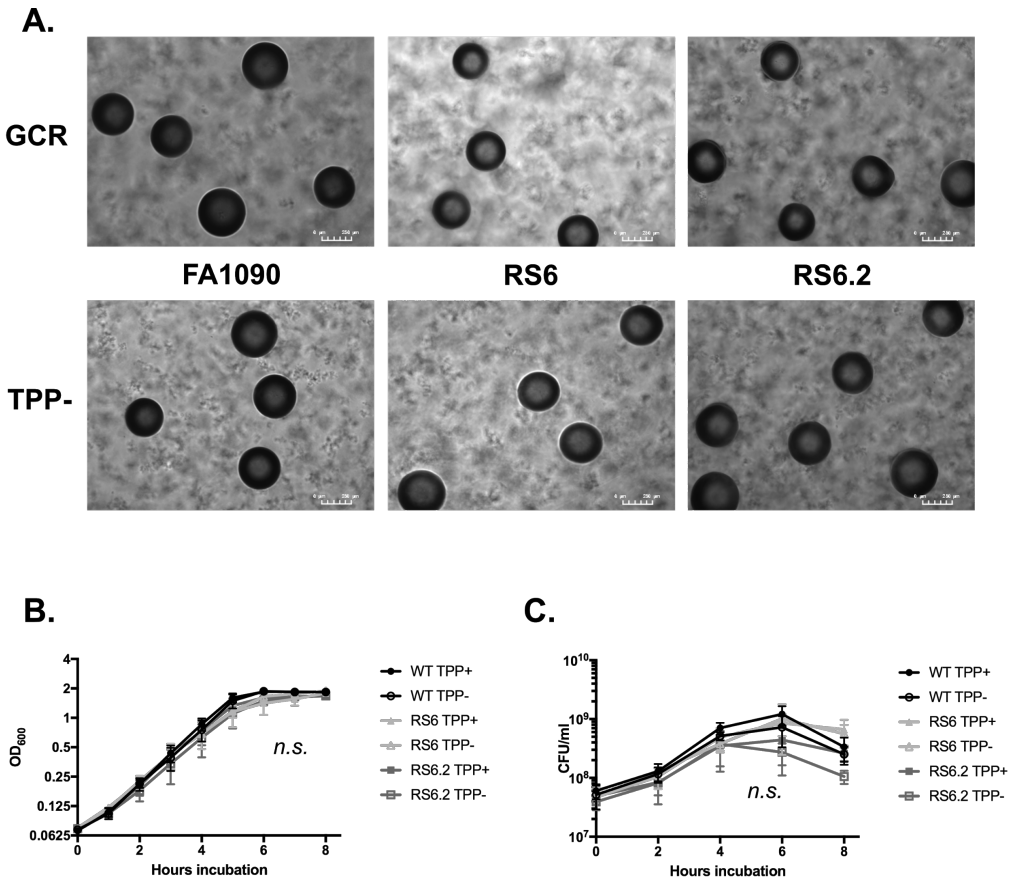

Figure 12. Growth of WT, RS6, and RS6.2 in TPP-supplemented or un-supplemented culture conditions.

WT, RS6, and RS6.2, bacteria were grown on TPP-supplemented agar (GCR) and un-supplemented agar (TPP-) for 24 hours before imaging (A). There were no significant differences in the colony diameters of the three strains on the un-supplemented medium. Bacteria were also harvested from GC agar plates and used to inoculate cultures of GC broth that either contained or lacked TPP (B). Cultures in which TPP was present are indicated by filled symbols, and cultures where TPP was absent are indicated by open symbols. Shown are the averages for OD_{600} measurements (B) and recovered CFUs (C) of three independent experiments, ± SEM.

Expression of the gonococcal *thiB* rescued growth in a TPP transport-deficient mutant of *Salmonella enterica*. We therefore sought to examine functionality of the putative ThiB and ThiP components by heterogenous complementation of a TPP transport-deficient strain of *Salmonella enterica*. The gonococcal *thiB* and *thiP* genes were cloned into an inducible plasmid vector and transformed into a THZ-auxotrophic strain of *Salmonella enterica* that is also deficient in TPP transport due to mutations in *thiB* or *thiP*. The strains were then tested for their ability to grow on minimal medium in the presence of TPP, the THZ moiety, or thiamine B1. Provision of either the THZ moiety or thiamine B1, which can be imported through another transporter, in the minimal medium will allow for the growth the *Salmonella* mutant; in contrast, TPP will only rescue growth if the strain is producing a functional ThiBPQ transporter. As shown in Figure 13, expression of the gonococcal *thiB* gene in the *Salmonella thiB* mutant allowed growth in the presence of TPP, indicating that it can perform as the substrate-binding component of a functional transporter. The putative gonococcal *thiP* gene, however, did not complement the *Salmonella* permease mutant (data not shown).

As a negative control, *Salmonella* carrying the empty complementation vector was tested in parallel on THZ, TPP, and B1 media, and as expected, the negative control grew on medium with THZ but not with medium containing TPP or B1 (Figure 13). As a positive control, an HMP-auxotrophic strain with a wild-type ThiB was also tested on the different media types. This strain can grow in the presence of TPP or B1, but cannot grow on the minimal medium containing only THZ, as its nutritional requirement is solely for the other moiety, HMP, which was the observed phenotype. We conclude that Gc ThiB

can substitute for the *S. enterica* ThiB protein to make a functional transporter for TPP, but the Gc ThiP protein cannot substitute for *S. enterica* ThiP.

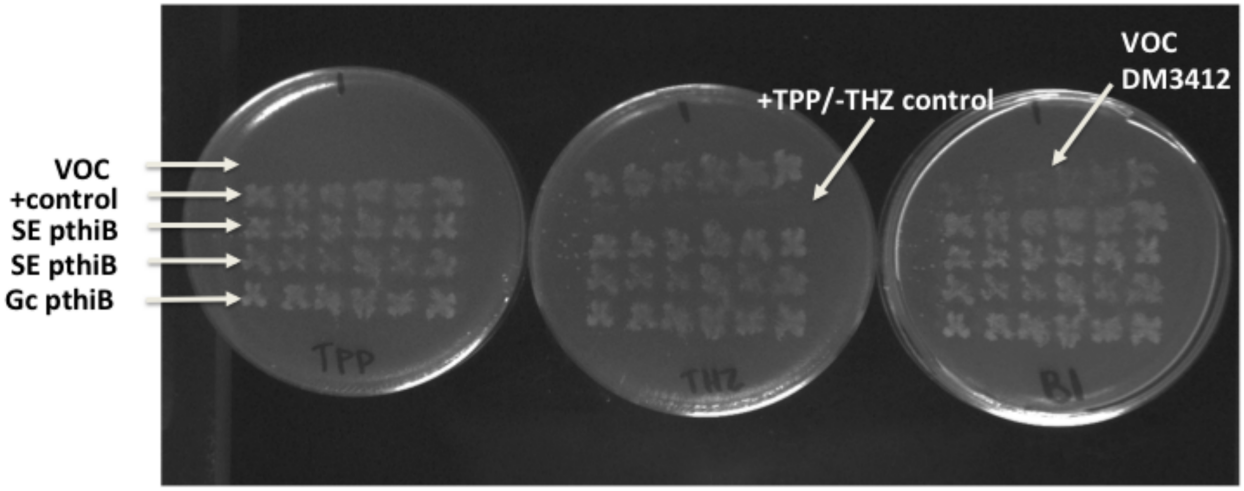

Figure 13. Complementation of TPP-transport deficient *Salmonella enterica* with the gonococcal *thiB* gene.

The gonococcal *thiB* gene was cloned into a plasmid and transformed into a TPP-transport and THZ-biosynthesis mutant of *S. enterica* (labeled Gc pthiB in the figure). The transformants were then tested on minimal media containing thiamine B1, TPP, or THZ. *Salmonella* carrying an empty vector (vector only control; VOC) was tested, as was *Salmonella* with the WT *Salmonella thiB* gene in order to ensure that complementation *in trans* could occur (SE pthiB). Additionally, an HMP *Salmonella* auxotroph was evaluated as a positive control (+control). Work performed by Jessica Lynn Irons in the laboratory of Dr. Diana Downs at the University of Georgia, Athens (USA).

Thiamine monophosphate rescued growth of a TPP-starved *thiO* mutant, but thiamine B1 could not be utilized. We then investigated the specificity of other substrates that can be potentially transported and utilized by Gc. The *thiO* mutant RS8, which is deficient in *de novo* biosynthesis of TPP, was starved for TPP before inoculating cultures containing serial dilutions of either thiamine monophosphate (TMP) or un-phosphorylated thiamine (B1). Cultures contained a range of concentrations from 10 nM to 10 pM of either substrate. Growth of TPP-starved RS8 was rescued by the addition of 10 nM TMP to the culture, but subsequent dilutions were only partially successful in restoring growth (Figure 14A). In contrast, none of the concentrations of thiamine B1 tested were able to support growth (Figure 14B). This result suggests that thiamine B1 may not be a substrate for ThiB, that it cannot be utilized by TPP salvage enzymes in Gc, or that the necessary components of a B1 salvage system are not present in Gc. We conclude that Gc has the capacity to transport TPP and the monophosphorylated ester TMP from its growth environment. Transport appears to be mediated by the ThiBPQ transporter system, but only the substrate-binding component ThiB has been identified at this time.

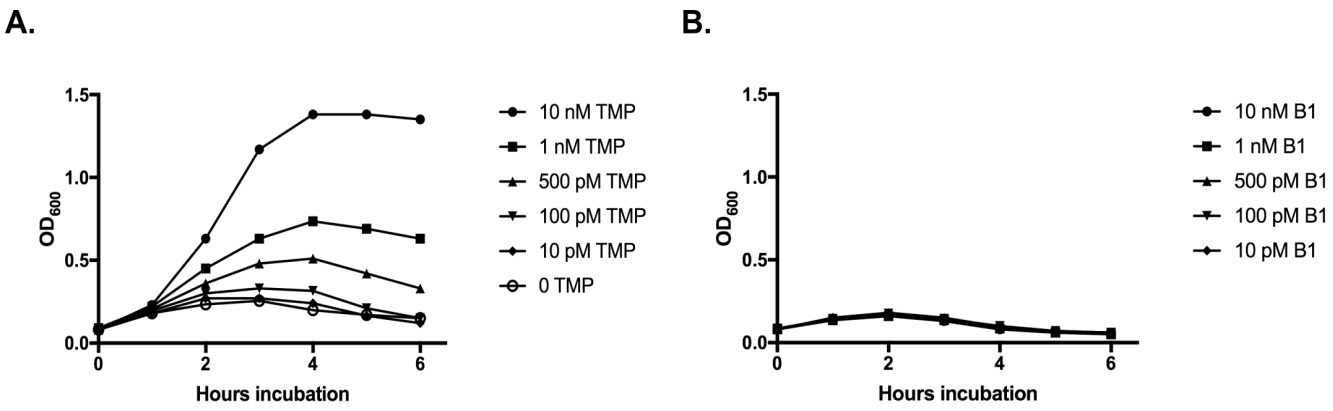

Figure 14. Thiamine monophosphate (TMP) and thiamine B1 usage in the gonococcal *thiO* mutant RS8.

RS8 bacteria were starved for TPP by growth in mini-cultures lacking TPP prior to the inoculation of large GC broth cultures. TMP (A) and B1 (B) stock solutions were titrated into the growth medium to measure the effectiveness of the different substrates for rescuing growth of the mutant strain in a dose-dependent manner.

Presence of the thiazole and pyrimidine moieties were detected in GC medium base, but a putative HMP transporter does not appear to be active in Gc. The capacity of Gc to utilize TMP suggests that other substrates could also be imported and recycled into the biosynthesis pathway to support bacterial growth. In addition, the ability of RS8 to survive repeated passages on TPP- led us to speculate that residual TPP or thiamine derivatives are present in GC medium base, which may also explain the successful growth of RS6 in GC media without added TPP. GC medium base consists in part of Proteose Peptone No.3, an enzymatic digest of porcine protein that can contain varying amounts of nutrients and vitamins. In order to examine whether TPP or any of its esters are present in the GC medium, a sample of GC Medium Base (BD Difco, DF0289-17-3) was reconstituted and evaluated as a growth medium for a number of *Salmonella* strains with mutations in the TPP biosynthetic pathway, specifically in the *thiC*, *thiH*, *thiE* or *thiL* genes. ThiC is involved in an early step in the formation of the pyrimidine moiety HMP, and ThiH (replaced by ThiO in *Bacillus* and Gc) is involved in the formation of the thiazole moiety THZ. ThiE and ThiL are active at the end of the pathway and are responsible for condensing the moieties together to form TMP, and phosphorylating TMP to produce TPP, respectively. We found that broth containing 0.2% of the GC medium base restored growth of a *thiC* mutant to the same level as 100 nM thiamine B1 (Figure 15A). A concentration of 0.02% BD medium supported growth better than glucose alone in the cultures (minimal conditions), but was not as effective as the 0.2% condition. These results were suggestive of the presence of TPP or thiamine B1 in the GC medium base. With further analysis, we found that supplementation of agar plates with 0.2% GC medium base allowed growth of a *thiC* and *thiH* mutant, indicating the presence of HMP

and THZ, but not the *thiE* or *thiL* mutants, which have a requirement for TPP or B1 (Figure 15B). From these results, we conclude that the HMP and THZ moieties, rather than TPP or B1, are present in the GC medium base and can be used to support the growth of bacterial mutants defective in TPP moiety biosynthesis.

Initially, sequence analysis indicated that NGO_2009, which is found directly upstream of and is transcribed in the same direction as *thiO* in FA1090, is a putative HMP transporter named *cytX*. This putative transporter was originally identified based on similarity to the *codB* cytosine permease in *E. coli*, and orthologs have been found in *Mannheimia haemolytica* and *Thermoanaerobacter* (130). NGO_2009 had 28% amino acid similarity with 77% coverage to CodB in *E. coli*. We therefore hypothesized that interruption of this sequence, combined with a mutation in the HMP biosynthesis gene *thiC*, would result in cell death in the absence of exogenous TPP. To test this hypothesis we inactivated NGO_2009 (*cytX*) with a kanamycin-resistance encoding cassette and *thiC* with a chloramphenicol resistance gene, and created a *cytX* single (RS12) and *thiC*, *cytX* double mutant (RS13) as described in the Materials and Methods (Table 1). However, when these strains were grown on TPP- agar they did not display an inhibited growth phenotype (Table 1), indicating that this transporter does not appear to be necessary for the acquisition of HMP from the growth medium. This result was perhaps surprising based on the homology and location of NGO_2009, as it appears to be the first gene in the operon containing *thiO* and other biosynthesis genes. The function of NGO_2009 is not clear and the mechanism which enables Gc to grow on media containing only HMP and THZ is not known. No other transporters specific for the TPP moieties have been

identified as of yet in Gc, suggesting that the gonococcal ThiB component may be able to mediate the transport of HMP or THZ from the environment in addition to TPP. We conclude that Gc may be able to import TPP intermediates to rescue growth when the biosynthesis pathway is inactivated, but the specific mechanism by which this occurs is still unclear.

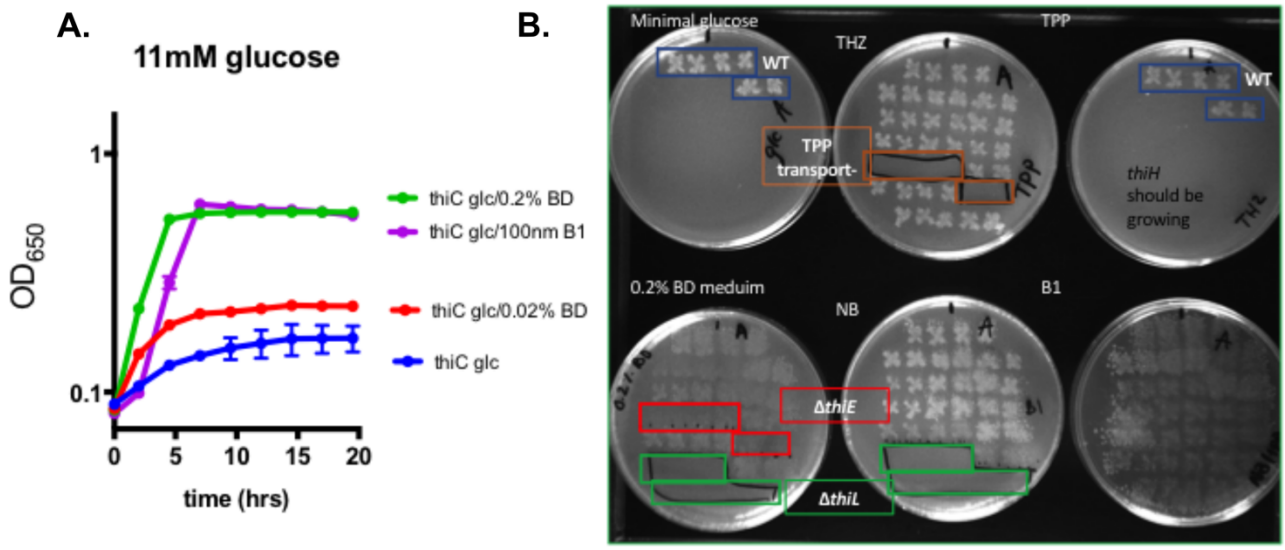

Figure 15. Analysis of GC Medium Base for the presence of the thiazole (THZ) and pyrimidine (HMP) moieties of TPP.

GC Medium Base powder was reconstituted, diluted, and evaluated for its ability to rescue the growth of several *Salmonella* TPP biosynthesis and transport mutants. Analysis was done in liquid cultures (A), with a *thiC* mutant defective in HMP production, and on agar media (B) with the other strains tested. Work performed by Jessica Lynn Irons in the laboratory of Dr. Diana Downs at the University of Georgia, Athens (USA).

A gonococcal *thiB*, *thiO* double mutant did not grow in the absence of TPP and required high concentrations of exogenous TPP for growth *in vitro*. We next sought to further confirm functionality of the gonococcal ThiB with the creation of a *thiB*, *thiO* double mutant in strain FA1090. The *thiB::cat* gene from RS6 was transformed into the *thiO* mutant RS8, and transformants were selected on Gc agar containing chloramphenicol and a high concentration of TPP (1 mM, GC+ agar). The presence of the mutated *thiB* and *thiO* genes in one transformant (RS9) was confirmed by PCR analysis. The RS9 mutant required 1 mM TPP to achieve normal growth (Figure 16A), and was unable to grow on GC agar plates without added TPP (TPP- agar, Figure 16C). When cultured on solid GCR agar, which contains ~4.3 uM TPP, the RS9 mutant produced colonies with drastically reduced diameters (Figure 16B).We then examined the growth of RS9 bacteria in GC broth to which increasing concentrations of TPP were added. The RS9 mutant exhibited normal growth kinetics in the presence of 1 mM TPP, but in the presence of 100 uM TPP or lower concentrations the mutant bacteria showed a brief logarithmic phase and did not reach the same absorbance readings (Figure 16D). With these results, we confirmed the functionality of the product of the gonococcal *thiB* gene, and conclude that this component is required in Gc to mediate transport of TPP from the growth medium.

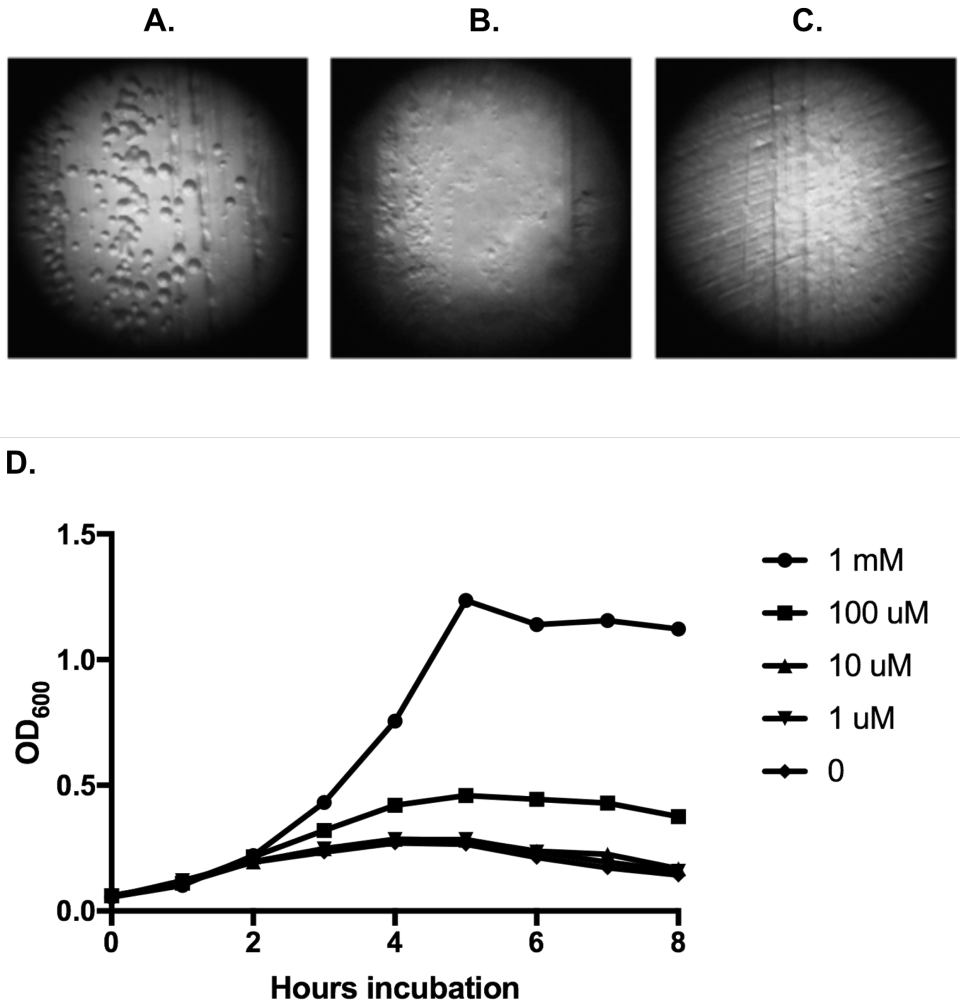

Figure 16. Growth of the *thiB*, *thiO* double mutant RS9 in the presence of decreasing concentrations of exogenous TPP.

The interrupted *thiB* gene from RS6 was transformed into the *thiO* mutant RS8 to create strain RS9. RS9 was grown on GC+ (A), GC (B), and TPP- (C) agar to determine the nutritional requirements of the double mutant. Cultures of RS9 were titrated with 1 mM to 1 uM of TPP in ten-fold dilutions, and the OD_{600} was assessed every hour (D).

The *thiB* mutant RS6 showed no attenuation in the murine lower genital tract, but the *thiB*, *thiO* double mutant RS9 was unable to establish infection. We previously showed that inactivation of the *thiO* gene in strain FA1090 was accompanied by a fitness cost during experimental murine infection, but that an active *thiO* gene and the capacity to produce TPP was not required for infection. In order to examine the importance of TPP transport for adaptation in this surrogate model of female lower genital tract infection (73), we performed competitive co-infections with WT, RS6 and RS9. Female BALB/c mice were inoculated intravaginally with an equal mixture of either RS6 and WT bacteria, or RS9 and WT bacteria. Vaginal swabs were then cultured onto selective and non-selective agar every other day for seven days, and the competitive index (CI) for each mouse at each time point was calculated as described in the Materials and Methods. On the first day of culture, RS6 appeared to demonstrate reduced fitness relative to the WT strain (mean CI = 0.14), but subsequently recovered to be equally fit as WT for the remaining days of culture (Figure 17A). We hypothesize that the early attenuation of the *thiB* mutant observed may be due to nutritional deficiencies before activation of the biosynthesis pathway. In contrast, mutant RS9, which is deficient in both TPP transport and biosynthesis, was highly attenuated relative to the WT FA1090 (Figure 17B). In fact, RS9 was not recovered from mice on any days of culture, suggesting that this strain is incapable of establishing infection. To test this hypothesis, we conducted non-competitive infections in BALB/c mice. The double mutant was not recovered on any of five consecutive days of culture from any of the five mice inoculated with the mutant, compared to high numbers of WT gonococci recovered from one mouse tested in parallel (data not shown). We conclude that ThiB-mediated TPP transport is not critical for

infection when TPP biosynthesis can occur, but when biosynthesis is impaired the presence of a functional TPP transporter becomes essential for genital tract infection.

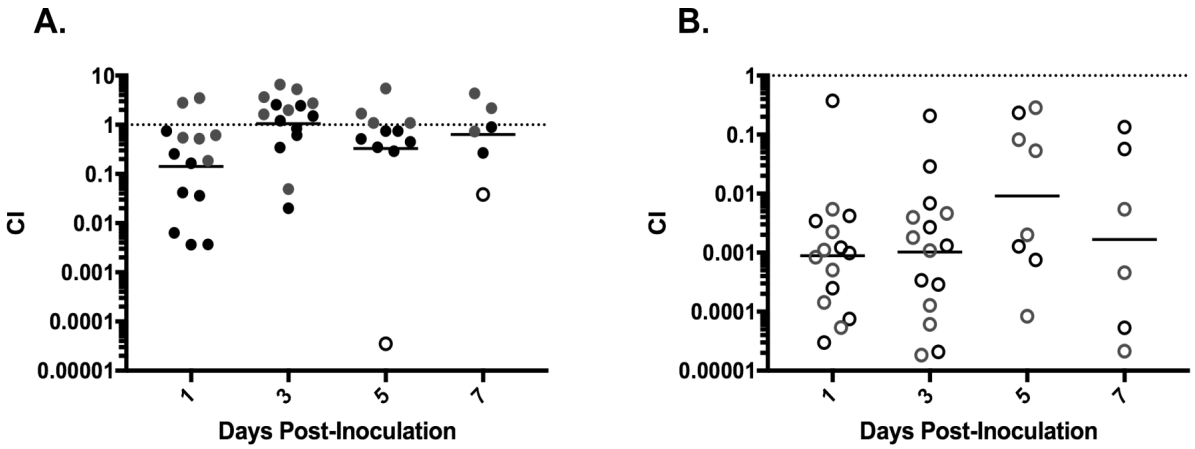

Figure 17. *In vivo* competitive infections of WT and RS6 or RS9 in female BALB/c mice.

WT and RS6 (A) or RS9 (B) gonococci were combined in equal numbers and used to inoculate the lower genital tract of estradiol-treated female BALB/c mice. Relative recovery of the mutant strains compared to WT over the course of infection was determined using GC agar with streptomycin (Sm) (total CFUs) and GC agar with Sm and chloramphenicol (Cm) (mutant CFUs). For infections containing RS9, GC+ agar containing the appropriate antibiotics was used. Results show the CI calculated for each mouse on the respective day of infection. Horizontal bars indicate the geometric mean. The marker at CI=1 indicates where WT and mutant bacteria were equally fit. A CI value below this line indicates decreased fitness relative to WT, whereas a value greater than 1 indicated increased fitness. Open symbols represent mice from which only WT bacteria was recovered; the limit of detection (1 CFU/ 100 uL of swab suspension) was used to calculate the number of mutant bacteria in these cases. Competitive infections were performed twice, the results of first experiment are shown in black, the second experiment is shown in gray.

CONCLUSIONS

TPP transport is critical for survival in the host when combined with deficiencies in the *de novo* production of TPP, suggesting that the ability of Gc to import TPP is an important step in adapting to and establishing infection in the host.

DISCUSSION

Gc is a highly adaptable pathogen capable of colonizing a variety of mucosal surfaces in the human host. Mechanisms of nutrient biosynthesis and transport are increasingly being recognized as critical elements of a pathogen's capacity to adapt to a host and successfully replicate under difficult or austere conditions. We previously described the presence of a biosynthesis pathway for the essential cofactor TPP in Gc. However, less is known about the capacity of Gc to import such necessary nutrients from its growth environment. In our previous study, we noted that Gc was able to replicate in the presence of very low levels of exogenously provided TPP, suggesting the presence of a system of active transport. Furthermore, investigation of the nutritional deficiencies of clinical Gc isolates have shown that TPP auxotrophs can survive in the genital tract, providing more evidence that active transport can occur.

In this study, we identified one critical component, ThiB, of a potential ThiBPQ TPP transporter in strain FA1090 of Gc. A *thiB* mutant strain did not display a phenotype when grown in the absence of TPP on agar and in broth cultures. However, functionality of this component was demonstrated by heterogeneous complementation in *Salmonella*. Furthermore, the construction of the gonococcal double mutant RS9, deficient in both

TPP transport and biosynthesis, showed that interruption of both systems resulted in highly attenuated growth and the requirement for high levels of exogenous TPP.

When these mutant strains were competed with WT bacteria in the female mouse model of Gc genital tract infection, the *thiB* mutant demonstrated a slight fitness disadvantage on the first day of culture. The attenuation subsequently resolved, with the *thiB* mutant ultimately displaying equal fitness to the WT strain for the remaining days of culture. It was previously shown that operons containing the biosynthetic *thi* genes are controlled by a TPP-responsive riboswitch element that can represses expression transcriptionally or translationally when TPP is available (106). The *thiB* sequence we identified is not preceded by any riboswitches and is presumed to be constitutively expressed, but the operon containing *thiO* does appear to be under the control of a TPP-responsive riboswitch element (125). Therefore, we hypothesize that this early fitness defect in the *thiB* mutant may be due to a delay in transcription of the genes for *de novo* biosynthesis as all strains were cultured on TPP-replete media prior to inoculation of mice.

The *thiB*, *thiO* double mutant showed a very different phenotype in mice. The double mutant was not recovered on any days of culture and was clearly unable to establish infection. It is not clear what the TPP availability of the murine genital tract might be, however, the requirement of 1 mM TPP for the *in vitro* culture of the double mutant is well beyond what physiological levels are likely to be based on the analysis of TPP content of human gynecologic tissues (51). In addition, we recently showed that a single *thiO* mutant is capable of surviving in mice in the absence of pressure from invading

PMNs (Chapter 3). These results reinforce the importance of possessing either a functional TPP transport or biosynthesis system for pathogenesis in Gc.

The evolution of transport systems may be driven by the available substrate repertoire in different environmental niches (78, 86, 134, 135). While our data clearly show that sufficient TPP is available in the murine lower genital tract for Gc to thrive and establish infection, other substrates may be present in the different body sites infected by Gc. In addition, TPP or intermediate biosynthesis products may be available within epithelial cells or neutrophils. In the murine model of genital tract infection utilized in this study, gonococci are mostly extracellular due to several host restrictions in known invasion receptors (133, 137). We therefore attempted to examine the importance of the ThiB transporter in the intracellular environment of ME-180 cervical epithelial cells. However, technical shortcomings prevented us from being able to test this hypothesis. The *thiB* mutant did not display any defects in the cell adhesion and invasion assay, but the *thiB*, *thiO* double mutant was unable to survive long enough in the assay medium to be evaluated (data not shown). Therefore, we could not determine whether stores of TPP or related compounds within host cells can be accessed by Gc within intracellular niches.

While assessing the ability of Gc to utilize substrates other than TPP, we found that a TPP-depleted *thiO* mutant could survive in the presence of TMP, but not thiamine B1. This is likely due to the presence of a functional ThiL gene in Gc (NGO_1575), which completes the biosynthetic pathway by phosphorylating TMP to the active cofactor TMP. Providing TMP in the culture media therefore circumvents the defects in thiazole

synthesis caused by the mutation in *thiO*. However, the inability of B1 to rescue growth suggests that there are gaps in the network of TPP salvage enzymes in Gc. In *E. coli* and *Salmonella*, salvage can occur at three main points. ThiM can salvage production of the thiazole moiety, ThiD works to recycle pyrimidine moiety precursors, and ThiK can convert thiamine B1 to TMP (39) (102). In *Bacillus subtilis*, ThiN can convert thiamine B1 directly into TPP (102) (140). In this functional assay, which we confirmed by genomic analysis in FA1090, Gc does not appear to possess *thiK* nor *thiN* homologs.

In addition, we found that the thiazole and pyrimidine moieties were present in the gonococcal medium base and may be utilized to support the growth of TPP auxotrophs and our *thiO* mutant strain. However, we were unable to confirm functionality of a putative HMP transporter in Gc, which would have indicated the capacity of Gc to salvage the pyrimidine moiety from the growth environment. At this time, no other transporters for TPP or related derivatives have been identified. The lack of identifiable TPP salvage genes is intriguing, and may be due in part to selection for a streamlined system of TPP usage in Gc. Thiamine B1 is found in low levels in serum, but not within cells and tissues (51) (164). Therefore, Gc would be considered to have limited opportunities to encounter this form of thiamine. These deficiencies in TPP salvage are striking, however, considering that Gc appears to possess the biosynthetic pathway components of Gram-positive organisms and the transport system of Gram-negative organisms. Overall, the variability in the completeness of these systems is reflective of the scattershot way Gc tends to obtain and incorporate DNA from diverse sources.

In summary, we have demonstrated the presence and functionality of the ThiB component of a system of TPP transport in pathogenic *Neisseria*. We have also shown that the ability to transport TPP, as mediated by ThiB, is important for promoting the establishment of infection in the host when biosynthesis is impaired. The study of this system is not complete, however, as we were unable to identify the remaining components of the putative ThiBPQ transporter. Furthermore, the *thiB* gene that we identified does not appear to be under the control of a Thi-element or TPP-riboswitch, making it unlikely that expression of this transport system is regulated the same way as those of *E. coli* and *Salmonella*.

Intriguingly, while other sexually transmitted pathogens as well as some commensals appear to have systems of TPP transport, few appear to have the components of a functional system of TPP biosynthesis (16) (152) (138). In a related example, the tsetse fly *Glossina* harbors two enteric symbionts which are required for vector survival. The symbiont *Wigglesworthia* is capable of producing TPP through a biosynthetic pathway while the secondary symbiont *Sodalis* lacks this capability, but possesses the ThiBPQ transporter (156) (157). It is thought that this form of complementation allows for the retention of both symbionts while avoiding competition between the two species. It is possible that the dual capabilities of Gc to both import and produce TPP play a similar role in the genital tract, promoting harmonious co-existence with commensal organisms that colonize the same niche while also representing an important nutritional advantage over other species. However, an exhaustive review of the TPP systems present in the

microbial flora of the genital tract that not been conducted, and this hypothesis has yet to be investigated.

Acknowledgments

We gratefully acknowledge Dr. Cara Olsen for her help with statistical analyses and Afrin A. Begum for assistance with the noncompetitive mouse infections. This work was supported by the National Institutes of Health grant ROI A142053, Uniformed Services University of the Health Sciences intramural grant USU MIC-73 2493, and USU Student Award T073377116.

CHAPTER 5: DISCUSSION

GENERAL SUMMARY

Gonococcal infections occur commonly in sexually active young people and can cause severe pathology if left untreated. The body's ability to prevent re-infections via the induction of the adaptive immune response is limited, and, although PMNs are an important component of innate immunity in limiting and clearing infections, Gc has many mechanisms that enable it to resist oxidative and non-oxidative pathways of PMN killing. Further complicating the ability to control infections is the persistent development of resistance to antibiotics by Gc, which has decreased the treatment options available for gonorrhea and raised the specter of untreatable gonococcal infections in the near future. So far, no successful vaccine candidates against gonorrhea have advanced to clinical trials, ensuring that antibiotic resistance will remain a serious threat for years to come. When faced with a public health crisis such as this, it is clear that every effort to understand the relationship between the pathogen and the host should be undertaken in order to identify new opportunities for microbial control.

In addition to providing the cell with critical nutrients for growth, metabolic pathways can be important for bacterial resistance to host immune effectors. Several metabolic processes have been described in Gc, but there are many details that remain to be investigated. Thiamine pyrophosphate (TPP) is an essential coenzyme that can be produced by bacteria through a *de novo* pathway of biosynthesis. TPP biosynthesis pathways in Gc had not been previously described, and the implications of this pathway

for bacterial virulence and survival in the host was not clear. Here we sought to examine the importance of the metabolic pathway that produces the essential coenzyme TPP for gonococcal growth *in vitro*, fitness *in vivo*, and resistance to effector molecules such as ROS and antimicrobial peptides produced by innate immune cells.

In order to learn how Gc functions within the body and possibly determine new avenues of treatment, it is crucial to understand the metabolic processes that are vital for supporting bacterial growth. In addition, differences in metabolic capabilities appear to separate Gc from other organisms, both pathogenic and commensal, that colonize the same niches. It is important to investigate the advantages these metabolic processes confer to Gc in order to understand how this pathogen has become equipped to be so successful in colonizing a hostile environment such as the genital tract.

SUMMARY OF CHAPTER 3 (SPECIFIC AIM 1)

Here we created a mutant strain of Gc that contains an insertion in the nucleotide sequence of the glycine oxidase gene *thiO*. ThiO is an essential enzyme in TPP biosynthesis because of its role in the production of the thiazole moiety THZ. We also complemented this mutant by insertion of the WT *thiO* sequence at a secondary location in the gonococcal chromosome. In our *in vitro* analyses of growth, we observed that the *thiO* mutant experienced a severe reduction in colony diameter when grown on agar that lacked TPP. No reduction of colony sizes was noted for the WT strain, FA1090, or for the complemented mutant in the absence of TPP. Furthermore, we characterized the growth kinetics in broth cultures, and found that the *thiO* mutant failed to double in

media that lacked TPP, and the number of viable colonies recovered declined. Importantly, however, prior TPP-starvation of the mutant was required to observe this phenotype. The *thiO* mutant was supported by exogenous TPP provided in nanomolar concentrations, but growth was inhibited if picomolar levels of TPP were provided. In an *in vitro* co-culture assay, the *thiO* mutant, but not the complemented strain, was less fit than the WT parent strain at later growth phases. Therefore, we conclude that Gc possesses a functional pathway of TPP biosynthesis, and production of TPP is important for bacterial growth under nutrient-limited conditions *in vitro*.

In a female mouse model of lower genital tract infection, the *thiO* mutant was attenuated relative to WT bacteria as the infection period progressed, but the complemented mutant was equally as fit as the WT strain. The observed attenuation was associated with the influx of vaginal PMNs. Interestingly, no significant fitness defects of the mutant or complemented mutant were seen in C57BL6 mice, which do not have an influx of vaginal PMNs in response to Gc infection. To assess the response of the *thiO* mutant to PMN-associated effector compounds released during infection, we examined susceptibility to two sources of ROS; H_2O_2 and paraquat. The *thiO* mutant was more susceptible to both H_2O_2 and internal ROS generated by paraquat compared to the WT and complemented strains, but was not more susceptible to penicillin, suggesting specificity of the effect. When challenged with exposure to isolated murine PMNs in an opsonic phagocytic killing assay, the *thiO* mutant experienced a drop in recovered viable bacteria when previously starved for TPP. However, the same effect was observed in PMNs that lack the capability to produce ROS, suggesting that oxidative killing is not responsible

for the increased killing of the mutant strain. Finally, we evaluated susceptibility to antimicrobial peptides, a mechanism of non-oxidative killing in PMNs. We found a slight increase in susceptibility of the *thiO* mutant when starved for TPP to polymyxin B, a bacterial antimicrobial peptide that is frequently used as a prototype antimicrobial peptide for testing bacterial resistance mechanisms. However, the effect was subtle, and the increments of the concentrations tested were reduced in order see an effect. From these results, we conclude that TPP biosynthesis confers a fitness advantage during symptomatic infections, but not in the absence of an inflammatory response. However, the mechanism by which TPP production protects Gc from exposure to PMN killing during infection remains somewhat unclear.

Limitations

One important limitation of this work is our lack of knowledge regarding the regulation of *thiO* and the other TPP biosynthesis genes. In other model organisms, operons containing *thi* genes are typically controlled by a TPP-responsive riboswitch that represses expression when thiamine levels are high. *In silico* analysis of Gc reveals that a predicted TPP-riboswitches is found upstream of the operon containing *thiO*, as well as upstream of the *thiC* gene, a critical enzyme for the production of the HMP moiety. However, the functionality of this riboswitch mechanism has not been validated in Gc. Therefore, it has not yet been confirmed that Gc regulates the production of TPP in response to the starvation conditions that we examined in this study. Furthermore, the lack of information on *thi* gene expression levels limits our ability to understand the phenotype observed *in vivo*. Results showing attenuation of the *thiO* mutant in a

symptomatic model, but not an asymptomatic model of infection indicate that the *thiO* mutant is more susceptible to PMN killing or other innate effectors within the inflammatory exudate. However, the decrease in bacterial fitness may also be due to nutritional deficiencies from prolonged exposure to the low TPP environment of the lower genital tract.

The availability of TPP in the murine lower genital tract is unknown. Studies of the TPP content in homogenized human tissues have indicated that thiamine is present in lower amounts in the tissues of the female lower genital tract relative to the upper reproductive tract organs. However, the availability of TPP on the mucosal surfaces of epithelial cells and in the cell secretions is not known. This information would enhance our ability to understand the dynamics between nutritional deficiencies, bacterial growth or survival, and PMN killing in our studies. In addition, we note that bacteria were starved for TPP by growth on TPP- agar for many of the *in vitro* assays. Due to the severe attenuation of the mutant after 20+ hours growth without TPP, it is difficult to ensure that the population of bacteria subsequently used in the assay are viable. Finally, we note that murine PMNs were used in our killing assays instead of human PMNs. One host-restriction that could influence the results is the lack of Opa protein-mediated uptake by PMNs through the human carcinoembryonic antigen cell adhesion molecule (CEACAM) receptors, which are not present on murine PMNs (137). In addition, the PMNs may be stimulated prior to the addition of bacteria in the assay by the inflammatory response to the thioglycollate broth that is administered to induce inflammation in the peritoneum, which could influence the observed results.

Future Directions

In order to understand the relationship between growth conditions and TPP biosynthesis, transcription of the *thi* genes should be examined. This could be investigated by qRT-PCR analysis of target genes encoding essential biosynthesis enzymes. Prior qRT-PCR analysis of *thiO* expression in Gc showed that transcription was low and thus difficult to detect, and could not be used to validate RNA-seq results that showed increased expression of *thiO* in a mutant bacterium with intermediate resistance to fluoroquinolone antibiotics (36). However, the analysis was conducted on bacteria that were grown under TPP-replete conditions when expression would be expected to be low. Therefore, qRT-PCR should be performed on bacteria grown under both TPP-replete and TPP-starved conditions in order to examine the effect of nutrient restriction on expression levels. In addition to the operons containing TPP biosynthesis genes, it was also observed that the expression of genes encoding proteins that utilize TPP as a cofactor can be controlled by TPP-responsive mechanisms (131). Therefore, RNA-seq analysis of the global transcriptomic changes that occur when Gc is starved for TPP would reveal the overall cellular response to nutrient deprivation.

In order to investigate the TPP milieu of the murine lower genital tract, HPLC analysis of the tissue of the reproductive organs could be performed. This process can detect the other phosphorylated esters of thiamine in addition to TPP, helping us to understand which substrates Gc may have access to during colonization of the host. In addition, HPLC could be performed on vaginal washes from female mice to describe the TPP

availability of the extracellular environment. In addition, as an indirect measure of TPP availability in the genital tract, *thiO* activation during infection could possibly be assessed by RT-PCR analysis of bacteria recovered from the vaginal swabs of inoculated female mice.

To apply more biological relevance to understanding the dynamic between innate immune cells and bacteria, PMN killing assays could be repeated using human cells. PMN assays that utilize adherent human cells rather than isolated cells better mimic the interaction between Gc and PMNs during infection. In these assays, PMNs are isolated from venous blood of human subjects and allowed to become adherent in the presence of IL-8, an important factor for PMN activation. This process more accurately represents the broad array of PMN-derived immune effector molecules that Gc is likely to be exposed to during the inflammatory response. Here we ruled out PMN-generated ROS as being responsible for decreased survival of the *thiO* mutant in the phagocytic PMN killing assays. Non-oxidative mechanisms of killing include the release of degradative enzymes and antimicrobial peptides from PMN granules during an inflammatory response. We assessed the susceptibility of the *thiO* mutant to polymyxin B, a bactericidal compound which is frequently used as a surrogate for PMN-derived antimicrobial peptides. The effect of exposure to polymyxin B using the agar dilution assay was subtle and difficult to characterize. Therefore, it might be more effective to use a more sensitive, quantitative measure of susceptibility to antimicrobial peptides such as determining the ED_{50} (effective dose at which 50% of the bacteria are killed). Finally, as a technical matter, it is recommended that further analysis of the *thiO* mutant should utilize bacteria that have

been starved in liquid cultures in order to more precisely control the number of bacteria, growth phase, and starvation status of the cells.

The reasons why TPP may be important for antimicrobial peptide resistance are not clear at this time. Given the complexity of these metabolic networks, it is possible that TPP plays a role in the transcriptional activation of genes that increase resistance to antimicrobial peptides. We also propose that TPP may be important for quenching or suppressing intracellular ROS that is produced as a stress response to growth in nutrient-poor conditions. Intracellular ROS can cause peroxidation of the fatty acid portion of membrane lipids, resulting in damage to those cell membranes, which would increase susceptibility to the harmful effects of antimicrobial peptides (79). In order to investigate this hypothesis, alterations in the bacterial cell membranes could be observed by electron microscopy, which would provide a great deal of information about the structural integrity of the *thiO* mutant, particularly during growth in low TPP conditions.

SUMMARY OF CHAPTER 4 (SPECIFIC AIM 2)

In the second aim of this project, we sought to identify a mechanism for the transport of TPP from the growth environment in Gc. We identified the components of a potential TPP transporter by *in silico* analysis and comparison to the nucleotide and predicted amino acid sequences of the ThiBPQ transporter from *Salmonella*. We identified two components, a putative TPP-binding subunit (ThiB) and permease/transmembrane channel (ThiP). We inactivated the putative ThiB and ThiP subunits by insertion of an antibiotic resistance-encoding cassette and evaluated the *in vitro* growth phenotype of our

mutant strains. There were no differences in growth of either the *thiB* or *thiP* mutant in the absence of TPP on agar media or in liquid cultures. In the absence of a phenotype, we attempted to complement a TPP-transport deficient *Salmonella* mutant using the gonococcal transporter components. We found that the gonococcal ThiB could rescue growth of the *Salmonella* mutant, but the ThiP candidate did not. We further confirmed the functionality of the gonococcal ThiB with the creation of a *thiB, thiO* double mutant, which is deficient in both TPP transport and biosynthesis. As predicted, the double mutant produced drastically reduced colony sizes on regular GC agar, required a high concentration of exogenous TPP (1 mM TPP) to achieve optimal growth, and failed to grow in media supplemented with no TPP or concentrations of 100 uM or less.

We also evaluated the fitness of the transporter mutants *in vivo*. The *thiB* mutant appeared slightly attenuated on the first day of infection compared to WT bacteria, but subsequently recovered to equal fitness. Conversely, the *thiB, thiO* double mutant was completely out-competed by the WT strain within one day of infection, was not recovered on any subsequent culture day, and thus did not appear to be infectious. This result was confirmed in a non-competitive infection experiment in female mice in which the *thiB*, *thiO* mutant was not recovered on any of five consecutive days of culture. We conclude that either a functional system of TPP biosynthesis or transport is essential for infection in this surrogate model of genital tract infection.

Finally, we also note that we assessed the GC medium base for the presence of thiamine, TPP, and the thiazole and pyrimidine moieties. Analysis showed that thiamine and TPP

were not present in the medium base but the THZ and HMP moieties were present. These moieties may be substrates for the transport system that we identified in Gc and could help to support the growth of our TPP mutants in culture. Initially, we identified a promising candidate for the proposed HMP transporter CytX based on homology to sequences in *Bacillus subtilis*. However, *cytX* single and *cytX*, *thiC* double mutants grew well on TPP- agar, where we would have expected death of the double mutant to occur. We conclude that Gc does not rely on CytX to obtain HMP from its growth environment but may have alternate transporter systems or salvage mechanisms for the TPP moieties.

Limitations

Our study of the transporter systems of Gc is incomplete due to our inability to locate the other components of a ThiBPQ transporter in Gc. Although the identity of the putative ThiP compared to sequences in *E. coli* and *Salmonella* was not as high as ThiB, basic sequence analysis indicated that it should have been able to function as the TPP permease to allow transport. Failure of the gonococcal ThiP to complement a TPP transport-deficient *Salmonella* mutant was surprising, and the reasons why complementation was unsuccessful are unknown. Furthermore, the lack of a good candidate for the ThiQ component, the ATPase subunit of the active transporter, leaves many questions unanswered about the nature of the mechanism for TPP transport in Gc.

In addition, the presence of the THZ and HMP moieties in the GC medium base was suggestive that these compounds could be transported and utilized to rescue the growth of the TPP biosynthesis mutant. However, although we were able to demonstrate the ability

of TPP and TMP to rescue growth, we were unable to test the effect of providing the individual moieties as they are not commercially produced. Therefore, it is not certain that THZ or HMP are substrates of ThiB, and whether they could be salvaged from the media to support bacterial growth is unknown.

We also investigated the ability of the transporter mutants to adhere to and invade cervical epithelial cells in order to test whether TPP within epithelial cells could be utilized by Gc. We found no differences in the capacity of the *thiB* single mutant to adhere to and invade cells (Figure 18). However, the *thiB*, *thiO* double mutant was not recovered from the assay medium. The severe attenuation of the *thiB*, *thiO* double mutant made it difficult to accurately interpret the results of our adhesion and invasion assays. We were unable to test our hypothesis that TPP is available within host cells as the mutant was unable to survive long enough in the assay medium for adherence to occur, which is required for subsequent cellular invasion. The assay medium is highly thiamine-restricted, and the failure to recover the double mutant, even at relatively early time points, is evidence of bacterial cell death due to nutritional deprivation. Without clarity on this issue, it is difficult to interpret the results of our *in vivo* experiments.

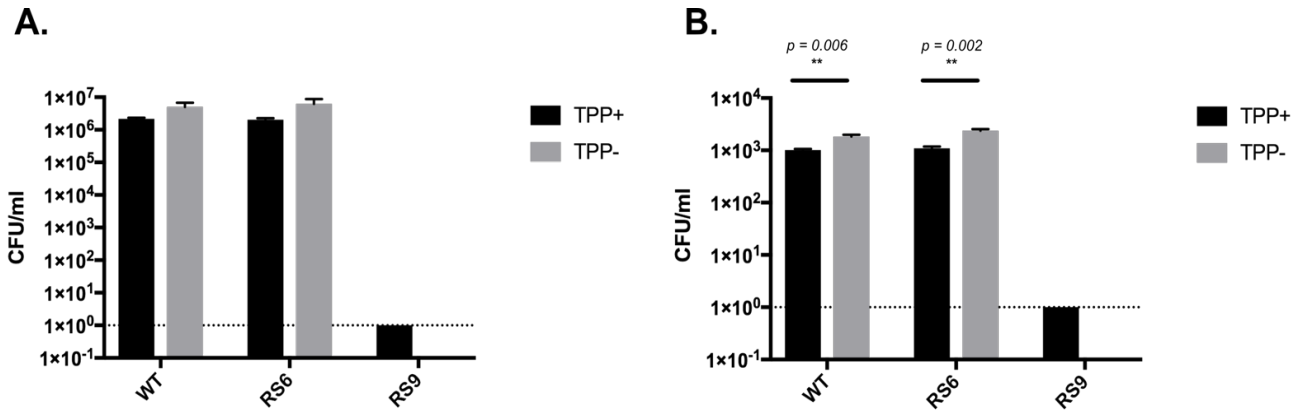

Figure 18. Adherence and invasion of ME-180 epithelial cells by WT, RS6, and RS9. WT, RS6, and RS9 bacteria were incubated with ME-180 human cervical epithelial carcinoma cells for 4 hours to allow adhesion to take place. Afterwards, samples were lysed with 1% saponin and plated on GC or GC+ media (A). For invasion assays, samples were allowed to incubate for an additional 2 hours before the addition of gentamicin to kill extracellular bacteria. After the gentamicin treatment, samples were lysed and plated as before (B). Statistical significance was determined by the unpaired t-test to compare the average CFUs recovered for TPP-replete and starved growth conditions within WT and RS6.

Future Directions

In order to locate the other components of the ThiBPQ transporter in Gc, co-immunoprecipitation could be used to find proteins that interact with the ThiB substrate-binding subunit in FA1090. Although antibodies are available for the thiamine transporter systems found in mammals, these transporters are fundamentally different from those found in prokaryotes and would not be expected to have cross-reactivity with our bacterial proteins. Unfortunately, there are no commercially available antibodies for the bacterial transporter, therefore it would be necessary to generate an antibody against purified ThiB protein in order to perform co-IP experiments.

To investigate expression more closely, transcriptional fusions could be constructed to determine when and under what growth conditions activation of this system occurs. RNA-seq could also be used to detect changes in expression. Also, we were unable to test the effects of providing the individual moieties to the *thiB*, *thiO* mutant bacteria due to the fact that these compounds are not commercially produced. Collaboration with a laboratory that is more established in research of TPP biosynthesis and transport and has the capability to produce these compounds in-house could potentially allow investigation of this angle. Interestingly, when WT FA1090 and the *thiB* mutant were starved for TPP in an epithelial adherence and invasion assay, there was a significant increase in the recovery of bacteria from the assay medium (Figure 18). We speculate that this increase may be due to activation of the TPP biosynthesis pathway during growth under TPP-starvation conditions prior to beginning the assay. In order to examine this finding in more detail, the expression of *thiO* and the other biosynthesis genes should be

investigated as previously suggested. These results would have interesting implications for understanding the metabolic response of Gc to the host environment during infection.

Finally, in order to investigate the use of intracellular TPP by Gc, the severe attenuation of the *thiB*, *thiO* double mutant in the adherence and invasion assays could be overcome by including increased exogenous TPP in the assay medium. Our titration experiments indicate that 100 uM of provided TPP was able to marginally support growth of the double mutant, and this may be a suitable concentration to support bacterial survival in our assay. In addition, an investigation of the TPP content of the murine genital tract as mentioned in the previous section will also help inform our interpretation of the results observed with the transport mutants.

GENERAL CONCLUSIONS

Although thiamine pyrophosphate has long been recognized as an important nutrient for gonococcal growth and recovery, this work represents the first known investigation into pathways of TPP metabolism and transport in Gc. Here we were able to show that Gc has the components of a *de novo* TPP biosynthetic pathway, and activity of this pathway is important for supporting bacterial growth under nutrient-poor conditions. While the TPP availability of the lower genital tract is not well described, we were able to show that TPP metabolism is important for bacterial survival *in vivo* and may contribute to resistance to infiltrating host PMNs. Gc has many mechanisms for resisting damage caused by exposure to ROS *in vitro*, most of which appear to be redundant to one another, but these systems are largely not critical for surviving challenges from PMNs. We observed a

similar effect, suggesting that TPP is not critical for resisting the selective pressure of oxidative killing. However, there were subtle differences apparent in the resistance of a TPP biosynthesis mutant to antimicrobial peptides, suggesting that TPP may play a role in gonococcal resistance to non-oxidative mechanisms of killing. This work adds to the body of knowledge surrounding gonococcal resistance to the host immune system and describes a new *de facto* virulence factor that may be integrated into previously studied system of host resistance.

In addition, we confirmed for the first time the functionality of a component of a TPP transporter system in Gc. This system could transport at least one other thiamine derivative and may be active with others, supplementing the TPP biosynthesis pathway and acting in concert with potential salvage enzymes to support bacterial growth in a variety of environmental niches. Our results also indicate that either TPP transport or biosynthesis must be present in order to support bacterial survival at the genital mucosa long enough to allow adherence and invasion of the host cells during colonization. This is new information that enhances our understanding of the infectious process of Gc, and shines a new light on the importance of metabolic pathways in the pathogenesis of agents of disease.

It is increasingly being recognized that it is no longer sufficient to study pathogens in isolation from the environment they colonize during infection. This is particularly true of sexually transmitted infections, which frequently occur in the non-sterile environment of the lower genital tract where bacterial diversity is rich (96). In this milieu, the factors that

drive pathogen virulence and disease progression are complex and poorly understood. Increasing our knowledge regarding the interplay between pathogens, host factors, and other bacterial flora is critical to illuminating the processes that influence disease outcomes.

Major efforts to characterize the microbial diversity in the human body have been pursued, especially for the vaginal microbiome (64, 124). These studies have found that the host and the microbial communities that populate it exist in a delicate balance of mutualism (89). Disturbances to this balance can result in the development of urogenital diseases, as the vaginal ecosystem acts as a first line of defense against infection by outcompeting invasive, pathogenic microorganisms (118). Despite the clear importance of these bacterial communities for reproductive health, there is still much that remains to be discovered regarding the factors that direct the interactions of these communities with potential pathogens. One such way to investigate these interactions is through the study of microbial metabolism.

Microbial metabolic mutants have long been studied as a successful way to gather information on specific genes, enzymes, and biochemical activities in a cell. However, these approaches were limited in that they assumed a “one gene one function” type of mechanism, and frequently did not consider the global importance of bacterial metabolism. More recently, the adoption of sophisticated methods and a focus on systems biology has highlighted the importance of metabolic integration- the ways in which metabolic pathways interact with each other to meet the needs of the cell. These systems

are complicated and our knowledge regarding their many products and functions is often incomplete. However, understanding how metabolism is integrated within a cell is vital to understanding how microorganisms adapt to changes in their environment.

Additionally, the vaginal ecosystem is thought to have developed as a co-evolutionary process between the host cells and the colonizing microbial flora (96). However, the specific factors behind the emergence of this close and critical mutualistic relationship are not entirely clear. Here, too, metabolism may play an important role, as the evolutionary changes that result in the generation and subsequent retention of metabolic pathways can be necessary for allowing microorganisms to adapt to new and stressful environmental conditions. The metabolic capacity of an invading pathogen may therefore be considered a selective factor that permits successful colonization of the host environment, much as Gc does in the urogenital tract.

The availability of micronutrients in the genital tract is not well understood and has primarily been studied in the context of fetal health. The creation of a series of microbial mutants deficient in select metabolic pathways, and their evaluation in *in vivo* models of infection, are important tools to deciphering the complex role of metabolism in pathogenesis. Here we have created just such a set of tools that may be employed with other microorganisms in continued efforts to characterize the interplay of metabolism and pathogen virulence.

Finally, there is considerable interest in pursuing metabolic pathways as a source of targets for novel antimicrobial therapeutics. This is especially true for those microorganisms that no longer respond to traditionally employed treatments, such as Gc. The prokaryotic TPP biosynthesis pathway is a particularly relevant example, as humans do not possess the capacity to synthesize TPP *de novo*. Therefore, the biosynthesis pathway components in prokaryotes are pathogenic-specific, making them attractive options for pharmacological inhibition.

Altogether, this work opens the possibility of further investigation into these pathways as a way to increase our understanding of the unique dynamic between the pathogen and host responses in the context of metabolism and nutritional requirements. Furthermore, it represents an intriguing snapshot of the evolutionary processes that have permitted Gc to become so successful a pathogen. This new knowledge may be applied to creating novel interventions for Gc, and may be applied to other organisms to advance our understanding of the role played by the production and acquisition of metabolites in STI pathogenesis.

APPENDIX A: Identification of Potential TPP and AHU Auxotrophs in a Repository of Clinical *Neisseria gonorrhoeae* Isolates

INTRODUCTION

Historically, auxotyping analysis was performed on clinical isolates of *N. gonorrhoeae* to determine specific nutritional requirements. This work led to the identification of several different forms of auxotrophy. Commonly identified gonococcal auxotrophs include proline, citrulline, and uracil (PCU) auxotrophs, as well as arginine-, hypoxanthine-, and uracil (AHU)-requiring strains (26, 115). PCU auxotrophs are commonly found in Canada, and while antibiotic resistance is typically infrequent in these strains, some PCU auxotrophs carry resistance-encoding genes on plasmids or chromosomally (115). Interestingly, AHU auxotrophy is correlated with increased serum resistance, invasive disease, and DGI (117). However, it is thought that these isolates possess other virulence factors that are responsible for the disease outcome, and that AHU deficiencies are simply a marker in a considerable percentage of serum-resistant strains that exhibit a high degree of clonality (56).

Other forms of auxotrophy have been identified. An analysis of North American and European gonococcal isolates collected in the pre- and post-penicillin era found several auxotypes, including those for thiamine B1, thiamine pyrophosphate, and glutamine (27). Interestingly, thiamine pyrophosphate and glutamine are routinely provided in media supplements in order to increase recovery of Gc from clinical samples. One notable result from the auxotyping studies was the discovery that methionine auxotrophy was linked to sulfonamide resistance, demonstrating the complex effects of the metabolic networks

(27). The recent establishment of a gonococcal isolate repository at the Uniformed Services University has given us the opportunity to conduct a similar analysis on currently circulating strains, some of which may have developed resistance to a broad array of antibiotics. With this resource, we examined a panel of isolates to determine whether AHU, TPP, and glutamine auxotrophs are circulating in the population and whether they are associated with specific body sites or antibiotic resistance profiles.

Classical techniques for auxotyping are labor-intensive and time consuming, and involve the preparation of many media formulations in order to specifically exclude micronutrients of interest. Furthermore, while these techniques can identify growth phenotypes, they do not provide information on the specific mutations that cause the nutritional defects. However, in the current world of advanced techniques for DNA sequence analysis, better options for evaluating the nutritional profiles of these strains could be developed. Therefore, an additional objective of this work was to investigate whether traditional auxotyping could be combined with whole-genome sequencing to provide a genetic rationale for observed auxotrophic phenotypes. With this work we hope to add to the body of knowledge regarding the nutritional requirements of currently circulating gonococcal isolates, as well as facilitate the development of a sequence-based method for identifying potential auxotrophs.

MATERIALS AND METHODS

Bacterial strains

Gonococcal isolates were obtained from clinical samples collected at health centers on both continental United States (CONUS) and non-US (OCONUS) military bases. Isolates were confirmed to be Gc through laboratory analysis and were tested for antimicrobial susceptibility before storage in the repository. Wild-type FA1090 was originally isolated from a case of DGI and is an AHU auxotroph. FA1090 was used as a positive control for the AHU screening, and as a negative control for the TPP and glutamine screens. Strain RS8 is a TPP biosynthesis-deficient *thiO* mutant in the FA1090 background and was used as a positive control for the TPP screen. Strain MS11 was originally isolated from an uncomplicated lower genital tract infection and has no known auxotrophies. MS11 was used as a negative control for the AHU screen.

Differential media

For conducting the screen for TPP auxotrophs, GC agar was prepared in which TPP was excluded from Kellogg's Supplement I. For the glutamine screen, L-glutamine was excluded from Supplement I instead of TPP. To screen for potential AHU auxotrophs, starch-free (ST-) media was prepared using the formulation for GC broth and added agar. Per liter: 15 g Proteose Peptone No. 3; 4 g potassium phosphate dibasic (anhydrous); 1 g potassium phosphate monobasic; 5 g sodium chloride; 15 g bacto-agar.

Auxotyping analysis

Repository isolates and the appropriate control strains were sub-cultured and passaged once on GC agar. After passage, strains were patched with toothpicks onto both the appropriate differential media and GC agar, in order to compare growth. Plates were

incubated at 37°C with 5% CO_2. After 24 hours, plates were removed from the incubator, and patches were examined on both plates to identify potential auxotrophs. For all the TPP-, gln-, and ST- plates on which growth occurred, the strains were patched a second time onto the corresponding nutrient deficient agar to deplete any potential internal reservoirs of micronutrients.

RESULTS

In the initial screen of 89 strains, we identified five potential TPP auxotrophs and four potential glutamine auxotrophs. One of these strains appeared to be deficient in both TPP and glutamine biosynthesis. To identify potential AHU auxotrophs that may be associated with invasive infections, we developed an agar medium that does not support growth of AHU auxotrophs. This medium, which we called starch-free (ST-) medium, is based on the observation by others that AHU auxotrophs are more susceptible to free fatty acids in the growth environment (10, 13, 179) and the knowledge that starch, typically in the form of cornstarch, is included in agar media formulations to neutralize free fatty acids produced during bacterial growth that are toxic when accumulated. The usefulness of ST- agar in selecting against AHU auxotrophs was confirmed by comparing the growth of FA1090 and MS11 bacteria on ST- agar (Figure 19). We expected that any AHU strains in the repository would display similarly poor growth on ST- plates. We screened 89 repository isolates on GC and ST- agar using FA1090 and MS11 as our growth controls. The analysis was performed three times, and strains that were identified in at least two out of the three screens were considered to be strong candidates for AHU auxotrophy.

With this method, we identified 12 isolates as potential AHU auxotrophs (Table 4). These strains were found among both CONUS and OCONUS isolates.

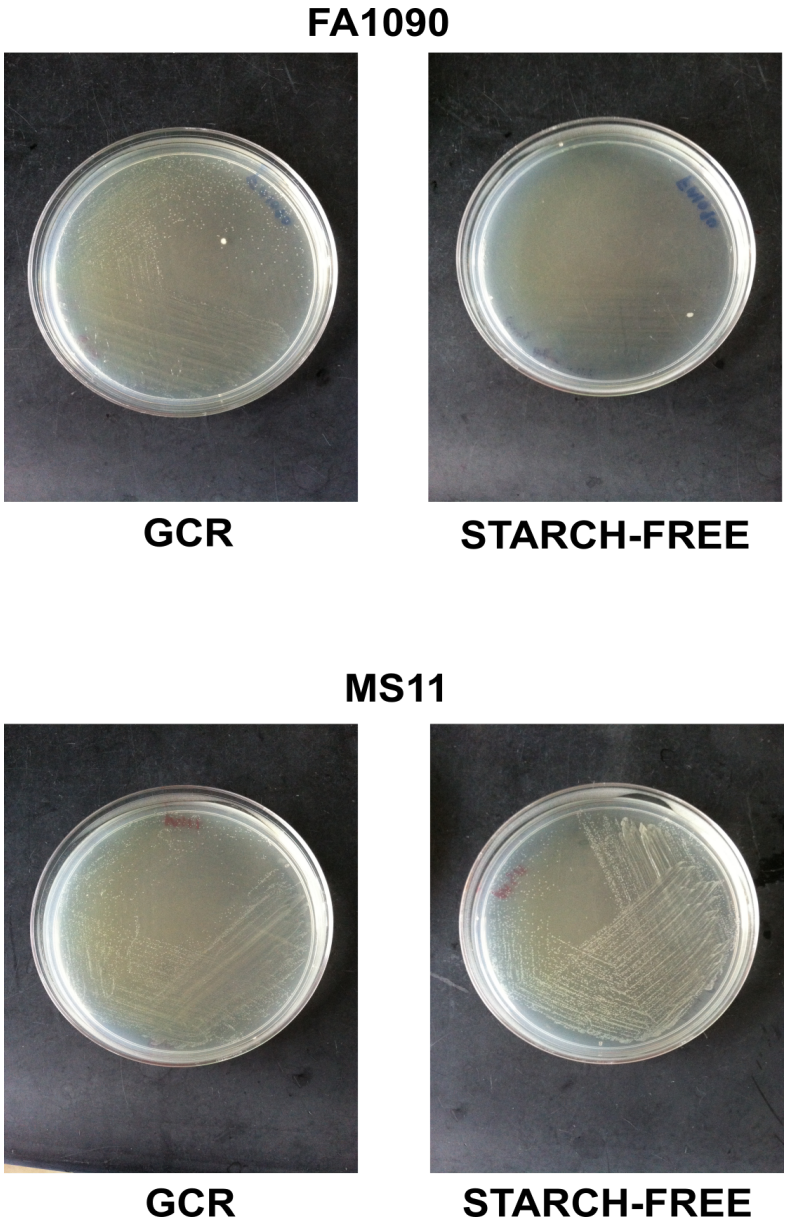

Figure 19. The AHU auxotroph FA1090 does not grow on GC agar in the absence of starch.

The inclusion of cornstarch in agar media detoxifies fatty acid byproducts of bacterial growth. The AHU auxotroph FA1090 is more susceptible to the effects of fatty acids when starch is excluded from the agar medium formulation and will fail to grow (top row). In contrast, the strain MS11 is not an AHU auxotroph, and was able to grow in the absence of starch (bottom row). The phenotype observed when starch was excluded from GC agar was the basis for our screen for AHU auxotrophs in our collection of gonococcal isolates.

Table 4. Distribution of potential AHU auxotrophs identified in starch-free screening of GEIS isolates.

Origin	Total isolates	Potential auxotrophs	% auxotrophs
CONUS	32	5	15.6
OCONUS	57	7	12.28

CONCLUSIONS

Based on this analysis, it is evident that TPP, glutamine, and AHU auxotrophs can be identified among currently circulating clinical isolates of *N. gonorrhoeae*. These potential auxotrophs were identified among both domestically and internationally obtained samples. The five TPP auxotrophs identified represent approximately 5.8% of the total panel of isolates screened, which is relatively consistent with previous studies, in which 4% of a panel of 74 isolates were identified as having a requirement for TPP (25). One isolate identified in the AHU screen was also identified in the glutamine screen, and the finding that TPP and glutamine double auxotrophs may also be present was intriguing. This discovery reflects previous observations that gonococcal isolates can carry multiple nutritional deficiencies and still successfully colonize the lower genital tract.

The results of the starch-free screening also support this observation, as a relatively high percentage of CONUS (15.6%) and OCONUS (12.28%) isolates were identified as potential AHU auxotrophs (Table 4). No isolates from known cases of DGI were included in the panel of repository strains, however, the serum resistance of these strains has not yet been determined and may yet reveal that some isolates have the potential to cause invasive disease. Interestingly, AHU auxotrophs were noted to have become the predominant auxotype recovered in some locations- up to 50% in some American cities- in the post-penicillin era of the late 1970s-1980s (85, 136). Whether this is still the case is unknown.

We conclude that the prevalence of TPP auxotrophs is similar in different geographic locations and does not appear to be considerably different from studies done some 45 years ago. As a future direction, we hope to confirm these results with the use of whole-genome sequencing, which will also provide a genetic basis for the observed growth defects. In the case of TPP auxotrophs, identifying the points in the biosynthetic pathway that are mutated will provide more information about the TPP biosynthesis genes in Gc to elaborate on the phenotypes that were identified in our investigation of *thiO*. Furthermore, increased susceptibility to PMN killing and antimicrobial peptides, as observed in our study of the *thiO* mutant, may also occur in naturally isolated TPP auxotrophs. If this is the case, one might hypothesize that TPP auxotrophs are more likely to be isolated from asymptomatic infections in which the innate immune system does not challenge the Gc auxotrophs to clear infection.

APPENDIX B: *In silico* Investigation of Thiamine Pyrophosphate Metabolic Capacity in Other Pathogens of the Human Lower Genital Tract

INTRODUCTION

Studies in our laboratory indicate that Gc has a complete TPP biosynthesis pathway, and that *de novo* production is important to support bacterial growth *in vivo* and *in vitro* under conditions of TPP starvation. The female genital tract is inhabited by commensal flora and sometimes sexually transmitted pathogens, which frequently appear as co-infections (34, 144). Unexpectedly, when we searched the genomes of other bacteria that colonize the genital tract, we found none that possessed a complete TPP biosynthesis pathway. However, many did appear to encode a TPP transporter system, indicating that they are able to obtain the cofactor by importing it from their growth environment. Therefore, we hypothesized that the presence of Gc may support colonization of commensal and pathogenic organisms via metabolic "sharing" of synthesized TPP in the otherwise nutrient-poor microenvironment of the lower genital tract.

MATERIALS AND METHODS

A review of available literature was undertaken in an attempt to identify TPP biosynthesis genes or TPP transporter components in *Lactobacillus crispatus*, *Mycoplasma genitalium, Chlamydia trachomatis*, *Treponema pallidum*, and *Haemophilus ducreyi*. The results of the literature analysis are summarized in Table 5. For the identification of YkoE in *Lactobacillus*, a BLAST search was performed using the nucleotide and amino acid sequences of YkoE from *Bacillus subtilis*.

RESULTS

Our interest focused on interactions of Gc with *Mycoplasma genitalium* and *Lactobacillus crispatus* due to their complex, poorly understood relationships with Gc. *Mycoplasma genitalium* (MG) is a pathogen that causes urethritis and upper reproductive tract infections in women, and is increasingly recognized as a major cause of non-gonococcal urethritis (28). The genus *Mycoplasma* is somewhat unusual due to its lack of a cell wall, and is thought to be among the smallest free-living organisms capable of reproduction (144). In addition, MG has one of the smallest genomes studied to date- a mere 580 Kb (144). To date, the sequence of only one potential TPP biosynthetic enzyme, *thiI*, has been identified in MG (24). It should also be noted that ThiI is no longer thought to be specifically involved in TPP biosynthesis, and therefore MG does not appear to contain the capacity for *de novo* TPP production (12). However, structural analysis of MG289, an extracytoplasmic lipoprotein, predicted its function to be the substrate-binding component of a thiamine transporter system (152). The apparent sacrifice of TPP biosynthesis, but the retention of TPP transport, may represent a strategic evolutionary adaptation designed to maintain a streamlined genome that will still permit infectivity. Although MG can be isolated concurrently with Gc, nothing is known about how these pathogens interact or co-exist within the host. We propose that Gc may serve as a source of TPP for MG, which may explain the tendency of these organisms to co-infect in the genital tract.

Lactobacillus crispatus (LC) is a commensal organism that is an important member of normal vaginal flora (72). During experimental Gc infection of female mice, we have observed that murine *Lactobacillus* species are frequently isolated from mice that are highly colonized with Gc (72). Lactobacilli have a high requirement for exogenous thiamine, and their growth has even been used in assays to determine levels of thiamine in organic material (58). However, TPP biosynthesis pathways in the lactobacilli are incomplete: most of the enzymes identified are either accessory enzymes that are nonspecific to the TPP pathway, or are classified as salvage enzymes that are dependent on the uptake of pathway intermediates to feed TPP production (138). A thiamine transporter, ThiT, has been described in other strains but has not yet been identified in LC (44). However, sequence analysis indicates that YkoE, a component of different transporter, is present (38). Although functional analyses are limited, YkoE is predicted to be active with thiamine and the precursor molecule HMP. We postulate that Gc and LC may engage in a synergistic relationship based on production and uptake of TPP, allowing enhanced survival and colonization of both species in the genital tract.

This trend extends to several other organisms as well. Chlamydia, caused by infection with *Chlamydia trachomatis*, is the most common notifiable disease in the United States (28). Chlamydia also frequently occurs as a co-infection with gonorrhea, and increases the transmission of HIV (28). Although *C. trachomatis* infections can still be successfully treated with antibiotics, a large proportion of cases are asymptomatic and can result in serious health complications (41). Furthermore, the scope and scale of these infections is epic- in 2016, almost 1.6 million cases were reported to the CDC, the highest number

recorded since surveillance began (160). *C. trachomatis* is an obligate intracellular organism, and, similarly to MG, has a small, compact genome (1.04 Mb) (41). Because of these limitations, *C. trachomatis* is highly dependent on the host cell to provide essential nutrients (41). In an *in silico* screen, *C. trachomatis* did not appear to have any thiamine biosynthesis genes annotated. However, the ThiT protein of a potential thiamine energy-coupling factor (ECF) transporter was identified (UniProt Accession: A0A0E9GFR9). Therefore, we suggest that *C. trachomatis* relies on this transporter in order to obtain the TPP necessary for survival and replication within host cells.

Two more STI pathogens, *Treponema pallidum* and *Haemophilus ducreyi*, the causative agents of syphilis and chancroid (genital ulcer disease, GUD), respectively, have an unusual relationship with TPP. *T. pallidum* is a fragile organism that cannot be cultivated on artificial media and grows very slowly, a trait which is responsible for the long incubation period frequently seen in syphilis (63). *H. ducreyi* is a fastidious organism, requiring supplementation of culture media with factor X (hemin) and fetal calf serum or activated charcoal (2). Both pathogens are capable of infecting the skin away from the genital mucosa, and *T. pallidum* can invade the bloodstream to disseminate to the central nervous system (63, 66). The ability of these organisms to survive in diverse environments suggests that they must possess some mechanism for the acquisition of essential nutrients. There is no evidence of *de novo* TPP biosynthesis in *T. pallidum*, however, the TPP-binding subunit of a potential ThiBPQ transporter was identified in both *T. pallidum* and *T. denticola*, and is regulated by a TPP-responsive riboswitch (15, 16). In an *in silico* analysis, *H. ducreyi* appeared to have limited components of the TPP

biosynthesis pathway, most notably ThiL, which may be functioning as a salvage mechanism in this organism as no other major biosynthesis enzymes are present. However, a potential ThiBPQ transporter was identified, suggesting that *H. ducreyi* primarily relies on TPP transport to meet its metabolic needs.

Interestingly, of the STI pathogens that we evaluated, only Gc appeared to have both a complete, functional pathway of TPP biosynthesis as well as a system for TPP transport (Table 5). These systems may provide Gc with a metabolic advantage over other organisms colonizing the genital tract, and an increased ability to adapt to this harsh environment. However, we also hypothesize that the presence of these systems in Gc can complement other organisms that lack TPP biosynthesis, facilitating co-infections with these pathogens.

Table 5. Microorganisms that colonize the lower genital tract and their potential TPP biosynthesis (TBS) and transporting capabilities.

Organism	TBS	Transporter	THI Element	Comments
M. genitalium	No	Potential	No*, riboswitch predicted in *M. hominis*	MG298: thiamin-binding extracytoplasmic lipoprotein
C. trachomatis	No	Potential	No*	ThiT transporter annotated
Lactobacillus	Incomplete	Yes	Yes	ThiT transporter identified and characterized
T. pallidum	No*	Yes	No*, Thi-box found in analysis of *T. denticola*	TPP-binding component in *T. denticola* and *T. pallidum*
H. ducreyi	Potential/ Incomplete	Yes	Yes	ThiL, DxP synthase, cysteine desulferase**, ThiB, ThiPQ
N. gonorrhoeae	Yes	Yes	Yes	This study

*Rodionov, Vitreschak, Mironov, and Gelfand. Comparative genomics of thiamin biosynthesis in procaryotes: new genes and regulatory mechanisms. J. Biol. Chem., 2002. 277(50):p48949-48959

***H. ducreyi* TBS genes found in KEGG pathway, but not validated in publication

CONCLUSIONS

Genital tract co-infections are common and pose a significant public health concern. This work made a preliminary effort to examine the potential link between bacterial species found in the genital tract and their ability to acquire the essential nutrient TPP. If carried forward, this work will enhance our knowledge of the relationships between these co-infecting species and will begin an investigation of a new paradigm in polymicrobial sexually transmitted infections.

REFERENCES

1. Aho EL, Cannon JG. 1988. Characterization of a silent pilin gene locus from Neisseria meningitidis strain FAM18. *Microb. Pathog.* 5(5):391–98
2. Alfa M. 2005. The laboratory diagnosis of Haemophilus ducreyi. *Can. J. Infect. Dis. Med. Microbiol.* 16(1):31–34
3. Anderson JE, Hobbs MM, Biswas GD, Sparling PF. 2003. Opposing selective forces for expression of the gonococcal lactoferrin receptor. *Mol. Microbiol.* 48(5):1325–37
4. Anderson MT, Seifert HS. 2011. Opportunity and means: horizontal gene transfer from the human host to a bacterial pathogen. *MBio.* 2(1):e00005–00011
5. Anishetty S, Pulimi M, Pennathur G. 2005. Potential drug targets in Mycobacterium tuberculosis through metabolic pathway analysis. *Comput. Biol. Chem.* 29(5):368–78
6. Apicella MA, Ketterer M, Lee FK, Zhou D, Rice PA, Blake MS. 1996. The pathogenesis of gonococcal urethritis in men: confocal and immunoelectron microscopic analysis of urethral exudates from men infected with Neisseria gonorrhoeae. *J. Infect. Dis.* 173(3):636–46
7. Arko RJ. 1989. Animal models for pathogenic Neisseria species. *Clin. Microbiol. Rev.* 2 Suppl:S56–59
8. Arko RJ, Kraus SJ, Brown WJ, Buchanan TM, Kuhn US. 1974. Neisseria gonorrhoeae: effects of systemic immunization on resistance of chimpanzees to urethral infection. *J. Infect. Dis.* 130(2):160–64
9. Ball LM, Criss AK. 2013. Constitutively Opa-expressing and Opa-deficient

neisseria gonorrhoeae strains differentially stimulate and survive exposure to human neutrophils. *J. Bacteriol*. 195(13):2982–90

10. Barth K, Clark VL. 2008. Differences in nitric oxide steady states between arginine, hypoxanthine, uracil auxotrophs (AHU) and non-AHU strains of Neisseria gonorrhoeae during anaerobic respiration in the presence of nitrite. *Can. J. Microbiol*. 54(8):639–46
11. Begley TP, Downs DM, Ealick SE, McLafferty FW, Van Loon AP, et al. 1999. Thiamin biosynthesis in prokaryotes. *Arch. Microbiol*. 171(5):293–300
12. Bender RA. 2011. The Danger of Annotation by Analogy: Most "thiI" Genes Play No Role in Thiamine Biosynthesis. *J. Bacteriol*. 193(18):4574–75
13. Bergsson G, Steingrímsson O, Thormar H. 1999. In vitro susceptibilities of Neisseria gonorrhoeae to fatty acids and monoglycerides. *Antimicrob. Agents Chemother*. 43(11):2790–92
14. Bettendorff L, Wins P. 2013. Biochemistry of Thiamine and Thiamine Phosphate Compounds. In *Encyclopedia of Biological Chemistry*, ed WJ Lennarz, MD Lane, pp. 202–9. Waltham: Academic Press
15. Bian J, Shen H, Tu Y, Yu A, Li C. 2011. The riboswitch regulates a thiamine pyrophosphate ABC transporter of the oral spirochete Treponema denticola. *J. Bacteriol*. 193(15):3912–22
16. Bian J, Tu Y, Wang S-M, Wang X-Y, Li C. 2015. Evidence that TP_0144 of Treponema pallidum is a thiamine-binding protein. *J. Bacteriol*. 197(7):1164–72
17. Bjerknes R, Guttormsen HK, Solberg CO, Wetzler LM. 1995. Neisserial porins inhibit human neutrophil actin polymerization, degranulation, opsonin receptor

expression, and phagocytosis but prime the neutrophils to increase their oxidative burst. *Infect. Immun.* 63(1):160–67

18. Bonin P, Tanino TT, Handsfield HH. 1984. Isolation of Neisseria gonorrhoeae on selective and nonselective media in a sexually transmitted disease clinic. *J. Clin. Microbiol.* 19(2):218–20
19. Borregaard N. 2010. Neutrophils, from marrow to microbes. *Immunity*. 33(5):657–70
20. Borregaard N, Cowland JB. 1997. Granules of the human neutrophilic polymorphonuclear leukocyte. *Blood*. 89(10):3503–21
21. Boslego JW, Tramont EC, Chung RC, McChesney DG, Ciak J, et al. 1991. Efficacy trial of a parenteral gonococcal pilus vaccine in men. *Vaccine*. 9(3):154–62
22. Britigan BE, Klapper D, Svendsen T, Cohen MS. 1988. Phagocyte-derived lactate stimulates oxygen consumption by Neisseria gonorrhoeae. An unrecognized aspect of the oxygen metabolism of phagocytosis. *J. Clin. Invest.* 81(2):318–24
23. Buck P, Rest RF. 1981. Effects of human neutrophil granule extracts on macromolecular synthesis in Neisseria gonorrhoeae. *Infect. Immun.* 33(2):426–33
24. Butt AM, Tahir S, Nasrullah I, Idrees M, Lu J, Tong Y. 2012. Mycoplasma genitalium: a comparative genomics study of metabolic pathways for the identification of drug and vaccine targets. *Infect. Genet. Evol.* 12(1):53–62
25. Carifo K, Catlin BW. 1973. Neisseria gonorrhoeae auxotyping: differentiation of clinical isolates based on growth responses on chemically defined media. *Appl. Microbiol.* 26(3):223–30
26. Catlin BW. 1973. Nutritional profiles of Neisseria gonorrhoeae, Neisseria

meningitidis, and Neisseria lactamica in chemically defined media and the use of growth requirements for gonococcal typing. *J. Infect. Dis.* 128(2):178–94

27. Catlin BW, Reyn A. 1982. Neisseria gonorrhoeae isolated from disseminated and localised infections in pre-penicillin era. Auxotypes and antibacterial drug resistances. *Br. J. Vener. Dis.* 58(3):158–65
28. Centers for Disease Control and Prevention. 2016. Sexually Transmitted Disease Surveillance 2015. U.S. Department of Health and Human Services, Atlanta, GA, United States of America
29. Chan PA, Janvier M, Alexander NE, Kojic EM, Chapin K. 2012. Recommendations for the diagnosis of Neisseria gonorrhoeae and Chlamydia trachomatis, including extra-genital sites. *Med. Health R. I.* 95(8):252–54
30. Chatterjee A, Li Y, Zhang Y, Grove TL, Lee M, et al. 2008. Reconstitution of ThiC in thiamine pyrimidine biosynthesis expands the radical SAM superfamily. *Nat. Chem. Biol.* 4(12):758–65
31. Chen CJ, Elkins C, Sparling PF. 1998. Phase variation of hemoglobin utilization in Neisseria gonorrhoeae. *Infect. Immun.* 66(3):987–93
32. Cole JG, Fulcher NB, Jerse AE. 2010. Opacity proteins increase Neisseria gonorrhoeae fitness in the female genital tract due to a factor under ovarian control. *Infect. Immun.* 78(4):1629–41
33. Cornelissen CN, Kelley M, Hobbs MM, Anderson JE, Cannon JG, et al. 1998. The transferrin receptor expressed by gonococcal strain FA1090 is required for the experimental infection of human male volunteers. *Mol. Microbiol.* 27(3):611–16
34. Creighton S, Tenant-Flowers M, Taylor CB, Miller R, Low N. 2003. Co-infection

with gonorrhoea and chlamydia: how much is there and what does it mean? *Int. J. STD AIDS*. 14(2):109–13

35. Criss AK, Seifert HS. 2008. Neisseria gonorrhoeae suppresses the oxidative burst of human polymorphonuclear leukocytes. *Cell. Microbiol*. 10(11):2257–70
36. D'Ambrozio JA. 2015. *Insights into the Enhanced in vivo Fitness of Neisseria gonorrhoeae Driven by a Fluoroquinolone Resistance-Conferring Mutant DNA Gyrase*. PhD thesis. Uniformed Services University of the Health Sciences
37. Dempsey JA, Litaker W, Madhure A, Snodgrass TL, Cannon JG. 1991. Physical map of the chromosome of Neisseria gonorrhoeae FA1090 with locations of genetic markers, including opa and pil genes. *J. Bacteriol*. 173(17):5476–86
38. Du Q, Wang H, Xie J. 2011. Thiamin (vitamin B1) biosynthesis and regulation: a rich source of antimicrobial drug targets? *Int. J. Biol. Sci*. 7(1):41–52
39. Du Q, Wang H, Xie J. Thiamin (Vitamin B1) Biosynthesis and Regulation: A Rich Source of Anti- microbial Drug Targets?
40. Edwards JL, Apicella MA. 2004. The molecular mechanisms used by Neisseria gonorrhoeae to initiate infection differ between men and women. *Clin. Microbiol. Rev*. 17(4):965–81, table of contents
41. Elwell C, Mirrashidi K, Engel J. 2016. Chlamydia cell biology and pathogenesis. *Nat. Rev. Microbiol*. 14(6):385–400
42. Emmerson AM, Jones AM. 2003. The quinolones: decades of development and use. *J. Antimicrob. Chemother*. 51 Suppl 1:13–20
43. Erkens GB, Slotboom DJ. 2010. Biochemical characterization of ThiT from Lactococcus lactis: a thiamin transporter with picomolar substrate binding affinity.

Biochemistry. 49(14):3203–12

44. Eudes A, Erkens GB, Slotboom DJ, Rodionov DA, Naponelli V, Hanson AD. 2008. Identification of genes encoding the folate- and thiamine-binding membrane proteins in Firmicutes. *J. Bacteriol*. 190(22):7591–94
45. Exley RM, Wu H, Shaw J, Schneider MC, Smith H, et al. 2007. Lactate acquisition promotes successful colonization of the murine genital tract by Neisseria gonorrhoeae. *Infect. Immun*. 75(3):1318–24
46. Fattal-Valevski A. 2011. Thiamine (Vitamin B1). *J. Evid. Based Complementary Altern. Med*. 16(1):12–20
47. Faulstich M, Böttcher J-P, Meyer TF, Fraunholz M, Rudel T. 2013. Pilus phase variation switches gonococcal adherence to invasion by caveolin-1-dependent host cell signaling. *PLoS Pathog*. 9(5):e1003373
48. Faurschou M, Borregaard N. 2003. Neutrophil granules and secretory vesicles in inflammation. *Microbes Infect*. 5(14):1317–27
49. Fisher DJ, Fernández RE, Adams NE, Maurelli AT. 2012. Uptake of biotin by Chlamydia Spp. through the use of a bacterial transporter (BioY) and a host-cell transporter (SMVT). *PLoS One*. 7(9):e46052
50. Forest KT, Tainer JA. 1997. Type-4 pilus-structure: outside to inside and top to bottom--aminireview. *Gene*. 192(1):165–69
51. Gangolf M, Czerniecki J, Radermecker M, Detry O, Nisolle M, et al. 2010. Thiamine status in humans and content of phosphorylated thiamine derivatives in biopsies and cultured cells. *PLoS One*. 5(10):e13616
52. Genee HJ, Bali AP, Petersen SD, Siedler S, Bonde MT, et al. 2016. Functional

mining of transporters using synthetic selections. *Nat. Chem. Biol.* 12:1015

53. Gliszczyńska-Świgło A. 2006. Antioxidant activity of water soluble vitamins in the TEAC (trolox equivalent antioxidant capacity) and the FRAP (ferric reducing antioxidant power) assays. *Food Chem.* 96(1):131–36
54. Gould RG, Kane LW, Mueller JH. 1944. On the Growth Requirements of Neisseria Gonorrhoeae. *J. Bacteriol.* 47(3):287–92
55. Gunn JS, Stein DC. 1996. Use of a non-selective transformation technique to construct a multiply restriction/modification-deficient mutant ofNeisseria gonorrhoeae. *Mol. Gen. Genet.* 251(5):509–17
56. Gutjahr TS, O'Rourke M, Ison CA, Spratt BG. 1997. Arginine-, hypoxanthine-, uracil-requiring isolates of Neisseria gonorrhoeae are a clonal lineage with a non-clonal population. *Microbiology.* 143 (Pt 2):633–40
57. Handing JW, Criss AK. 2015. The lipooligosaccharide-modifying enzyme LptA enhances gonococcal defence against human neutrophils. *Cell. Microbiol.* 17(6):910–21
58. Hankin L, Squires S. 1960. An evaluation of the Lactobacillus viridescens assay for thiamine. *Appl. Microbiol.* 8:209–11
59. Higashi-Okai K, Nagino H, Yamada K, Okai Y. 2006. Antioxidant and prooxidant activities of B group vitamins in lipid peroxidation. *J. UOEH.* 28(4):359–68
60. Hill SA, Davies JK. 2009. Pilin gene variation in Neisseria gonorrhoeae: reassessing the old paradigms. *FEMS Microbiol. Rev.* 33(3):521–30
61. Hobbs MM, Anderson JE, Balthazar JT, Kandler JL, Carlson RW, et al. 2013. Lipid A's structure mediates Neisseria gonorrhoeae fitness during experimental infection

of mice and men. *MBio*. 4(6):e00892–13

62. Hobbs MM, Sparling PF, Cohen MS, Shafer WM, Deal CD, Jerse AE. 2011. Experimental Gonococcal Infection in Male Volunteers: Cumulative Experience with Neisseria gonorrhoeae Strains FA1090 and MS11mkC. *Front. Microbiol*. 2:123
63. Hook EW Rd. 2017. Syphilis. *Lancet*. 389(10078):1550–57
64. Huang B, Fettweis JM, Brooks JP, Jefferson KK, Buck GA. 2014. The changing landscape of the vaginal microbiome. *Clin. Lab. Med*. 34(4):747–61
65. Jaehme M, Singh R, Garaeva AA, Duurkens RH, Slotboom D-J. 2018. PnuT uses a facilitated diffusion mechanism for thiamine uptake. *J. Gen. Physiol*. 150(1):41–50
66. Janowicz DM, Li W, Bauer ME. 2010. Host-pathogen interplay of Haemophilus ducreyi. *Curr. Opin. Infect. Dis*. 23(1):64–69
67. Jean S, Juneau RA, Criss AK, Cornelissen CN. 2016. Neisseria gonorrhoeae Evades Calprotectin-Mediated Nutritional Immunity and Survives Neutrophil Extracellular Traps by Production of TdfH. *Infect. Immun*. 84(10):2982–94
68. Jenkins AH, Schyns G, Potot S, Sun G, Begley TP. 2007. A new thiamin salvage pathway. *Nat. Chem. Biol*. 3(8):492–97
69. Jerse AE. 1999. Experimental gonococcal genital tract infection and opacity protein expression in estradiol-treated mice. *Infect. Immun*. 67(11):5699–5708
70. Jerse AE, Bash MC, Russell MW. 2014. Vaccines against gonorrhea: current status and future challenges. *Vaccine*. 32(14):1579–87
71. Jerse AE, Cohen MS, Drown PM, Whicker LG, Isbey SF, et al. 1994. Multiple gonococcal opacity proteins are expressed during experimental urethral infection in the male. *J. Exp. Med*. 179(3):911–20

72. Jerse AE, Crow ET, Bordner AN, Rahman I, Cornelissen CN, et al. 2002. Growth of Neisseria gonorrhoeae in the female mouse genital tract does not require the gonococcal transferrin or hemoglobin receptors and may be enhanced by commensal lactobacilli. *Infect. Immun.* 70(5):2549–58

73. Jerse AE, Wu H, Packiam M, Vonck RA, Begum AA, Garvin LE. 2011. Estradiol-Treated Female Mice as Surrogate Hosts for Neisseria gonorrhoeae Genital Tract Infections. *Front. Microbiol.* 2:107

74. Johnson AP, Tuffrey M, Taylor-Robinson D. 1989. Resistance of mice to genital infection with Neisseria gonorrhoeae. *J. Med. Microbiol.* 30(1):33–36

75. Johnson MB, Criss AK. 2011. Resistance of Neisseria gonorrhoeae to neutrophils. *Front. Microbiol.* 2:77

76. Jones CL, Napier BA, Sampson TR, Llewellyn AC, Schroeder MR, Weiss DS. 2012. Subversion of host recognition and defense systems by Francisella spp. *Microbiol. Mol. Biol. Rev.* 76(2):383–404

77. Jordan PW, Snyder LAS, Saunders NJ. 2005. Strain-specific differences in Neisseria gonorrhoeae associated with the phase variable gene repertoire. *BMC Microbiol.* 5(1):21

78. Jørgensen ME, Xu D, Crocoll C, Ramírez D, Motawia MS, et al. 2017. Origin and evolution of transporter substrate specificity within the NPF family. *Elife.* 6:

79. Joshi SG, Cooper M, Yost A, Paff M, Ercan UK, et al. 2011. Nonthermal dielectric-barrier discharge plasma-induced inactivation involves oxidative DNA damage and membrane lipid peroxidation in Escherichia coli. *Antimicrob. Agents Chemother.* 55(3):1053–62

80. Juneau RA, Stevens JS, Apicella MA, Criss AK. 2015. A thermonuclease of Neisseria gonorrhoeae enhances bacterial escape from killing by neutrophil extracellular traps. *J. Infect. Dis.* 212(2):316–24

81. Jung IL, Kim IG. 2003. Thiamine protects against paraquat-induced damage: scavenging activity of reactive oxygen species. *Environ. Toxicol. Pharmacol.* 15(1):19–26

82. Kampmeier RH. 1983. Introduction of sulfonamide therapy for gonorrhea. *Sex. Transm. Dis.* 10(2):81–84

83. Kawasaki T, Miyata I, Esaki K, Nose Y. 1969. Thiamine uptake in Escherichia coli. I. General properties of thiamine uptake system in Escherichia coli. *Arch. Biochem. Biophys.* 131(1):223–30

84. Kellogg DS Jr, Peacock WL Jr, Deacon WE, Brown L, Pirkle DI. 1963. NEISSERIA GONORRHOEAE. I. VIRULENCE GENETICALLY LINKED TO CLONAL VARIATION. *J. Bacteriol.* 85:1274–79

85. Knapp JS, Thornsberry C, Schoolnik GA, Wiesner PJ, Homes KK. 1978. Phenotypic and epidemiologic correlates of auxotype in Neisseria gonorrhoeae. *J. Infect. Dis.* 138(2):160–65

86. Lam VH, Lee J-H, Silverio A, Chan H, Gomolplitinant KM, et al. 2011. Pathways of transport protein evolution: recent advances. *Biol. Chem.* 392(1-2):5–12

87. Lankford CE, Scott V, Cox MF, Cooke WR. 1943. Some Aspects of Nutritional Variation of the Gonococcus. *J. Bacteriol.* 45(4):321–27

88. Lankford CE, Skaggs PK. 1946. Cocarboxylase as a growth factor for certain strains of Neisseria gonorrhoeae. *Arch. Biochem.* 9:265–83

89. Larsen B. 1993. Vaginal flora in health and disease. *Clin. Obstet. Gynecol.* 36(1):107–21

90. Leonardi R, Roach PL. 2004. Thiamine Biosynthesis in Escherichia coli: IN VITRO RECONSTITUTION OF THE THIAZOLE SYNTHASE ACTIVITY. *J. Biol. Chem.* 279(17):17054–62

91. Lewis DA. 2010. The Gonococcus fights back: is this time a knock out? *Sex. Transm. Infect.* 86(6):415–21

92. Lewis LA, Choudhury B, Balthazar JT, Martin LE, Ram S, et al. 2009. Phosphoethanolamine substitution of lipid A and resistance of Neisseria gonorrhoeae to cationic antimicrobial peptides and complement-mediated killing by normal human serum. *Infect. Immun.* 77(3):1112–20

93. Lonsdale D. 2006. A review of the biochemistry, metabolism and clinical benefits of thiamin(e) and its derivatives. *Evid. Based. Complement. Alternat. Med.* 3(1):49–59

94. Lorenzen DR, Günther D, Pandit J, Rudel T, Brandt E, Meyer TF. 2000. Neisseria gonorrhoeae porin modifies the oxidative burst of human professional phagocytes. *Infect. Immun.* 68(11):6215–22

95. Lukienko PI, Mel'nichenko NG, Zverinskii IV, Zabrodskaya SV. 2000. Antioxidant properties of thiamine. *Bull. Exp. Biol. Med.* 130(9):874–76

96. Ma B, Forney LJ, Ravel J. 2012. Vaginal microbiome: rethinking health and disease. *Annu. Rev. Microbiol.* 66:371–89

97. Madeo M, O'Riordan N, Fuchs TM, Utratna M, Karatzas KAG, O'Byrne CP. 2012. Thiamine plays a critical role in the acid tolerance of Listeria monocytogenes. *FEMS Microbiol. Lett.* 326(2):137–43

98. Maness MJ, Sparling PF. 1973. Multiple antibiotic resistance due to a single mutation in Neisseria gonorrhoeae. *J. Infect. Dis.* 128(3):321–30

99. Mayor MT, Roett MA, Uduhiri KA. 2012. Diagnosis and management of gonococcal infections. *Am. Fam. Physician.* 86(10):931–38

100. McGee DJ, Rest RF. 1996. Regulation of gonococcal sialyltransferase, lipooligosaccharide, and serum resistance by glucose, pyruvate, and lactate. *Infect. Immun.* 64(11):4630–37

101. McQuillen DP, Gulati S, Ram S, Turner AK, Jani DB, et al. 1999. Complement processing and immunoglobulin binding to Neisseria gonorrhoeae determined in vitro simulates in vivo effects. *J. Infect. Dis.* 179(1):124–35

102. Melnick J, Lis E, Park J-H, Kinsland C, Mori H, et al. 2004. Identification of the two missing bacterial genes involved in thiamine salvage: thiamine pyrophosphokinase and thiamine kinase. *J. Bacteriol.* 186(11):3660–62

103. Ménard R, Sansonetti PJ, Parsot C. 1993. Nonpolar mutagenesis of the ipa genes defines IpaB, IpaC, and IpaD as effectors of Shigella flexneri entry into epithelial cells. *J. Bacteriol.* 175(18):5899–5906

104. Mickelsen PA, Blackman E, Sparling PF. 1982. Ability of Neisseria gonorrhoeae, Neisseria meningitidis, and commensal Neisseria species to obtain iron from lactoferrin. *Infect. Immun.* 35(3):915–20

105. Mickelsen PA, Sparling PF. 1981. Ability of Neisseria gonorrhoeae, Neisseria meningitidis, and commensal Neisseria species to obtain iron from transferrin and iron compounds. *Infect. Immun.* 33(2):555–64

106. Miranda-Ríos J, Navarro M, Soberón M. 2001. A conserved RNA structure (thi

box) is involved in regulation of thiamin biosynthetic gene expression in bacteria. *Proc. Natl. Acad. Sci. U. S. A.* 98(17):9736–41

107. Morse SA, Bartenstein L. 1974. Factors affecting autolysis of Neisseria gonorrhoeae. *Proc. Soc. Exp. Biol. Med.* 145(4):1418–21

108. Morse SA, Stein S, Hines J. 1974. Glucose metabolism in Neisseria gonorrhoeae. *J. Bacteriol.* 120(2):702–14

109. Müller IB, Hyde JE, Wrenger C. 2010. Vitamin B metabolism in Plasmodium falciparum as a source of drug targets. *Trends Parasitol.* 26(1):35–43

110. Napier BA, Meyer L, Bina JE, Miller MA, Sjöstedt A, Weiss DS. 2012. Link between intraphagosomal biotin and rapid phagosomal escape in Francisella. *Proc. Natl. Acad. Sci. U. S. A.* 109(44):18084–89

111. Natera J, Massad WA, García NA. 2011. Vitamin B1 as a scavenger of reactive oxygen species photogenerated by vitamin B2. *Photochem. Photobiol.* 87(2):317–23

112. Nelson M. 1973. Uncomplicated male gonorrhea--a review. *Calif. Med.* 118(1):10–13

113. Ngampasutadol J, Ram S, Blom AM, Jarva H, Jerse AE, et al. 2005. Human C4b-binding protein selectively interacts with Neisseria gonorrhoeae and results in species-specific infection. *Proc. Natl. Acad. Sci. U. S. A.* 102(47):17142–47

114. Ngampasutadol J, Ram S, Gulati S, Agarwal S, Li C, et al. 2008. Human factor H interacts selectively with Neisseria gonorrhoeae and results in species-specific complement evasion. *J. Immunol.* 180(5):3426–35

115. Ng L-K, Lau A, Martin I, Tsang R. 2006. Characterization of proline, citrulline, and uracil auxotrophic plasmid-carrying Neisseria gonorrhoeae strains in Canada,

1993-2003. *Sex. Transm. Dis.* 33(11):688–90

116. Ng L-K, Martin IE. 2005. The laboratory diagnosis of Neisseria gonorrhoeae. *Can. J. Infect. Dis. Med. Microbiol.* 16(1):15–25

117. Noble RC, Reyes RR, Parekh MC, Haley JV. 1984. Incidence of disseminated gonococcal infection correlated with the presence of AHU auxotype of Neisseria gonorrhoeae in a community. *Sex. Transm. Dis.* 11(2):68–71

118. Noyes N, Cho K-C, Ravel J, Forney LJ, Abdo Z. 2018. Associations between sexual habits, menstrual hygiene practices, demographics and the vaginal microbiome as revealed by Bayesian network analysis. *PLoS One*. 13(1):e0191625

119. Okai Y, Higashi-Okai K, F Sato E, Konaka R, Inoue M. 2007. Potent radical-scavenging activities of thiamin and thiamin diphosphate. *J. Clin. Biochem. Nutr.* 40(1):42–48

120. Packiam M, Veit SJ, Anderson DJ, Ingalls RR, Jerse AE. 2010. Mouse strain-dependent differences in susceptibility to Neisseria gonorrhoeae infection and induction of innate immune responses. *Infect. Immun.* 78(1):433–40

121. Packiam M, Yedery RD, Begum AA, Carlson RW, Ganguly J, et al. 2014. Phosphoethanolamine decoration of Neisseria gonorrhoeae lipid A plays a dual immunostimulatory and protective role during experimental genital tract infection. *Infect. Immun.* 82(6):2170–79

122. Park J-H, Dorrestein PC, Zhai H, Kinsland C, McLafferty FW, Begley TP. 2003. Biosynthesis of the thiazole moiety of thiamin pyrophosphate (vitamin B1). *Biochemistry*. 42(42):12430–38

123. Petersen LA, Downs DM. 1997. Identification and characterization of an operon

in Salmonella typhimurium involved in thiamine biosynthesis. *J. Bacteriol.* 179(15):4894–4900

124. Ravel J, Gajer P, Abdo Z, Schneider GM, Koenig SSK, et al. 2011. Vaginal microbiome of reproductive-age women. *Proc. Natl. Acad. Sci. U. S. A.* 108 Suppl 1:4680–87

125. Remmele CW, Xian Y, Albrecht M, Faulstich M, Fraunholz M, et al. 2014. Transcriptional landscape and essential genes of Neisseria gonorrhoeae. *Nucleic Acids Res.* 42(16):10579–95

126. Report on Global Sexually Transmitted Infection Surveillance 2015. 2016. World Health Organization

127. Rest RF. 1979. Killing of Neisseria gonorrhoeae by human polymorphonuclear neutrophil granule extracts. *Infect. Immun.* 25(2):574–79

128. Rodionov DA, Hebbeln P, Eudes A, ter Beek J, Rodionova IA, et al. 2009. A novel class of modular transporters for vitamins in prokaryotes. *J. Bacteriol.* 191(1):42–51

129. Rodionov DA, Leyn SA, Li X, Rodionova IA. 2017. A Novel Transcriptional Regulator Related to Thiamine Phosphate Synthase Controls Thiamine Metabolism Genes in Archaea. *J. Bacteriol.* 199(4):

130. Rodionov DA, Vitreschak AG, Mironov AA, Gelfand MS. 2002. Comparative genomics of thiamin biosynthesis in procaryotes. New genes and regulatory mechanisms. *J. Biol. Chem.* 277(50):48949–59

131. Rodríguez RM. 2002. Importance of water-soluble vitamins as regulatory factors of genetic expression. *Rev. Invest. Clin.* 54(1):77–83

132. Rouquette-Loughlin C, Dunham SA, Kuhn M, Balthazar JT, Shafer WM. 2003. The NorM efflux pump of Neisseria gonorrhoeae and Neisseria meningitidis recognizes antimicrobial cationic compounds. *J. Bacteriol.* 185(3):1101–6

133. Sadarangani M, Pollard AJ, Gray-Owen SD. 2011. Opa proteins and CEACAMs: pathways of immune engagement for pathogenic Neisseria. *FEMS Microbiol. Rev.* 35(3):498–514

134. Saier MH Jr. 2003. Tracing pathways of transport protein evolution. *Mol. Microbiol.* 48(5):1145–56

135. Saier MH Jr, Ren Q. 2006. The bioinformatic study of transmembrane molecular transport. *J. Mol. Microbiol. Biotechnol.* 11(6):289–90

136. Sarafian SK, Knapp JS. 1989. Molecular epidemiology of gonorrhea. *Clin. Microbiol. Rev.* 2 Suppl:S49–55

137. Sarantis H, Gray-Owen SD. 2012. Defining the roles of human carcinoembryonic antigen-related cellular adhesion molecules during neutrophil responses to Neisseria gonorrhoeae. *Infect. Immun.* 80(1):345–58

138. Saulnier DM, Santos F, Roos S, Mistretta T-A, Spinler JK, et al. 2011. Exploring metabolic pathway reconstruction and genome-wide expression profiling in Lactobacillus reuteri to define functional probiotic features. *PLoS One.* 6(4):e18783

139. Schauer K, Stolz J, Scherer S, Fuchs TM. 2009. Both thiamine uptake and biosynthesis of thiamine precursors are required for intracellular replication of Listeria monocytogenes. *J. Bacteriol.* 191(7):2218–27

140. Schyns G, Potot S, Geng Y, Barbosa TM, Henriques A, Perkins JB. 2005. Isolation and characterization of new thiamine-deregulated mutants of Bacillus

subtilis. *J. Bacteriol*. 187(23):8127–36

141. Seib KL, Simons MP, Wu H-J, McEwan AG, Nauseef WM, et al. 2005. Investigation of oxidative stress defenses of Neisseria gonorrhoeae by using a human polymorphonuclear leukocyte survival assay. *Infect. Immun*. 73(8):5269–72

142. Seib KL, Wu H-J, Kidd SP, Apicella MA, Jennings MP, McEwan AG. 2006. Defenses against oxidative stress in Neisseria gonorrhoeae: a system tailored for a challenging environment. *Microbiol. Mol. Biol. Rev*. 70(2):344–61

143. Senapati SK, Dey S, Dwivedi SK, Patra RC, Swarup D. 2000. Effect of thiamine hydrochloride on lead induced lipid peroxidation in rat liver and kidney. *Vet. Hum. Toxicol*. 42(4):236–37

144. Sethi S, Singh G, Samanta P, Sharma M. 2012. Mycoplasma genitalium: an emerging sexually transmitted pathogen. *Indian J. Med. Res*. 136(6):942–55

145. Settembre EC, Dorrestein PC, Park J, Augustine A, Begley TP, Ealick SE. 2003. Structure of ThiO (glycine oxidase) from Bacillus subtilis

146. Settembre EC, Dorrestein PC, Park J-H, Augustine AM, Begley TP, Ealick SE. 2003. Structural and mechanistic studies on ThiO, a glycine oxidase essential for thiamin biosynthesis in Bacillus subtilis. *Biochemistry*. 42(10):2971–81

147. Shafer WM. 1988. Lipopolysaccharide masking of gonococcal outer-membrane proteins modulates binding of bacterial cathepsin G to gonococci. *J. Gen. Microbiol*. 134(3):539–45

148. Shafer WM, Datta A, Kolli VSK, Rahman MM, Balthazar JT, et al. 2002. Phase variable changes in genes lgtA and lgtC within the lgtABCDE operon of Neisseria gonorrhoeae can modulate gonococcal susceptibility to normal human serum. *J.*

Endotoxin Res. 8(1):47–58

149. Shafer WM, Qu X, Waring AJ, Lehrer RI. 1998. Modulation of Neisseria gonorrhoeae susceptibility to vertebrate antibacterial peptides due to a member of the resistance/nodulation/division efflux pump family. *Proc. Natl. Acad. Sci. U. S. A*. 95(4):1829–33

150. Shafer WM, Yu EW, Rouquette-Loughlin C, Golparian D, Jerse AE, Unemo M. 2016. Efflux Pumps in Neisseria gonorrhoeae: Contributions to Antimicrobial Resistance and Virulence. In *Efflux-Mediated Antimicrobial Resistance in Bacteria*, pp. 439–69. Adis, Cham

151. Simons MP, Nauseef WM, Apicella MA. 2005. Interactions of Neisseria gonorrhoeae with adherent polymorphonuclear leukocytes. *Infect. Immun*. 73(4):1971–77

152. Sippel KH, Venkatakrishnan B, Boehlein SK, Sankaran B, Quirit JG, et al. 2011. Insights into Mycoplasma genitalium metabolism revealed by the structure of MG289, an extracytoplasmic thiamine binding lipoprotein. *Proteins*. 79(2):528–36

153. Skaar EP, Tobiason DM, Quick J, Judd RC, Weissbach H, et al. 2002. The outer membrane localization of the Neisseria gonorrhoeae MsrA/B is involved in survival against reactive oxygen species. *Proc. Natl. Acad. Sci. U. S. A*. 99(15):10108–13

154. Smirnov A, Daily KP, Criss AK. 2014. Assembly of NADPH oxidase in human neutrophils is modulated by the opacity-associated protein expression State of Neisseria gonorrhoeae. *Infect. Immun*. 82(3):1036–44

155. Smithline HA, Donnino M, Greenblatt DJ. 2012. Pharmacokinetics of high-dose oral thiamine hydrochloride in healthy subjects. *BMC Clin. Pharmacol*. 12:4

156. Snyder AK, Deberry JW, Runyen-Janecky L, Rio RVM. 2010. Nutrient provisioning facilitates homeostasis between tsetse fly (Diptera: Glossinidae) symbionts. *Proc. Biol. Sci.* 277(1692):2389–97

157. Snyder AK, McLain C, Rio RVM. 2012. The tsetse fly obligate mutualist Wigglesworthia morsitans alters gene expression and population density via exogenous nutrient provisioning. *Appl. Environ. Microbiol.* 78(21):7792–97

158. Soler-García AA, Jerse AE. 2004. A Neisseria gonorrhoeae catalase mutant is more sensitive to hydrogen peroxide and paraquat, an inducer of toxic oxygen radicals. *Microb. Pathog.* 37(2):55–63

159. Soler-García AA, Jerse AE. 2007. Neisseria gonorrhoeae catalase is not required for experimental genital tract infection despite the induction of a localized neutrophil response. *Infect. Immun.* 75(5):2225–33

160. *STD Surveillance Report Press Release: 2015 National Data for Gonorrhea, Chlamydia and Syphilis | 2016 | Newsroom | NCHHSTP | CDC*. https://www.cdc.gov/nchhstp/newsroom/2016/std-surveillance-report-2015-press-release.html

161. Stohl EA, Criss AK, Seifert HS. 2005. The transcriptome response of Neisseria gonorrhoeae to hydrogen peroxide reveals genes with previously uncharacterized roles in oxidative damage protection. *Mol. Microbiol.* 58(2):520–32

162. Stohl EA, Dale EM, Criss AK, Seifert HS. 2013. Neisseria gonorrhoeae metalloprotease NGO1686 is required for full piliation, and piliation is required for resistance to H2O2- and neutrophil-mediated killing. *MBio.* 4(4):

163. Stohl EA, Seifert HS. 2006. Neisseria gonorrhoeae DNA recombination and

repair enzymes protect against oxidative damage caused by hydrogen peroxide. *J. Bacteriol*. 188(21):7645–51

164. Tallaksen C, Bøhmer T, Bell H, Karlsen J. 1990. High performance liquid chromatographic (HPLC) determination of thiamine and thiamine phosphate esters in human blood and serum. *Clin. Nutr*. 9:47

165. Taylor-Robinson D, Furr PM, Hetherington CM. 1990. Neisseria gonorrhoeae colonises the genital tract of oestradiol-treated germ-free female mice. *Microb. Pathog*. 9(5):369–73

166. Thorgersen MP, Downs DM. 2009. Oxidative stress and disruption of labile iron generate specific auxotrophic requirements in Salmonella enterica. *Microbiology*. 155(Pt 1):295–304

167. Tunc-Ozdemir M, Miller G, Song L, Kim J, Sodek A, et al. 2009. Thiamin confers enhanced tolerance to oxidative stress in Arabidopsis. *Plant Physiol*. 151(1):421–32

168. Turner PC, Thomas CE, Elkins C, Clary S, Sparling PF. 1998. Neisseria gonorrhoeae heme biosynthetic mutants utilize heme and hemoglobin as a heme source but fail to grow within epithelial cells. *Infect. Immun*. 66(11):5215–23

169. Unemo M, Nicholas RA. 2012. Emergence of multidrug-resistant, extensively drug-resistant and untreatable gonorrhea. *Future Microbiol*. 7(12):1401–22

170. Unemo M, Shafer WM. 2011. Antibiotic resistance in Neisseria gonorrhoeae: origin, evolution, and lessons learned for the future. *Ann. N. Y. Acad. Sci*. 1230:E19–28

171. Uysal HB, Dağlı B, Yılmaz M, Kahyaoğlu F, Gökçimen A, et al. 2016.

Biochemical and Histological Effects of Thiamine Pyrophosphate against Acetaminophen-Induced Hepatotoxicity. *Basic Clin. Pharmacol. Toxicol.* 118(1):70–76

172. Vander Horn PB, Backstrom AD, Stewart V, Begley TP. 1993. Structural genes for thiamine biosynthetic enzymes (thiCEFGH) in Escherichia coli K-12. *J. Bacteriol.* 175(4):982–92

173. Vonck RA, Darville T, O'Connell CM, Jerse AE. 2011. Chlamydial infection increases gonococcal colonization in a novel murine coinfection model. *Infect. Immun.* 79(4):1566–77

174. Walker CK, Sweet RL. 2011. Gonorrhea infection in women: prevalence, effects, screening, and management. *Int. J. Womens Health.* 3:197–206

175. Warner DM, Folster JP, Shafer WM, Jerse AE. 2007. Regulation of the MtrC-MtrD-MtrE efflux-pump system modulates the in vivo fitness of Neisseria gonorrhoeae. *J. Infect. Dis.* 196(12):1804–12

176. Warner DM, Shafer WM, Jerse AE. 2008. Clinically relevant mutations that cause derepression of the Neisseria gonorrhoeae MtrC-MtrD-MtrE Efflux pump system confer different levels of antimicrobial resistance and in vivo fitness. *Mol. Microbiol.* 70(2):462–78

177. Webb E, Claas K, Downs D. 1998. thiBPQ encodes an ABC transporter required for transport of thiamine and thiamine pyrophosphate in Salmonella typhimurium. *J. Biol. Chem.* 273(15):8946–50

178. Webb E, Febres F, Downs DM. 1996. Thiamine pyrophosphate (TPP) negatively regulates transcription of some thi genes of Salmonella typhimurium. *J. Bacteriol.*

178(9):2533–38

179. Wesley Catlin B. 1977. Nutritional Requirements and Auxotyping. In *The Gonococcus*, ed RB Roberts, pp. 91–109. John Wiley & Sons, Inc.

180. Wiesner PJ, Thompson SE 3rd. 1980. Gonococcal diseases. *Dis. Mon.* 26(5):1–44

181. Wiesner PJ, Thompson SE. 1982. Gonococcal Infections. In *Bacterial Infections of Humans*, pp. 235–57. Springer, Boston, MA

182. Wu H, Jerse AE. 2006. Alpha-2,3-sialyltransferase enhances Neisseria gonorrhoeae survival during experimental murine genital tract infection. *Infect. Immun.* 74(7):4094–4103

183. Wu H, Soler-García AA, Jerse AE. 2009. A strain-specific catalase mutation and mutation of the metal-binding transporter gene mntC attenuate Neisseria gonorrhoeae in vivo but not by increasing susceptibility to oxidative killing by phagocytes. *Infect. Immun.* 77(3):1091–1102

184. Yang QL, Gotschlich EC. 1996. Variation of gonococcal lipooligosaccharide structure is due to alterations in poly-G tracts in lgt genes encoding glycosyl transferases. *J. Exp. Med.* 183(1):323–27

185. Zhang Y, Taylor SV, Chiu HJ, Begley TP. 1997. Characterization of the Bacillus subtilis thiC operon involved in thiamine biosynthesis. *J. Bacteriol.* 179(9):3030–35

186. Zhang YJ, Reddy MC, Ioerger TR, Rothchild AC, Dartois V, et al. 2013. Tryptophan biosynthesis protects mycobacteria from CD4 T-cell-mediated killing. *Cell.* 155(6):1296–1308