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Regulatory mechanisms that impact *Neisseria gonorrhoeae* survival of host innate immunity and antibiotics: The roles of LptA, TbpBA, and MisR

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Abstract

Regulatory mechanisms that impact *Neisseria gonorrhoeae* survival of host innate immunity and antibiotics: The roles of LptA, TbpBA, and MisR

By Justin L. Kandler

Neisseria gonorrhoeae is a Gram-negative bacterium and causes the sexually transmitted infection, gonorrhea. Gonococci can be cultured from purulent exudate in symptomatic individuals, which also contains numerous polymorphonuclear cells (neutrophils). This potent proinflammatory response is effective largely due to the killing action of cationic antimicrobial peptides (CAMPs) and proteins, but not all gonococci are killed. To further explore how gonococci survive the innate immune response, we investigated the importance of a two component regulatory system response regulator, MisR, for gonococcal resistance to CAMPs. Though loss of MisR did not affect the function of the LptA lipid A phosphoethanolamine transferase required for gonococcal resistance to CAMPs, we observed that *lptA* can be transcribed from two distinct promoters, and is post-transcriptionally regulated by a phase variable poly-T_8 tract present within the *lptA* open reading frame. Importantly, *lptA*-deficient gonococci are much less fit than WT gonococci in competitive infections of mice and men. RNA-Seq analysis demonstrated that MisR significantly regulates the transcription of nearly 100 genes (including the transferrin-binding protein genes *tbpB* and *tbpA*, which are essential for *in vivo* survival). Interestingly, we found a previously unknown RNA species that impacts *tbpBA* transcript and TbpBA protein levels by an undefined mechanism. Phagocytosis of gonococci by human macrophage-like monocytic cells greatly upregulated transcription of *tbpBA* (and other iron-responsive genes), and did not kill 100% of the internalized bacteria, suggesting that the iron-limiting environment of the macrophage interior can be sensed by gonococci to increase survival during the iron-limiting innate immune response. Loss of MisR increased susceptibility to CAMPs and aminoglycosides by approximately the same factor (4-8 fold). Furthermore, function (but not expression) of the MtrCDE antimicrobial efflux pump was impaired by loss of MisR in both WT and *mtrCDE*-overexpressor genetic backgrounds, which are common among gonococcal clinical isolates. We propose that loss of MisR increases membrane permeability due to misfolded protein accumulation. The characterization herein of a novel multi-antimicrobial resistance mechanism in gonococci is of special interest in light of the dwindling number of curative antibiotics for gonorrhea, and the approaching implementation of an aminoglycoside (gentamicin) as a first-line therapy in the United States.

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Chapter 1: Introduction

I. *Neisseria gonorrhoeae*—Ancient records, organism discovery, and notable characteristics

Neisseria gonorrhoeae is a Gram-negative diplococcus and the causative agent of the sexually transmitted infection (STI) termed gonorrhea. Early historical accounts suggest that *N. gonorrhoeae* (the gonococcus) has been plaguing humans for millenia. The Greek physician Galen first coined the term "gonorrhea" in the late 2nd century A.D. as the synthesis of the Greek words *gonos* (seed/semen) and *rhoia* (flux/flow) that literally means "flow of seed" in reference to the purulent exudate classically associated with acute male gonococcal urethritis (Galen mistook leukocytic and gonococcal exudate matter for semen) (1, 2). Another account, from the biblical Book of Leviticus in the Old Testament (*King James Bible*, Leviticus 15:2-3), refers to the uncleanliness of "issue/discharge" from the flesh. Perhaps the oldest recording of gonorrhea dates to 2600 B.C., when the Chinese Emperor Huang Ti described a disease resembling gonorrhea in his medical textbook (3). Though vague in their descriptions, it could be argued that these references to what are probably symptoms of gonococcal urethritis are historical evidence that mankind and gonococci have been coevolving since the Exodus out of Egypt or likely earlier.

Like many other microscopic pathogens, the causative agent of gonorrhea remained unknown until the late 19th century when pioneering advances in the science of microbiology directly linked microbes and disease. *N. gonorrhoeae* was first discovered in 1879 when Albert Ludwig Sigesmund Neisser, a German physician in his 20's, demonstrated that diplococci could be reliably observed in purulent exudate from women,

men, and the eyes of infants, using a simple methyl violet staining technique and microscopic observation (4-6). These small, figure 8-shaped (diplococcal) micrococci were subsequently named after Neisser, and the strategy of staining exudate smears remains a commonly utilitized diagnostic for gonococcal infection to this day.

The gonococcus is a notable pathogen in many ways, the most clinically important of which is its ability to reinfect the host an indeterminate number of times without eliciting a protective memory immune response (7) [the reader is also referred to the history of James Boswell, an 18th century Englishman who contracted gonorrhea more than 10 separate times and recorded it all in his journals (8)]. It is now thought that this phenomenon mainly occurs for two reasons: i) the suppression of the host T_H1-/T_H2-dependent adaptive immune response (9) and ii) phase (10) and antigenic (11) variation that occurs at the genetic level, which greatly increases the number of antigenic combinations that can be displayed by any given isolate of gonococci (12). Much of this variation is due to the highly recombinant nature of gonococci and their considerable propensity towards transformation by foreign DNA from other *Neisseria* and closely related species like *Haemophilus influenzae* (13, 14). Transformation can be initiated by donation of DNA via autolysis or Type IV secretion. Generally, gonococci are very competent organisms (that is, they are easily transformed by external DNA). However, DNA is unlikely to enter the gonococcus efficiently unless it contains a 10 nucleotide DNA uptake sequence (DUS) that is recognized by the neisserial ComP receptor (15). Thus, *Neisseria* have evolved to keep much foreign DNA out. However, in an unexpected turn of events, this highly competent pathogen incorporated a piece of *Homo sapiens* DNA into its own genome; remarkably, this human addition was found to be present in

11% of gonococcal strains tested (16). To the author's knowledge, the results of the Anderson and Seifert study are the first recorded instance of inter-domain horizontal gene transfer from a mammalian host to a prokaryotic pathogen, and have broad implications for our general understanding of evolution (17). Furthermore, *N. gonorrhoeae* are reported to be homozygous diploid organisms, and thus each self-contained gonococcal colony-forming unit (cfu) may have more than one copy of each gene (18)—this arrangement is thought to make it less likely for deleterious mutations to produce a phenotype. Additionally, gonococcal infection is often asymptomatic for reasons that are not yet clear, and as a result this sexually-transmitted pathogen has flourished in large part by limiting host awareness of its presence.

II. Gonococcal Disease

a. Epidemiology

Gonorrhea is currently the 2nd most reported infection in the United States and >106 million people are newly infected each year globally (19-21). These numbers are unlikely to be accurate, however, given that many gonococcal infections in women (and some in men) do not produce noticeable symptoms and so go unreported. Furthermore, cases of gonorrhea in high risk populations [sex workers, men who have sex with men (MSM), injectable drug users] are also underreported (21, 22). In the United States, a sentinel program called the "Gonococcal Isolate Surveillance Project" (GISP) monitors rates of gonococcal infection and changes in gonococcal susceptibility to antimicrobial chemotherapies by sampling from male patients with gonococcal infection. As of 2012, rates of gonococcal infection in the U.S. are approximately equal among men and women (about 1 in 1000 of each gender are infected), adolescents and young adults between the

ages of 15 and 24 carry the majority of the reported infections, and blacks have a 4-5 fold higher infection rate than the other ethnicities; reported infections are highest in the Southeastern U.S. and least prevalent in Idaho, Montana, New Hampshire, Vermont, and Wyoming (23).

High transmissibility is a hallmark of any successful pathogen. Generally, the chances of gonococcal transmission from an infected woman to a healthy man are 1 in 4, and from an infected man to a healthy woman nearly 3 in 4 (probability of infection is independent of the number of exposures) (24). In comparison, HIV transmission rates from an infected to a healthy individual are relatively low (1 in 100 to 1 in 1000), but can be greatly increased by the presence of preexisting sexually transmitted infections such as chlamydia, syphilis, and gonorrhea (25, 26). As a master pathogen of humans, *N. gonorrhoeae* can reside undetected in the host; lower genital tract infections are asymptomatic in 50-80% of women (and occasionally in men) (24). Matters are further complicated by the asymptomatic nature of pharyngeal infections and the ability of gonococci to transmit from the penetrative to the receptive partner and vice versa (27). Infection can even be transmitted vertically from an infected mother to the eyes of her child during birth (a condition known as opthalmia neonatorum). Thus, it is essential to consider the anatomical sites involved (e.g. conjunctiva, pharynx, rectum, urogenital tract) when reporting transmission rates.

b. Pathogenesis

As mentioned above, gonococcal transmission events from one person to another can occur between numerous mucosal sites and are likely to be much more diverse than the canonical male-to-female/female-to-male vaginal intercourse. However, much of the

research into the pathogenesis of gonococcal disease has focused on the host tissues involved in that scenario, and a brief summary of the Edwards and Apicella model of gonococcal pathogenesis (which consolidates a vast body of work) will be described below [see (24)]:

Men. Upon infection of the male urethra, gonococci bind to epithelial cells through an interaction between the gonococcal outer membrane lipooligosaccharide (LOS) and the host asialoglycoprotein receptor (ASGP-R). This invasion of the host epithelium results in expression of cytokines and chemokines, most notably TNF-α, IL-1β, IL-6, and IL-8, which participate in the proinflammatory response and aid in the recruitment of a large influx of polymorphonuclear leukocytes (PMNs). Composed primarily of neutrophils, this wave of PMNs generates its own cytokines and chemokines in addition to those produced by the epithelium, and this process collectively results in the symptomatic inflammatory response seen in men. Initial distant interactions are thought to be due to binding of Type IV pili to PMNs (28), and subsequent close interactions with PMNs occur through the binding of gonococcal opacity proteins (Opa) and PMN carcinoembryonic antigen-related cell adhesion molecules (CEACAMs), which typically results in phagocytosis. A small percentage of gonococci survive PMN killing as shown by culturing from purulent exudates [see (29, 30)] and are sialylated during intracellular residence within PMNs. However, *de*sialylation of the gonococcal LOS is necessary for gonococcal invasion of urethral epithelial cells, and occurs due to the presence of neuraminidases on the surface of host neutrophils, macrophages, and sperm. In this way, close external contact with PMNs may ironically exacerbate the spread of

infection. Desialylated gonococci also bind to ASGP-R present on sperm, enhancing the chances of transmission by "hitching a ride".

Women. Upon infection of the lower female genital tract, gonococcal pili bind to host complement receptor 3 (CR3) present on cervical epithelial cells. Complement is activated, and the alternative complement pathway factor C3b is deposited on the gonococcal surface. Gonococcal Opa proteins attract host factor H, which enhances the cleavage of C3b to its inactive form (iC3b) by host factor I. iC3b, gonococcal porin, and CR3 all become tightly associated at the interface of the gonococcal surface and the cervical cell, triggering gonococcal internalization through macropinocytosis [an "actin-dependent process initiated from surface membrane ruffles which give rise to large endocytic vacuoles called macropinosomes"; see (31)]. Depending on the environmental status of the lower female genital tract (which can be impacted by menses, commensal flora, and pH), gonococci may ascend into the upper genital tract via binding of gonococcal ribosomal protein L12 to host lutropin receptor (LHr). The expression of LHr increases in an ascending fashion from the endometrial to the Fallopian tube epithelia. Here, ciliated cells are lost due to LOS and peptidoglycan toxicity which is further exacerbated by TNF-α release; this loss of ciliary function can have impacts on female fertility and passage of eggs down the Fallopian tubes. Nonciliated cells serve as entry points for gonococcal transcytosis to the basal lateral surface, where they may subsequently enter into subepithelial tissue. Gonococci that have survived to this point are sometimes able to initiate disseminated infection by gaining access to the bloodstream, but will only survive the killing action of host complement if they have the necessary complement resistance characteristics [e.g. have porin-associated factor H or

C4 binding protein (C4bp), sialylated LOS, phosphoethanolamine (PEA)-modified LOS, can bind but not be killed by complement membrane attack complexes; see (32)].

c. Symptoms

While disseminated gonococcal infection is rare [previous estimates at 0.5-3% of untreated infections (33, 34)] its symptoms are severe and include bacteremia-induced swelling of the skin, tendons, and joints. If the infection is not treated, serious sequelae such as endocarditis, meningitis, and osteomyelitis may occur (35). A.H. Harkness writes in detail of the various anatomical sites accessed by disseminated gonococci; of historical note, Harkness' introduction mentions that much of the knowledge we have about gonococcal colonization sites was gathered by examination of cadavers or the unethical inoculation of the blind in the late 19th and early 20th centuries (36).

Proven incidence of fatal complications from gonococcal infection is exceedingly rare (37), though possible. More clinically relevant are instances of ectopic (Fallopian tube) pregnancy that can occur due to pelvic inflammatory disease (PID) and are potentially fatal to both mother and child (38); such mortality probably occurs with some frequency in under-developed countries due to complications of ectopic pregnancy. However, the majority of gonococcal infections result in uncomplicated lower genital tract symptoms (men) or no symptoms at all (women). Nevertheless, it is important to emphasize the risk to reproductive health (in some cases, sterility) posed by gonococcal colonization.

In symptomatic men, early indicators of gonococcal infection are pain or a burning sensation during urination and penile discharge (white, yellow or green in color). Less often, swelling of the epididymis in the testicles can leave permanent damage and

lead to infertility. In symptomatic women, early indicators of gonococcal infection include pain or a burning sensation during urination, increased vaginal discharge, or bleeding between periods. Ascending gonococcal infections in women can damage to the Fallopian tubes and cause infertility and PID (39). During PID, inflammation from gonococcal infection opens a window of opportunity for microbes that are normally sequestered in the vaginal cavity to ascend into the upper genital tract. This condition has a high morbidity; approximately 20% of women with PID become infertile, and twice that number have chronic pelvic pain. About 1% of women with PID that conceive develop ectopic pregnancy (40). In both sexes, rectal infection (though sometimes asymptomatic) can lead to anal itching or discharge, rectal soreness and bleeding, and painful bowel movements (39). Though antibiotic therapy has limited gonococcal infections to their highly curable, uncomplicated origins in the lower genital tract, rectum, and pharynx in much of the world, the looming threat of antibiotic-resistant untreatable gonorrhea can no longer be ignored (19).

III. A history of treatment strategies

a. Classical therapy-Renaissance treatments

Gonococci have been surviving in humans for a very long time, during which they have evolved numerous known and unknown mechanisms to stay alive. In return, we humans have (for the most part unsuccessfully) experimented with numerous substances in an effort to keep gonococci out of our bodies. Prior to the use of sulfa drugs in the 1930's, some of these "therapies" seemed to do more harm than good, and many were arguably intended to be penance for the sinful soul as much as cures for the body. Early biblical history suggests that Moses' people, if they had unclean discharges, were

segregated away from others for a week, made to wash themselves and contaminated objects with water, and tasked with giving up two turtle doves or pigeons to the priest for an offering (*King James Bible*, Leviticus 15:1-15).

During the 1st and early 2nd millennia A.D., naturalistic treatments seemed to be the norm, often consisting of urethral injections composed of seawater, goat's milk, barley water, sugar, almond milk, honey, or various botanical extracts. These relatively mild homeopathic remedies went out of vogue in the Renaissance when it was discovered that mercury (in the form of mercury chloride or "calomel") could be used to treat syphilis, and thus logic dictated that mercury would be beneficial for treatment of gonorrhea as well (though these two diseases were often found together or confused with one another). Then followed venesection (bleeding), Spanish fly (cantharides—blistering agents extracted from insects), and urethral irrigations of lead, turpentine, and silver nitrate (sometimes in concentrations up to 4% w/v!)(41). These treatments could hurt the body and the bank; one account from the American colonial period estimated "treatment for simple gonorrhea, with salivation" at £2, 5 shillings (a hefty sum, given that farmhands were only paid about £30/year) (42).

b. The 20th century—temporary victory

In the 1920's, the continuing lack of a reproducibly effective therapy for gonococcal infection left some clinicians fumbling for a cure. There were instances of using 1% w/v saline, ether, formaldehyde, or iodine injections into the urethra (41); these treatments could be referred to as "punitive therapy". Even as late as 1941, heat therapy—wherein the patient would be totally encapsulated (save the head) within a heat cabinet to mimic fever—was still a medically-approved option, despite the availability of

sulfa drugs (43). Fortunately, a revolutionary new treatment for bacterial infections, penicillin, would hit the market in time for WWII.

Alexander Fleming[1] published the potent antibacterial (and antigonococcal) effect of *Penicillium* filtrates in 1929 (45), but the bactericidal activity of penicillin would likely have remained academic without the help of Ernst Boris Chain and Howard Walter Florey, who successfully purified the molecule and proved its safety and efficacy *in vivo* (46). Fleming, Chain, and Florey would share the 1945 Nobel Prize in Physiology or Medicine for their discoveries. But it would take more than three people to make the first true "wonder drug" concentrated, plentiful, and cheap—indeed, the advent of penicillin required a revolutionary marriage of academic science and industry never before seen on such a scale (47). In its wartime heyday, public service posters claimed that penicillin could cure gonorrhea in as little as 4 hours [Figure 1A; (48) http://medicalhistory.blogspot.com/2012/06/clap.html]—which was no doubt a welcome change from posters declaring "A minute with Venus…a year with Mercury!" [Figure 1B; (48) http://medicalhistory.blogspot.com/2012/06/clap.html]. After the magnificent success of penicillin and later antibiotics in treating infections, someone in 1967 made the tragically premature statement that "It is time to close the book on infectious diseases, and declare the war against pestilence won."[2] The next decades would come to show, not least of all through the evolution of gonococcal antibiotic resistance, the error of that statement.

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[1]Contrary to popular belief, the observation that fungi could have curative and more specifically, antibacterial properties was made prior to Fleming's studies of *Penicillium* [see (44)].

[2]This quote is often attributed to former US Surgeon General Dr. William H. Stewart, but a recent investigation suggests that he said nothing of the kind and was, in contrast, well aware of the threat of antibiotic resistance [see (49)].

Penicillin was first introduced in the United States in 1943 for treatment of gonorrhea, but by 1958 there were reports of penicillin resistance and by 1987 (44 years later), penicillin was no longer recommended as a first-line anti-gonococcal chemotherapy due to the high risk of treatment failure. Similarly, the gonococcus had previously evolved and continues to evolve resistance mechanisms to all of the other drugs used to treat gonorrhea, including the silver-based compound termed protargol (1897-1938), sulfonamides (1938-1949), erythromycin (1952-1977), tetracycline (1962-1986), fluoroquinolones (1987-2007), azithromycin (1983-2007), and cefixime (1983-2012) [drug introduction and retraction dates summarized from Figure 1 of the Ph.D. thesis of Paul J.T. Johnson (50)].

c. Present day—a return to flawed options

Today, recommended treatment for uncomplicated lower genital tract gonococcal infections in the United States is governed by the CDC, and currently consists of an injectable cephalosporin, ceftraxione, given in conjunction with one of two oral antibiotics—azithromycin or doxycycline—in order to slow the spread of resistant isolates (51). A recent clinical trial by the CDC suggests dual treatment with gentamicin plus azithromycin OR gemifloxacin plus azithromycin is >99.5% efficacious in clearing gonococcal infection, but a large percentage (up to 10%) of study participants experienced unpleasant gastrointestinal side effects including vomiting, nausea, abdominal discomfort/pain, and diarrhea (52, 53). In all likelihood, it will one day become necessary to transition from a ceftriaxone-based treatment to the gentamicin- or gemifloxacin-based treatments tested in the aforementioned clinical trial. However, patients are less likely to follow their treatment regimens if they experience unpleasant

side effects, and such noncompliance could hasten the development of gonococcal isolates resistant to this new therapy. Furthermore, updated CDC-approved treatment regimens may not be feasible in other parts of the world due to excessive cost, lack of policy implementation, poor surveillance, drug scarcity, noncompliance, or any combination of these factors. In order to break the untenable cycle of drug change→gonococcal resistance→drug change, it is essential that a vaccine be developed to prevent gonococcal infection and transmission.

IV: Gonococcal vaccinology—failed trials and hope for the future

As of 2014, there is no approved protective vaccine against gonococcal infection or colonization. In the 1970's, hopes were high that a killed whole-cell vaccine would provide protection, and a study was conducted among a population of Inuit in Northern Canada that had a high rate of gonococcal infection. Unfortunately, despite promisingly mild reactions to the high concentration of gonococcal endotoxin in the vaccine, there was no protection against later challenge with gonococci and this strategy was abandoned (54-57). Subsequently, efforts to vaccinate a high-risk group of US military personnel stationed in South Korea using purified pilin, a major surface antigen on the gonococcal exterior, also failed. It was later determined that antigenic variation due to changes in the coding sequence of the expressed pilin gene *pilE* [see (11) and (58)] likely rendered any anti-pilin antibodies useless against antigenically-different challenge strains (57, 59, 60).

Currently, several other antigens have become promising vaccine candidates due to their superficial location, high conservation among gonococcal strains, and necessity for gonococcal pathogenesis and/or survival in the host. Some examples of these targets include MtrE, which is the outer membrane channel component of the MtrCDE and

FarAB-MtrE efflux pumps and is required for *in vivo* fitness in the face of host antimicrobials; TbpBA, the transferrin binding proteins required for survival in the human male infection model due to their role in iron piracy; AniA, a nitrite reductase required for anaerobic growth and biofilm formation; and OmpA, an outer membrane protein that mediates invasion of cervical and endometrial cells [vaccine targets are reviewed in (61)]. Another potential candidate is a multi-antigen peptide mimetic of the gonococcal 2C7 oligosaccharide epitope, which accelerates clearance of gonococci in a mouse model (62).

However, one of the biggest challenges blocking the way to an effective gonococcal vaccine is the ability of gonococci to modulate the host immune response, often eliciting a symptomatic proinflammatory innate immune response and minimizing the generation of immune memory. This modulatory effect is thought to be accomplished by several immunosuppressive mechanisms; broadly, these mechanisms include the inhibition of T-cell proliferation, the induction of macrophage pyronecrosis, and suppression of T_H1-/T_H2-dependent adaptive immune responses [see (61, 63)]. It will be crucial to ensure that these mechanisms are accounted for during future vaccine trials.

Of note, the administration of microcapsules containing the proinflammatory, pro-T_H1 cytokine IL-12 during gonococcal infection of female BALB/c mice resulted in the generation of an enhanced anti-gonococcal immune memory and was protective against reinfection (64). The authors suggest that administration of IL-12 microcapsules in patients with active gonococcal infection may, in essence, transform a natural infection into a live vaccine, and have the added benefit of targeting high-risk populations. However, as only a single gonococcal strain (FA 1090) was used in these experiments,

the protective effect of IL-12 will need to be proven by challenge with multiple, antigenically-distinct gonococcal strains before vaccination studies should be continued further. Additionally, the propensity of gonococcal infections to often be asymptomatic in women and when located in the pharynx begs the question: will IL-12 treatment still result in immune memory when there are no symptoms?

V: Mechanisms of antibiotic resistance

In the absence of an effective vaccine, antibiotic therapy remains the only option to combat gonococcal infection and stop spread of the disease in the community. Unfortunately, the gonococcus has evolved resistance mechanisms to all antibiotic chemotherapies used for its treatment. Some of these mechanisms [reviewed in (65-69)] increase gonococcal fitness in the host (e.g. enhanced expression of *mtrCDE*), and some even increase fitness without antibiotic pressure (changes to *gyrA*) (70). Importantly, this contrasts with the generally accepted dogma that antibiotic resistance comes at a fitness cost, and raises the possibility that treatment-resistant gonococci will have an advantage over their susceptible counterparts in the wild—such strains could spread with greater efficiency. Antibiotic resistance determinants germane to this work are briefly described below. For a more comprehensive review, readers are referred to (69).

a. β-lactams

Perhaps the most studied mechanisms of drug resistance in the gonococcus are those that impact treatment with β-lactam antibiotics. These drugs kill actively dividing bacteria by binding to their peptidoglycan transpeptidase enzymes (penicillin binding proteins) and inhibiting their function, resulting in the breakup of peptidoglycan and cell lysis (71, 72). It has been shown that gonococcal β-lactam resistance evolved by the step-

wise acquisition of changes to multiple chromosomal loci (73) and the presence (in some cases) of a plasmid encoding a β-lactamase (74). Ongoing changes to the mosaic *penA* gene that encodes the peptidoglycan transpeptidase PBP2, which are thought to be acquired through numerous transformation events between commensal *Neisseria* and pathogenic *Neisseria,* greatly impair the ability of β-lactam drugs to prevent cell wall synthesis by changing the efficacy of β-lactam binding to PBP2 enzyme (75-77). Furthermore, mutations in the genes encoding the porin PorB1b (*penB*) and the Type IV pilus secretin PilQ (*pilQ*) can decrease the permeability of the gonococcal outer membrane to β-lactams and hinder their entry into the cell (78, 79). The mutation in another PBP allele called *ponA1* (which encodes the peptidoglycan transpeptidase PBP1) cannot independently increase resistance to β-lactams, but does so 2-fold when present in a gonococcal strain bearing the *pilQ2* (previously known as *penC*) allele, which encodes an E666K-mutant allele of the PilQ secretin with a decreased ability to import β-lactams (79, 80).

Finally, β-lactam drugs can be excreted by the well-characterized MtrCDE efflux pump, which can be overexpressed due to single base pair mutations in the promoter region of *mtrCDE* (81-83), induction by the transcriptional regulatory protein MtrA (84, 85), or derepression of *mtrCDE* due to loss-of-function mutations in the *mtrCDE* transcriptional repressor, MtrR (86-88). Recently, the importance of MtrCDE to the antibiotic resistance phenotype of extensively drug resistant (XDR) gonococcal strains was made clear by Golparian and colleagues, who demonstrated that penicillin, cefixime, and ceftriaxone resistance was variably but significantly decreased upon genetic

inactivation of *mtrCDE* in multiple strains, including the now infamous XDR strain H041 (89, 90).

Importantly, the resistance mechanisms described above are not strictly additive under all conditions; that is, the role of one resistance factor might not be evident unless certain others are present (as in the case of *ponA1*), and the degree to which any given resistance factor protects against β-lactams depends upon the antibiotic being tested (penicillin vs. extended spectrum cephalosporins). Interestingly, it is possible for resistance determinants to be generated in the laboratory that are not likely to be found in nature; for instance, the *pilQ2* allele renders gonococci unable to produce pili (79)—which are important in pathogenesis—and was not found in a genetic profiling study of 46 clinical isolates with reduced susceptibility to extended spectrum cephalosporins (91). Readers interested in the complex nature of gonococcal β-lactam resistance determinants are directed to reference (73) for more information.

b. Aminoglycosides and spectinomycin

The era of aminoglycoside antibiotics began with the characterization of streptomycin in 1944 as an anti-mycobacterial substance that might be used for treatment of tuberculosis (92). Numerous other aminoglycoside drugs (including amikacin, kanamycin, tobramycin, and gentamicin) were subsequently developed. These bactericidal molecules are thought to kill bacteria by binding to the 30S ribosomal subunit and decreasing the accuracy of codon recognition, which causes misreading of the mRNA (93). Misreading results in the production of incorrectly translated, misfolded proteins that cannot perform their normal function. Aminoglycoside exposure eventually leads to bacterial cell death due to nonfunctional ribosomes, an overabundance of

reactive oxygen species and loss of envelope integrity (94-96). Spectinomycin belongs to a closely related but functionally different class of antibiotics called “aminocyclitols”. While this drug also binds to the 30S ribosomal subunit, spectinomycin—in contrast to aminoglycosides—inhibits translocation of the peptidyl-tRNA from the “A” site to the “P” site of the ribosome, resulting in the truncation of nascent polypeptides rather than misreading, and is consequently bacteriostatic rather than bactericidal (93).

As aminoglycosides are positively charged, they cannot passively diffuse through the hydrophobic lipid bilayer that comprises much of the cell membrane(s). Rather, aminoglycosides must first bind to the negatively charged surfaces of bacteria, then enter through an energy dependent process known as “self-promoted uptake” (93, 97-99), whereby imperfections in the cell membrane allow a small amount of aminoglycoside to enter, cause mistranslation, and initiate the integration of misfolded proteins into the membrane. This process generates structural irregularities through which more aminoglycoside molecules may invade the cell envelope. Typical mechanisms of resistance to aminoglycosides include decreased import and accumulation, efflux, drug modification, and target modification (93, 100).

Gonococcal resistance to aminoglycosides, and mechanisms that explain such resistance, are now seldom-studied topics. The Atlanta ST-28 clinical strain isolated in 1963 displayed a very high streptomycin minimum inhibitory concentration (MIC of >5000 μg/mL) (101). Sarrubi, Maness, Sparling and colleagues later found that streptomycin and spectinomycin resistance in gonococcal strains FA5, FA47, and FA77 (and probably ST-28) was due to genetic modifications at loci encoding ribosomal genes whose products (rRNA or protein) are integrated into the 30S ribosomal structure (102,

103). Such ribosomal modifications reduce the capacity of streptomycin and spectinomycin to bind the ribosome and greatly increase resistance. Kanamycin resistance is genetically linked with streptomycin and spectinomycin resistance in *N. gonorrhoeae* (104). Gentamicin resistance is likely to be mediated by the same locus due to its structural similarity to kanamycin, though this has not been experimentally determined in gonococci (105).

Cell-free extracts of a different strain (ML5034) of streptomycin-resistant *N. gonorrhoeae* were able to inactivate streptomycin by an adenylylation mechanism that required ATP (106). However, this finding may have been unique, as a subsequent report could not establish the presence of any adenyl transferase (*aadA*) genes in streptomycin/spectinomycin resistant gonococcal strains (104). Interestingly, a 6 MDa conjugal plasmid carrying streptomycin phosphotransferase activity was found in a study of several strains of the commensal *Neisseria* species *N. subflava* and *N. sicca* (107), which raises the possibility that close associations between pharyngeal gonococci, meningococci, and commensal *Neisseria* could ultimately result in the presence of aminoglycoside-modifying enzymes in gonococci (though this has not yet been reported).

Spectinomycin was marketed specifically for the treatment of gonococcal infection in the 1960's and became first-line therapy among U.S. military personnel stationed in Korea in the early 1980's. However, treatment failures in that population reached 8.2% after only four years (105, 108). The first clinical isolate (strain 81-075927) of penicillinase-producing *N. gonorrhoeae* (PPNG) resistant to spectinomycin was found in 1981 (109). The emergence of this dually-resistant strain was significant because just 2 years previously, the CDC had recommended spectinomycin for use as an alternative

therapy for uncomplicated anogenital infections failing penicillin therapy (110). The spectinomycin-resistant phenotype of the strain 81-075927 was linked to streptomycin resistance, and as no spectinomycin-inactivating enzyme could be found, it was hypothesized that the resistance mechanism involved modification of the 30S ribosome due to chromosomal changes in genes encoding ribosomal components (see Sparling studies above). It was later found that decreased susceptibility to spectinomycin in *N. gonorrhoeae* is due specifically to one or more of the following: 1) a C1192U transition mutation in 16S rRNA helix 34 (111, 112) 2) deletion of codon 27 (valine) or a K28E mutation in the *rpsE* gene encoding ribosomal protein S5 (113), and 3) a T24P mutation in ribosomal protein S5 (114). Between the rapid onset of resistance, problems with drug availability (115), and limited efficacy for pharyngeal gonorrhea (116), it would seem that spectinomycin is no longer a reliable treatment alternative.

On the other hand, with the rise in gonococcal resistance to extended spectrum cephalosporins and widespread resistance to other classes of antibiotics, the aminoglycosides have recently become an important future option for first-line antigonococcal therapy for a number of reasons; they are readily available, relatively cheap when compared to cephalosporins (117), and have been used with great success for 14 years in the African country of Malawi (118) with no apparent development of resistance. Similar data showing the lasting efficacy of gentamicin and kanamycin in Indonesia are also available (119, 120). Furthermore, a Phase IV clinical trial conducted by the National Institute of Allergy and Infectious Diseases (121) showed that gentamicin (in combination with azithromycin) demonstrated a 100% cure rate against uncomplicated lower genital tract gonococcal infections (202/202 patients cured)(53).

Cure rates were also 100% for rectal and pharyngeal infections (n=1 and n=10, respectively), but those study groups were considerably smaller and should be revisited.

Importantly, there are no known gentamicin resistance mechanisms in the gonococcus. While there are some reports of decreased gentamicin and kanamycin susceptibility in Ethiopia and Mozambique (122), the lack of a standardized clinical MIC breakpoint makes it difficult to gauge what "resistance" actually means on a μg/mL scale (123), and the mechanisms providing resistance were not explored. One important drawback to aminoglycoside therapy is the risk of oto- and nephrotoxicity. Aminoglycosides are known to have toxic side effects on the inner ear and kidneys in a relatively large portion of recipients (up to 25%) (124), depending on dosage. Nevertheless, if used carefully, these drugs will undoubtedly become an essential tool for gonococcal antibiotic therapy in the approaching decade.

VI: Gonococcal resistance to host antimicrobial factors

As stated above, the gonococcus has evolved or acquired numerous mechanisms of resistance that have allowed it to persist as global health problem despite our best efforts at antibiotic chemotherapy. However, it is important to remember that this pathogen was able to survive the human immune system long before the discovery and use of antibiotic drugs or even chemical/botanical therapies. This is, in and of itself, a remarkable feat, given the large number of host defense mechanisms arrayed against *N. gonorrhoeae* infection. These include but are not limited to: mechanical removal by urination; competition with host flora; chemical defenses such as pH, fatty acids, and bile salts; the presence of lysozyme and other antimicrobial proteins and peptides on epithelial surfaces and in the body fluids; phagocytosis by phagocytes (e.g. neutrophils and

macrophages) that exposes gonococci to a wide variety of killing mechanisms (oxidative and nonoxidative); iron-limitation, nutrient starvation, and oxygen deprivation; secretory IgA antibody at mucosal surfaces, and complement-mediated killing.

Work from the Shafer lab has long been associated with advances in the field of gonococcal host-resistance mechanisms, especially those involving gonococcal iron-acquisition and survival of host antimicrobials, which are in large part the focus of this work. These topics will be discussed below.

a. Factors involved in iron-acquisition

All organisms require iron for growth, but the host restricts the availability of free iron through the use of iron-binding proteins. This element is an essential cofactor for numerous enzymes, metalloproteins, and prosthetic groups such as heme. In the laboratory, *in vitro* cultures of gonococci are routinely supplemented with iron nitrate [$Fe(NO_3)_3$] but *in vivo*, iron is not so easily obtained. In the host, iron is sequestered away from invading pathogens through the iron-limiting innate immune defense—in this way, the host can limit growth and replication of microbes by starving them for this essential nutrient. During infection, liver hepatocytes (and to a lesser extent macrophages and adipocytes) respond to pathogen-associated signals by increasing expression of the master iron-regulating hormone, hepcidin (a 25 amino acid peptide). Hepcidin acts to limit the amount of iron available in extracellular fluids by downregulating and initiating the degradation of the cell membrane iron exporter, ferroportin, in the small intestine, liver, and macrophages (125, 126). In addition to their role as innate immune cells, macrophages also play a key role in normal iron homeostasis by degrading old and senescent red blood cells (RBCs) in a process called erythrophagocytosis, during which

splenic and hepatic macrophages engulf nonfunctional RBCs and liberate the iron-containing heme.

Host iron restriction. The vast majority of iron required for generation of new red blood cells (erythropoeisis) is recycled during erythrophagocytosis (~25 mg/day). In contrast, only about 1 mg/day of iron comes from dietary uptake (127, 128). Because of this, macrophages are important sources of iron and as they are frequently in close contact with pathogens, several mechanisms exist to keep macrophagic iron away from microbes. In response to hepcidin, macrophages degrade ferroportin and decrease ferroportin expression (129), as mentioned above. Furthermore, a divalent cation transporter, NRAMP1—which transports iron between the cytosol and late endosomes/phagolysosomes—is important for limiting early replication of intracellular pathogens (130-132). Whether NRAMP1 works to keep iron in or out of the phagosomal compartment is unclear, but it has been hypothesized that movement of iron into this compartment could increase the production of ROS by Fenton and Haber-Weiss reactions, thus damaging engulfed microbes; alternately, removal of iron to the cytosol by NRAMP1 would keep this important nutrient away from iron-starved, phagocytosed microbes (133). Iron that has left the phagosomal compartment can be 1) safely stored in cytosolic ferritin cages, 2) incorporated into iron-sulfur clusters, or 3) chelated by the small-molecule iron-binding mammalian siderophore 2,5-DHBA[3] (136). Microbial siderophores are scavenged by the host protein NGAL, which binds extracellular siderophores and trafficks them to macrophages for degradation and iron release (137-139). Apart from these responses, the host routinely keeps a tight hold on iron by keeping

[3]Interestingly, the mammalian siderophore 2,5-DHBA differs from the *E. coli* siderophore 2,3-DHBA only by the position of a single hydroxy group, and both of these compounds can be trafficked by the host protein NGAL to control extracellular iron levels whether during health or infection [see (134-135)].

it bound up in transferrin (blood and extracellular fluids) (136) and lactoferrin (secreted body fluids such as saliva, tears, milk, and semen) (140). This iron containment scheme is essential for limiting the production of damaging reactive oxygen species (ROS) produced by the Fenton and Haber-Weiss reactions (141, 142) and keeping iron away from microorganisms.

Gonococcal iron-acquisition systems. Clearly, despite all of the ways the host attempts to keep iron away from pathogens, the gonococcus scavenges enough to grow and replicate *in vivo*, and it does this using a variety of "iron-piracy" mechanisms that enable it to steal this critical element from the host. Unlike some other bacteria, gonococci do not produce siderophores, and must steal iron directly from human iron-binding proteins such as transferrin (hTF), lactoferrin, hemoglobin. Gonococci use a series of surface-exposed iron transportation mechanisms to acquire iron from these host proteins (TbpA/B, LbpA/B, and HpuB/A, respectively, where the first protein listed is the TonB-dependent transporter)(143). The TbpBA transferrin binding proteins encoded by *tbpB* and *tbpA* are perhaps the best-studied iron transporter system in gonococci, and were recently structurally characterized using highly homologous meningococcal orthologues (144). TbpBA are of interest to this work and will be elaborated on further.

TbpA is an integral membrane protein comprised of 22 transmembrane beta-strands that form a β-barrel structure and an N-terminal periplasmic "plug" domain (143). While TbpA is essential for iron piracy from hTF (145), its partner, the surface-bound TbpB lipoprotein, is dispensable for growth on hTF as the sole iron source. However, TbpB greatly enhances the efficiency of this growth for reasons described below (146). The importance of this system for gonococcal pathogenesis was made clear by

experiments demonstrating that *tbp*-deficient gonococci do not cause disease in a human male volunteer model of lower genital tract infection (147).

Gonococci are thought to remove iron from hTF in 6 steps: 1) The C-terminal lobe of the bilobular hTF-Fe^{3+} is initially attracted to TbpB on the gonococcal surface and binds TbpA at numerous hTF-specific sites (this binding is enhanced by TbpB, which together with TbpA forms an enclosed chamber around the iron binding cleft of hTF-Fe^{3+}); 2) movement of a protruding TbpA loop and the TbpA L3 helix finger towards bound hTF-Fe^{3+} destabilizes the iron-binding cleft of the C-lobe; 3) the liberated Fe^{3+} ion is released directly into the TbpA transporter, where it is drawn towards the N-terminal plug domain by an acidic "EIEYE" 5-residue sequence; 4) TonB [an envelope protein that uses ExbB/ExbD-transferred energy derived from the proton motive force to affect changes at the outer membrane (148)] binds to the iron-loaded plug domain of TbpA at its TonB box sequence, drawing the plug downward to face the periplasmic side of the outer membrane; 5) the periplasmic ferric binding protein FbpA, which now has access to the iron-loaded TbpA plug domain, binds to TbpA and receives iron; 6) ferrated FbpA transports iron to the cytoplasmic permease FbpB, which is powered by the FbpC ATPase and transports iron into the cytoplasm (143, 144).

TbpB and TbpA are encoded in an operon (*tbpBA*) and are under the transcriptional control of the ferric uptake regulator, Fur (149, 150). When intracellular iron levels are high, Fur can be bound by ferrous (Fe^{2+}) iron, dimerize, and bind to the *tbpBA* promoter to silence transcription. In contrast, under low levels of intracellular iron Fur is not ferrated, releasing it from the *tbpBA* promoter and derepressing the operon (151). In *N. gonorrhoeae*, Fur+Fe^{2+} is directed to the *tbpBA* promoter by an ~15 bp

operator site known as a "Fur-box" (152-155). Aside from Fur-related regulation, little is known about the factors that influence expression of *tbpBA* genes. It has been hypothesized that the *tbpBA* transcript contains a hairpin loop between *tbpB* and *tbpA*, and that a 2-fold difference in transcript levels of these genes under iron-limiting conditions could be due to loop effects on transcription (156). The reader will recall that TbpBA is currently a vaccine target (see above), and knowledge of factors that influence its expression in the host would be most valuable for future vaccine studies.

Remarkably, gonococci can even acquire iron that has already been stolen from the host, by binding and transporting siderophores produced by other bacteria. One well-studied siderophore receptor is the ferric enterbactin transporter, FetA, which is capable of binding a variety of catecholate-type siderophores produced by other bacteria (a.k.a. "xenosiderophores")(157, 158). Once bound at the gonococcal surface, siderophore-associated iron is moved into the periplasm by the FetA TonB-dependent transporter, which releases the iron to the periplasmic binding proteins FetB or FetB2. Iron is then moved into the cytoplasm by an inner membrane ATP transport system comprised of FetCDEF (143). Under iron replete conditions, *fetA* is subject to repression by Fur+Fe^{2+} (150, 152), but is derepressed and can be activated by the AraC-family transcriptional regulatory protein, MpeR (159, 160) when iron levels are low (158). *fetA* is also phase variable due to a poly-C tract in its promoter that modulates transcription (161).

b. Factors mediating resistance to host cationic antimicrobial peptides

Antimicrobial peptides (AMPs) are one of the most ancient and effective means of killing microbes. These polypeptides can be found in nearly all forms of life (eukaryotes and prokaryotes), and range in size from 2 [gageotetrin A; (162)] to nearly

200 [TnGlv2; (163)] amino acids (aa) in length [see the Antimicrobial Peptide Database at http://aps.unmc.edu/AP/main.php; (164, 165)]. AMPs vary widely in their structure and function, and many (among other functions) can kill protozoa, bacteria, and viruses. In the war against bacteria, useful AMPs are usually cationic (CAMPs), which directs binding to the anionic surfaces of prokaryotes. CAMPs are usually small (12-50 aa), quite basic (+2 to +9 charge), and are partially hydrophobic (166). CAMPs are thought to kill bacteria by binding to the negatively-charged bacterial surface, entering into the membrane(s), and forming holes or pores (167). This process both destroys the electrochemical gradient between the cell exterior (or the periplasm in Gram-negatives) and the cytoplasm of the bacterium, and enables water to flow freely into the cell causing death by loss of proton motive force, leakage of cell contents, and/or lysis (166, 168). Interestingly, some CAMPs, e.g. pyrrhocoricin (169), can also kill bacteria through intracellular means, by binding to DNA, RNA, protein folding machinery, etc. and disrupting their normal functions (170).

During infection, gonococci are exposed to many CAMPs, which are constitutively produced by phagocytes and can be inducibly produced by both phagocytes and mucosal epithelial cells in response to injury or infection. There are 5 known ways in which gonococci resist killing by CAMPs: 1) downregulation of host CAMP expression, 2) delayed lysosomal fusion with gonococcal phagosomes, 3) hindrance of CAMP access to the gonococcal surface, 4) CAMP efflux, and 5) gonococcal surface modifications (see also Chapter 6 (Mechanisms and Significance of Bacterial Resistance to Human Antimicrobial Peptides).

Downregulation of host CAMP expression. In 2001, Islam and colleagues reported that enteric bacteria, in addition to their well known surface-mediated CAMP resistance, could actively downregulate host CAMP expression (171). This finding, when applied to gonococcal infection (which is able to persist despite a large influx of CAMP-producing PMNs), begged the question: can gonococci downregulate expression of CAMPs too? Seeking an answer to this question, Bergman and colleagues (172) tested whether gonococcal infection had any effect on the expression of the CAMP termed LL-37 (the α-helical human cathelicidin) in human cervicovaginal epithelial cells. Indeed, the Bergman study found that gonococcal infection downregulates LL-37 at the mRNA and protein level, and that this effect was dependent on the presence of an unknown heat labile factor associated with live gonococci (heat killed gonococci and gonococcal lysate could not reproduce the decrease in LL-37 expression). Furthermore, LL-37 expression was reduced by gonococcal infection even in the presence of the potent LL-37 inducer, butyrate. The authors hypothesized that the unknown heat labile gonococcal factor likely interrupts some step in the pathway between butyrate exposure and LL-37 upregulation. However, no further work has yet been published to more fully explain the mechanism of LL-37 downregulation by gonococci.

Delayed lysosomal fusion with gonococcal phagosomes. During studies aimed at determining cellular mechanisms that contribute to survival of gonococci in PMNs, a surprising discovery was made—GC can actually slow the fusion of primary (azurophilic) granules with the maturing phagolysosome (173). Primary granules are the last type to fuse with the phagolysosome, and contain powerful antimicrobials [for reviews, see (174, 175)]. Primary granule contents shown to possess direct antimicrobial

activity independent of any additional proteolytic function include lysozyme (176, 177), α-defensins (178), bactericidal/permeability-increasing protein (BPI)(179), cathepsin G (180-182), and neutrophil elastase (183). Thus, given the high CAMP-mediated antimicrobial potential of primary granules, gonococcal evasion of primary granule fusion would be expected to contribute to the viability of gonococci in the host.

Hindrance of CAMP access to the gonococcal surface. It is well known that many bacteria can produce attached or secreted polymers that slow or stop the movement of CAMPs toward the negatively-charged outer surface of the bacterium; some examples include staphylococcal polysaccharide intercellular adhesin (PIA), alginic acid production by *Pseudomonas aeruginosa*, capsular polysaccharide made by *N. meningitidis*, and generation of glycoprotein S-layers by both eubacteria and archaebacteria in the environment (for a review, see Chapter 6 Mechanisms and Significance of Bacterial Resistance to Human Antimicrobial Peptides). Unlike its close cousin *N. meningitidis*, the gonococcus does not produce capsule. However, it may utilize small, positively-charged, host-derived polyamines as a “cloaking” mechanism to shield itself against host CAMPs. Indeed, Goytia and Shafer found that biologically-relevant concentrations of spermine (a polyamine found in the human urogenital tract) could decrease the killing efficacy of the model CAMP polymyxin B (PMB) by 32-fold and the human CAMP LL-37 by 5-10 fold (184).

Furthermore, the formation of gonococcal biofilms (185) *in vivo* (186) may help keep CAMPs from getting close to the outer membrane. In contrast to the free-floating, planktonic bacteria of *in vitro* broth culture, in nature bacteria usually exist incorporated into biofilms (187). Biofilms are large ultrastructures formed by a combination of

bacterial cells and secreted/released components that form an extracellular matrix (188). Gonococcal biofilms are now known to be largely comprised of DNA and shed outer membrane vesicles, and likely contain many gonococci that are metabolically less active (189). Thus, decreased respiration or shielding by extracellular CAMP-binding molecules (e.g. DNA, lipid A) may provide some protection from the killing activities of CAMPs. Interestingly, spermine has been shown to inhibit gonococcal biofilm formation *in vitro* (190); this mechanism may exacerbate the dispersal of gonococci during transmission events and provide some protection from CAMPs [see above: (184)] until biofilms can be established.

Gonococcal efflux of CAMPs. Bacterial efflux (i.e. the energy-dependent movement of toxic substrates from the cell interior to the cell exterior through large envelope-bound protein machines) is one of the key factors contributing to the global antibiotic resistance crisis. Due to the complex nature of these structures and their ubiquity throughout nature, it is thought that they existed long before the clinical use of antibiotics as a way for bacteria to rid themselves of toxic chemicals present in the environment or antimicrobial substrates produced by competing microbes and host organisms (191). There are five known efflux systems expressed by *N. gonorrhoeae*. These are (in order of discovery), MtrCDE (spermicides, hydrophobic antibiotics and host antimicrobials, detergents, dyes, and CAMPs)(81), FarAB-MtrE (fatty acids)(192), Mef (found only in rare clinical isolates; exports macrolides)(193), NorM (quarternary ammonium compounds)(194), and MacAB (macrolides)(195). In addition, recent evidence suggests that MtrF can expel sulfa drugs from the cell interior (W.M. Shafer and E.W. Yu, personal communication).

Of these, only MtrCDE has been shown to be important for gonococcal resistance to CAMPs. Thus far, it has been demonstrated that loss of a functional MtrCDE pump results in enhanced susceptibility to porcine protegrin-1 (PG-1) and its linearized variant PC-8, horseshoe crab tachyplesin-1 (TP-1), the human cathelicidin LL-37 (196), and PMB [meningococcus: (197); unpublished observations in the gonococcus: see Chapter 7]. The MtrCDE efflux pump is powered by the proton motive force (198) and is constructed from three different proteins: (i) MtrD, the inner membrane pump (199), (ii) MtrE, the outer membrane channel through which antimicrobials are expelled to the extracellular milieu (200), and (iii) MtrC, which exists as six envelope-spanning adaptor proteins that connect the pump and the channel. This system is suggested to have an assembled stoichiometry of 3:6:3 [MtrD(3):MtrC(6):MtrE(3)] (201). The production of such a large multimeric complex is costly for the cell and must be tightly controlled. Below, I will discuss the current known mechanisms that impact MtrCDE expression and function.

In GC, the *mtrCDE* operon is repressed by MtrR, a TetR family transcriptional regulator (202-204). In contrast, MtrR's counterpart, MtrA, is an AraC/XylS family transcriptional activator of *mtrCDE*, and is required for inducible resistance (84). This family of proteins typically binds to ligands at an unconserved N-terminus or central region (205). Its homology to ligand-binding activators suggests that MtrA can activate *mtrCDE* in response to the presence of antimicrobials. In support of this, the MtrA binding site at the *mtrCDE* promoter was found to be near that of MtrR, and MtrA binding could be enhanced by preincubation of MtrA with a nonionic detergent, TX-100 (a known MtrCDE substrate)(85); the authors propose that MtrA and MtrR compete for

binding at the *mtrCDE* promoter, and that the presence of MtrCDE pump substrates leads to increased *mtrCDE* transcription through the above mechanism. Furthermore, susceptibility to MtrCDE pump substrates can be regulated indirectly through an iron-responsive, MpeR-dependent mechanism wherein low concentrations of iron release Fur from the *mpeR* promoter, leading to direct MpeR repression of the *mtrR* gene and thus derepression of *mtrCDE* (160, 206). These regulatory relationships facilitate the dynamic expression of the efflux pump to defend against antimicrobials when needed, and to limit needless metabolic costs when conditions are more favorable.

The *mtr* (multiple transferable resistance) system was discovered when a single mutation in GC was found to confer increased resistance to a panel of structurally diverse antimicrobials (207). This was the first of several mutations that were found to increase expression of the MtrCDE efflux pump. For instance, mutations in the *mtrR* open reading frame or its promoter can decrease or eliminate repression of the efflux pump by inhibiting MtrR binding or expression (81, 86, 208). Moreover, mutations in the *mtrR-mtrCDE* intergenic region, such as the deletion of a single nucleotide in a 13bp inverted repeat (81) and the presence of a second promoter 120 bp upstream of the *mtrC* start codon (83) can greatly enhance transcription of *mtrCDE*. For a complete description of these mutations and their effects on gonococcal fitness *in vitro* and *in vivo*, see (67, 88, 209).

As stated above, LOS structure is important for resistance to CAMPs. Interestingly, it was later found that gonococci with a truncated LOS structure (strain WS1) were unable to reach normal levels of resistance to the MtrCDE pump substrate TX-100 when overexpressing *mtrCDE* due to a deletion in the *mtrR-mtrCDE* 13bp

inverted repeat (198). However, this phenomenon did not apply to crystal violet or erythromycin, which are also MtrCDE substrates. The authors concluded that gonococci have different LOS structural requirements in order for MtrCDE to efficiently pump out various hydrophobic antimicrobials. Furthermore, the presence of a putative inner membrane permease, MtrF, is necessary for high level resistance to TX-100 (159, 210). Though the exact contribution of MtrF to MtrCDE-mediated resistance remains unclear, it is hypothesized that MtrF may synergistically interact with MtrCDE to provide inducible resistance to antimicrobials.

Gonococcal surface modifications. One of the most thoroughly studied mechanisms of bacterial CAMP resistance is modification of the bacterial surface. Generally, resistance is accomplished by decreasing the net-negative charge of the outermost surface of the bacterium, thus making it less attractive to positively-charged CAMPs. For Gram-negative organisms, the outer membrane (OM) is generally the first layer of the bacterial cell wall encountered by antimicrobials once they have passed any unattached bacterial products or capsular structures. The OM is composed primarily of lipopolysaccharide (LPS) in the outer leaflet and phospholipids in the inner leaflet (211). LPS is comprised of three domains; a hydrophobic lipid A anchor, a core oligosaccharide, and an O-antigen polysaccharide of variable length. Numerous modifications can be made to LPS (212) and are often targeted towards the lipid A domain (which has negatively-charged phosphate groups) but can also occur at the core oligosaccharide. Examples include the decoration of lipid A with positively-charged small molecules such as 4-amino-4-deoxy-L-arabinose (L-Ara4N) and PEA, dephosphorylation of the β-1',6-linked glucosamine disaccharide component of lipid A,

decoration of core oligosaccharide with zwitterionic phosphorylcholine, and acylation of lipid A to decrease membrane permeability (these and many other envelope modifications related to CAMP resistance are reviewed in Chapter 6: Mechanisms and Significance of Bacterial Resistance to Human Antimicrobial Peptides).

Gonococci are known to have a highly variable surface structure, a trait which has greatly contributed to the difficulty of making a vaccine and the inability of the host to mount a protective memory immune response to gonococcal infection. The importance of LOS oligosaccharide for gonococcal CAMP resistance was demonstrated in a series of reports by Shafer and colleagues in which it was shown that cathepsin G[4] could kill gonococci independently of any enzymatic activity (182), and that it could bind to (214) and kill (181) gonococci with a truncated LOS structure (strain WS1) much better than an isogenic parent strain with a normal LOS structure (strain FA102). These findings suggested that sugar residues might help to occlude negatively-charged sites at the gonococcal surface, hindering the binding of cathepsin G and thus decreasing its ability to kill.

Perhaps the most phenotypically dramatic mechanism that protects gonococci from CAMP attack is the decoration of the 4' (and rarely) 1 positions of lipid A with PEA. The amino group of PEA and the nature of the attachment at the phosphate moiety diminishes the negative charge of the gonococcal outer surface. This modification is carried out by the LptA lipid A phosphoethanolamine transferase (encoded by *lptA*), and gonococci that do not express *lptA* have greatly increased susceptibility to the model CAMP, PMB (215). PMB is a cyclic lipopeptide produced by the bacterium *Bacillus*

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[4] Interestingly, it was later found that a small peptide sequence within cathepsin G, HPQYNQR, is similar to a sequence within the cytotoxic T-cell protein granzyme B, and that both are bactericidal against gonococci [see (213)].

polymyxa, and has been used to characterize CAMP resistance mechanisms in numerous studies due to its ease of use and low cost. Amphipathic in nature, PMB is comprised of a positively-charged hexapeptide ring covalently bound to a hydrophobic fatty acid tail (216). Thus, PMB initiates entry into bacterial cells via ionic attractions between the PMB hexapeptide ring and lipid A phosphates, followed by invasion of the membrane by the PMB acyl tail, ultimately resulting in the destruction of the cell membrane and lysis [(217); for an excellent review of antimicrobial peptide killing models see (218)].

In other bacteria, modification of lipid A is known to be a highly-regulated process, and can be influenced by environmental factors like divalent cation (Mg^{2+}, Ca^{2+}) concentration, acidic conditions, and CAMP stress (212). The current paradigm for this is centered around the PhoP/PhoQ two component regulatory system (TCS)(219). TCSs are virtually ubiquitous in bacteria, and constitute one of the main mechanisms by which bacteria sense and response to changes in their microenvironment. They are usually composed of an integral cytoplasmic membrane sensor kinase and a cytoplasmic DNA binding response regulator. When the correct signal is detected, the sensor kinase is autophosphorylated at a conserved histidine residue. This phosphoryl group can then be transferred to the cytoplasmic response regulator at a conserved aspartic acid residue. This transfer changes the conformation of the response regulator such that it can bind to different sites on the chromosome and regulate genes as part of a coordinated response to the environmental stimulus (220).

Decreased levels of divalent cations cause envelope stress due to ionic repulsion between negatively charged phosphates in the outer membrane of Gram-negative bacteria. In *Salmonella*, it has been shown that the PhoQ sensor kinase possesses two

acidic α-helices (α-4 and α-5) that, under PhoQ-repressing conditions, lie close to the acidic outer leaflet of the cytoplasmic membrane. This interaction is only allowed to occur when divalent cations are present to act as metal bridges between these two negatively-charged surfaces. However, when 1) divalent cations are lacking, 2) proton levels are high (acidic conditions), or 3) CAMPs are present, these bridges are lost or disrupted. Once the α-helices are released from their quiescent state against the membrane, PhoQ is activated, autophosphorylates, and can phosphorylate PhoP to initiate regulation of genes to defend the bacterium from these unfavorable conditions (219). One of the phosphorylated PhoP (PhoP~P) targets is *pmrD*, which encodes a protein (PmrD) that stabilizes the phosphorylated state of another response regulator, PmrA. PmrA is part of a TCS termed PmrAB, which becomes indirectly activated by PhoQ-activating conditions due to this mechanism. PmrA~P in turn activates the expression of *pmrC* (a.k.a. *eptA*), which encodes a phosphoethanolamine transferase responsible for PEA decoration of lipid A. This modification greatly increases resistance of *Salmonella* to CAMPs such as PMB (221).

Currently, there are no published reports of *lptA* (the *pmrC* homologue) regulation in *Neisseria gonorrhoeae*. One possibility is that *lptA*, like *pmrC* in *Salmonella*, could become activated by a TCS under inducing conditions. There are 4 known TCSs in gonococci: MisRS [a conserved homologue of the meningococcal MisRS reported in (222-225)], NarPQ (226), BmrRS (a.k.a BasRS) (227), and NtrXY (228)[PilAB was originally thought to be a TCS that controlled pilus expression, but was later shown to be an FtsY homologue (229-232)]. They are involved in CAMP resistance, oxygen-limited

growth, outer membrane blebbing, and respiration, respectively. Of these, MisRS is the most likely to be involved in *lptA* regulation, and will be discussed further.

Despite equal or greater sequence similarity to other TCSs, MisRS was originally termed "PhoPQ" in *N. meningitidis* because like *Salmonella*'s PhoP, MisR appeared to be required for resistance to CAMPs and growth at low concentrations of Mg^{2+} (222), and was avirulent in a mouse model of intraperitoneal infection (223). However, it was subsequently renamed "MisRS" because of the regulatory impact of MisR on meningococcal LOS inner core structure (224) and experiments showing that unlike *Salmonella* PhoP, *N. meningitidis* MisR is not involved in resistance to acidic pH (222), does not regulate *mgtA*—one of the most strongly PhoP-regulated genes in *Salmonella* [see discussion sections in (224, 233)]—and is not induced by low Mg^{2+} concentrations (225). Studies also revealed that MisR is important for CAMP resistance (222-224), *in vivo* pathogenesis (223, 234), and *in vitro* toxicity (235, 236). Additionally, MisR has been shown to regulate numerous pathogenesis genes in meningococci encoding products involved in modification of LOS, iron-acquisition, and other important virulence factors (224, 233, 237, 238). Importantly, *misR*-deficient meningococci showed a modest but reproducible 2-fold decrease in transcription of *lptA* (224). Since the gonococcal and meningococcal *misR* genes are highly homologous, it is possible that MisR impacts gonococcal CAMP resistance and that *lptA* expression might be regulated by this TCS but evidence for this is lacking.

Specific Aims

Even with the vast body of research on the topics of gonococcal survival of the innate immune system, several questions still remain. What factors are important for gonococci to acquire iron when inside iron-rich macrophages? How is expression of *lptA*, and thus decoration of lipid A with PEA, controlled? Moreover, what other mechanisms might contribute to the survival of a small percentage of phagocytosed gonococci inside of CAMP-rich PMNs? I investigated these questions using the following specific aims:

1. Define the MisR regulon in gonococci and determine what regulatory targets contribute to CAMP resistance.
2. Determine if gonococcal survival in macrophages is associated with differences in expression of iron-acquisition genes.

Figures

Figure 1: The era of efficacious penicillin therapy

Figure 2: US Army Air Forces public service announcement warning soldiers about STIs (circa 1940)

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Chapter 2: Lipid A's structure mediates *Neisseria gonorrhoeae* fitness during experimental infection of mice and men

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Lipid A's Structure Mediates *Neisseria gonorrhoeae* Fitness during Experimental Infection of Mice and Men

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ABSTRACT **Phosphoethanolamine (PEA) on *Neisseria gonorrhoeae* lipid A influences gonococcal inflammatory signaling and susceptibility to innate host defenses in *in vitro* models. Here, we evaluated the role of PEA-decorated gonococcal lipid A in competitive infections in female mice and in male volunteers. We inoculated mice and men with mixtures of wild-type *N. gonorrhoeae* and an isogenic mutant that lacks the PEA transferase, LptA. LptA production conferred a marked survival advantage for wild-type gonococci in the murine female genital tract and in the human male urethra. Our studies translate results from test tube to animal model and into the human host and demonstrate the utility of the mouse model for studies of virulence factors of the human-specific pathogen *N. gonorrhoeae* that interact with non-host-restricted elements of innate immunity. These results validate the use of gonococcal LptA as a potential target for development of novel immunoprophylactic strategies or antimicrobial treatments.**

IMPORTANCE **Gonorrhea is one of the most common bacterial sexually transmitted infections, and increasing antibiotic resistance threatens the use of currently available antimicrobial therapies. In this work, encompassing *in vitro* studies and *in vivo* studies of animal and human models of experimental genital tract infection, we document the importance of lipid A's structure, mediated by a single bacterial enzyme, LptA, in enhancing the fitness of *Neisseria gonorrhoeae*. The results of these studies suggest that novel agents targeting LptA may offer urgently needed prevention or treatment strategies for gonorrhea.**

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Gonorrhea remains a global health problem. The worldwide incidence of *Neisseria gonorrhoeae* infection equaled or surpassed that of *Chlamydia trachomatis* as the most common bacterial sexually transmitted infection for the first time in 2008 (1). Increasing bacterial resistance to antibiotics used to treat gonorrhea, coupled with a dearth of new antimicrobial therapies in development, raises the specter of incurable *N. gonorrhoeae* infections (2). The potential of adverse reproductive health consequences of untreatable gonococcal infections as well as increased HIV transmission in areas where both infections are prevalent is alarming (3). Identification of new therapeutic and vaccine targets for gonorrhea may be more important now than ever before.

The most abundant lipid constituent of the *N. gonorrhoeae* outer membrane is lipooligosaccharide (LOS), a glycolipid comprised of an antigenically variable oligosaccharide core (4) attached to lipid A, which is frequently decorated by phosphoethanolamine (PEA) at the 4′ position (5, 6). The presence or absence of PEA-decorated lipid A (PEA-lipid A) profoundly influences inflammatory signaling (7, 8) and bacterial susceptibility to innate host defenses, including the bactericidal activities of normal human serum, complement, and cationic antimicrobial peptides (CAMPs) (5, 9). To assess the importance of this structure during human infection, we constructed PEA transferase (*lptA*) deletion mutants of *N. gonorrhoeae* strain FA1090, which cannot add PEA to lipid A, and tested FA1090 Δ*lptA* in competitive infections with isogenic *lptA*$^{+}$ gonococci in the female murine lower genital tract and the human male urethra.

Strains. *N. gonorrhoeae* FA1090 is a porin serotype PIB-3, streptomycin (Sm)-resistant strain that has been used extensively in experimental human infection studies (10). Bacteria were cultured on gonococcal agar (GC agar) supplemented with Kellogg's supplement I and ferric nitrate or in GC broth as described previously (11) with or without antibiotics as appropriate. Cultures were incubated at 35 to 37°C with 5 to 7% CO_2.

FA1090 Δ*lptA* was constructed without altering the antibiotic susceptibility of the wild-type strain, as previously described (12). Briefly, a two-gene cassette containing both a selectable marker (chloramphenicol [Cm] acetyltransferase [CAT] conferring Cm resistance) and a counterselectable marker (*rpsL*, conferring Sm sensitivity on the naturally resistant strain FA1090) was cloned

into the *lptA* gene and used to replace the wild-type gene in the FA1090 chromosome by allelic exchange; transformants were selected on GC agar with 1 μg Cm/ml. The resulting intermediate strain was Cm resistant and Sm sensitive. A second transformation replaced the CAT *rpsL* cassette with an unmarked deletion encompassing approximately 80% of the *lptA* coding sequence; transformants were selected on GC agar with 100 μg Sm/ml. The resulting strain, FA1090 Δ*lptA*, was Cm sensitive and Sm resistant, as is wild-type strain FA1090. PCR amplification of the *lptA* locus and analysis of the FA1090 Δ*lptA* genome sequence confirmed deletion of the gene in the mutant. The wild-type *lptA* gene from strain FA19 (identical to that of FA1090) was introduced into the FA1090 deletion mutant using the pGCC4 $lptA^+$ vector as previously described (5). The wild-type *lptA* gene is under the control of a *lac* promoter and is positioned between *lctP* and *aspC* in C′FA1090 Δ*lptA*.

Electrophoretic characterization of gonococcal LOS and lipid A biochemical analyses. Whole-cell proteinase K-digested lysates of gonococci were resolved by Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and LOS bands were identified by silver staining and Western blotting with monoclonal antibody 3F11 as previously described (9). Lipooligosaccharides were purified from *N. gonorrhoeae* strains grown in GC broth, and lipid A was isolated by mild acid hydrolysis as previously described (9). Lipid A was purified by extraction with chloroform and methanol; purified material was used for compositional analysis and determination of mass by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) using a model 4700 proteomics analyzer instrument (Applied Biosystems). Oligosaccharides were purified by size exclusion column chromatography; linkage analysis was performed by gas chromatography-mass spectrometry (GC-MS) using an Alltech AT-1 fused-silica capillary column on a Hewlett-Packard HP5890 gas chromatograph equipped with mass selective detector 5970 MSD.

PMB MIC determination. MICs of polymyxin B (PMB) were determined by spotting approximately 10^5 CFU of *N. gonorrhoeae* suspensions onto GC agar containing 2-fold differences in PMB, from 0.19 to 200 μg PMB/ml; the MIC was defined as the highest concentration of PMB at which bacterial growth was observed after a 24-h incubation.

Experimental murine infection. All animal experiments were conducted at the Uniformed Services University of the Health Sciences according to the guidelines of the Association for the Assessment and Accreditation of Laboratory Animal Care under a protocol that was approved by the University's Institutional Animal Care and Use Committee. Female BALB/c mice (6 to 8 weeks old; National Cancer Institute) were treated with water-soluble 17β-estradiol and antibiotics to increase susceptibility to *N. gonorrhoeae* as described previously (13). Groups of mice were inoculated intravaginally with wild-type FA1090 combined with similar numbers of FA1090 Δ*lptA* or C′FA1090 Δ*lptA* CFU (total dose, 10^6 CFU *N. gonorrhoeae*; 7 mice/group). Vaginal swabs were collected every other day for 6 days starting on day 2 postinoculation and suspended in 100 μl GC broth. Vaginal swab suspensions and inocula were cultured quantitatively on GC agar with 100 μg Sm/ml (total number of CFU) and GC agar with Sm plus 10 μg PMB/ml (FA1090 or C′FA1090 Δ*lptA* CFU).

Experimental human infection. Procedures for participant recruitment, informed consent, intraurethral inoculation, and antibiotic treatment were as previously described (11). Experimental human infections were conducted in the Clinical and Translational Research Center of the North Carolina Translational and Clinical Sciences Institute at the University of North Carolina at Chapel Hill according to the guidelines of the U.S. Department of Health and Human Services and the University's Institutional Review Board under a protocol that was approved as a U.S. investigational new device (IND). All study participants provided written informed consent. In two separate trials, volunteers received approximately equal numbers of *N. gonorrhoeae* FA1090 and FA1090 Δ*lptA* CFU (total dose, 10^5 to 10^6 CFU *N. gonorrhoeae*; 3 men/trial). Gonococci in the inoculum suspensions were predominantly opacity-associated adhesin (Opa) negative and piliated and expressed the same PilE sequence as previously characterized FA1090 variants used in experimental human infection studies (14). First-void urine specimens were obtained daily after inoculation. Urine sediment was cultured quantitatively on GC agar with 3 μg vancomycin, 12.5 units nystatin, and 5 μg trimethoprim lactate/ml, which permits the growth of FA1090 and FA1090 Δ*lptA*. Up to 96 colonies per subject per culture were replica plated on GC agar with and without 7.5 μg colistin/ml to distinguish the 2 strains; only wild-type FA1090 grows in the presence of the polycationic antibiotic colistin.

Competitive index calculations and statistics. For experimental murine and human infections, results were expressed as the competitive index (CI) for infected individuals using the equation CI = [mutant CFU (output)/wild-type CFU (output)]/[mutant CFU (input)/wild-type CFU (input)]. For murine infections, in which total CFU and wild-type CFU were enumerated directly from vaginal specimen cultures, the limit of detection of 1 CFU was assigned for a strain that was not recovered from an infected mouse. For human infections, in which total CFU were enumerated directly from urine sediment cultures and up to 96 CFU per specimen was subcultured to identify wild-type or mutant colonies, the limit of detection (1/96 CFU) was assigned for the output proportion of a strain that was not detected from an infected man. A CI of <1 indicates that the mutant is less fit than the wild-type strain. For experimental human infections, we also compared the proportion of wild-type FA1090 among recovered *N. gonorrhoeae* isolates on the final day of participation for each infected subject to the proportion in the inoculum using a single-sample *t* test, with the proportion for the null hypothesis equal to 0.54 (the mean proportion of strain FA1090 in the inoculum). With a clearly directional hypothesis that the $lptA^+$ strain would predominate, we used SigmaStat for Windows, v3.5 (Systat Software, Inc.), to calculate one-tailed statistics, with the type I error rate controlled at 0.05.

Lack of LptA-mediated PEA attachment to lipid A renders *N. gonorrhoeae* susceptible to a model CAMP *in vitro*. PEA addition at the 4′ position of FA1090 lipid A was abrogated in FA1090 Δ*lptA* and restored in the complemented strain C′FA1090 Δ*lptA* (see Fig. S1A to D in the supplemental material). All strains produced 4.2- and 4.5-kDa LOS species; the former reacted with monoclonal antibody 3F11 (data not shown), confirming the presence of the lacto-*N*-neotetraose epitope associated with naturally acquired gonococcal urethritis (15). As expected, deletion of *lptA* rendered FA1090 hypersusceptible to the model CAMP PMB (MICs, 100 μg PMB/ml for FA1090 versus 0.78 μg PMB/ml for FA1090 Δ*lptA*), which was reversed by 32-fold in the complemented strain (data not shown). Importantly, *in vitro* growth rates of FA1090 Δ*lptA* and wild-type FA1090 were indistinguishable

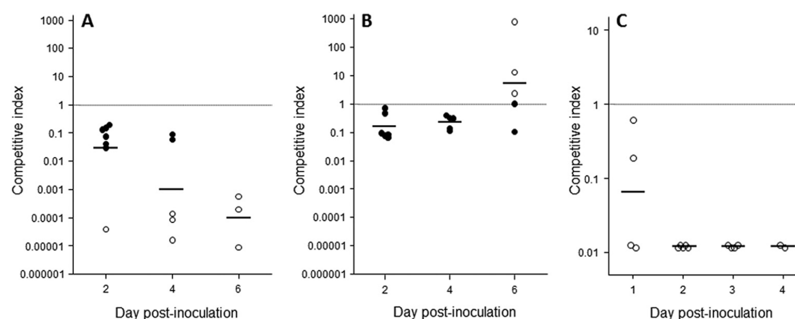

FIG 1 PEA decoration of *N. gonorrhoeae* lipid A confers a competitive advantage during genital tract infection. The competitive index (CI) was equal to [FA1090 Δ*lptA* or C′FA1090 Δ*lptA*/FA1090 (output)]/[FA1090 Δ*lptA* or C′FA1090 Δ*lptA*/FA1090 (input)]. (A) Competitive infections in female BALB/c mice ($n = 7$) with FA1090 Δ*lptA* and FA1090; (B) female BALB/c mice ($n = 7$) with C′FA1090 Δ*lptA* and FA1090; (C) male volunteers ($n = 6$) with FA1090 Δ*lptA* and FA1090. Note the different *y* axis scale in panel C, imposed by the limit of detection of 100-fold-decreased fitness in experimental human infections. (A and C) A CI of <1 indicates that the mutant is less fit than the wild type. Solid circles indicate mice from which both wild-type *N. gonorrhoeae* FA1090 and FA1090 Δ*lptA* were recovered (A) and both wild-type *N. gonorrhoeae* FA1090 and C′FA1090 Δ*lptA*/FA1090 were recovered (B). Open circles indicate infected mice or men from whom only wild-type *N. gonorrhoeae* FA1090 was recovered (A and C) or only C′FA1090 Δ*lptA* was recovered (B). Horizontal bars indicate geometric mean CIs.

(data not shown), and the Δ*lptA* mutant did not show a fitness difference from wild-type FA1090 when cocultured *in vitro* through stationary phase in the absence of selective pressure (data not shown). From mixtures containing similar numbers of Δ*lptA* mutant and wild-type FA1090 cells exposed to 5 μg PMB/ml for 45 min *in vitro*, only wild-type *N. gonorrhoeae* with PEA-lipid A survived (data not shown), confirming the importance of this structure in CAMP resistance.

LptA-mediated PEA attachment to lipid A confers a survival advantage to *N. gonorrhoeae* during competitive infection in mice. Experimental infection of female BALB/c mice has been used to study innate host defenses against *N. gonorrhoeae* and the mechanisms by which gonococci evade these defenses (16–18). Groups of female BALB/c mice were inoculated intravaginally with mixtures containing similar numbers of wild-type FA1090 and either FA1090 Δ*lptA* or C′FA1090 Δ*lptA* CFU (total dose, 5×10^6 to 7×10^6 CFU). The relative number of colonies of each strain recovered from vaginal swabs from infected mice was determined for up to 6 days after inoculation; output ratios of the two strains were normalized to the ratio of strains in the inoculum. We observed a 10- to 10,000-fold decrease in recovery of the *lptA* mutant relative to the wild-type parent strain FA1090 on days 2 to 6 of infection (Fig. 1A). No mutant bacteria were recovered from several mice on multiple days postinoculation, whereas wild-type gonococci were recovered from these mice at levels of $>10^3$ to $>10^6$ CFU/ml vaginal swab suspension (Fig. 1A, open circles). Complementation of *lptA* restored recovery of the mutant by day 6 postinoculation (Fig. 1B). Packiam et al. recently showed similar results with gonococcal strain FA19 during competitive infection in mice (19).

LptA-mediated PEA attachment to lipid A confers a survival advantage to *N. gonorrhoeae* in the male urethra. To determine potential influences of host restriction differences in mice and humans (as well as differences in female and male genital tracts) on the contribution of PEA-lipid A to *in vivo* gonococcal survival, we performed competitive infections with wild-type *N. gonorrhoeae* FA1090 and the *lptA* mutant in the human challenge model of gonococcal urethritis (10). Six subjects were inoculated intraurethrally with approximately equal mixtures of FA1090 and FA1090 Δ*lptA* (total dose, 10^5 to 10^6 CFU). Five subjects (83%) developed gonococcal urethritis 2 to 4 days after inoculation; one remained uninfected for 5 days after inoculation (Table 1). The relative number of each strain recovered was determined by subculturing up to 96 colonies from each positive urine sediment culture on selective media; output ratios of the two strains were normalized to the ratio of strains in the inoculum. Among colonies tested, only wild-type FA1090 was recovered (Table 1). We observed the maximum possible 100-fold decrease in the recovery of the *lptA* mutant relative to that of wild-type FA1090 on days 2 to 4 of infection (Fig. 1C).

In our competitive infection studies, PEA-lipid A clearly provided a substantial fitness benefit to gonococci during infection, both in the human male urethra and in the murine female genital tract, compared to *N. gonorrhoeae* lacking PEA attached to lipid A. The mouse model likely underestimates the importance of PEA-lipid A during infection due to differences in human and murine complement-binding proteins. However, some factors are apparently not host restricted (e.g., CAMP-mediated killing of the *lptA* mutant), confirming that the murine model can provide important information regarding the pathogenesis of gonococcal infection that is translatable to humans. In the mouse model, reduced infectivity resulting from *lptA* deletion in *N. gonorrhoeae* strain FA19 occurs only during competitive infections; noncompetitive infections with wild-type FA19, the FA19 Δ*lptA* mutant, or the complemented FA19 *lptA* mutant produce similar kinetics of vaginal colonization and bacterial recovery (19). However, only wild-type *N. gonorrhoeae* FA19 and the complemented FA19 *lptA* mutant induce proinflammatory host responses to gonococcal infection in female mice. John and colleagues previously demonstrated reduced inflammatory stimulation of human THP-1 cells by an *lptA* null mutant of the related pathogen *N. meningitidis* and by commensal *Neisseria* species that lack *lptA* (20). Thus, PEA decoration of gonococcal lipid A not only increases inflammatory responses *in vitro* and during murine infection but also protects *N. gonorrhoeae* from the consequences of inflammation, including increased CAMP production. Experimental human infection

TABLE 1 Competitive infection of male volunteers with *N. gonorrhoeae* FA1090 and FA1090 Δ*lptA*[h]

Trial	Subject identifier	Inoculum size[a]	Day of treatment[b]	Urethral swab culture	Bacteriuria[c]	Pyuria[d]	Urethritis[e]	% wild-type gonococci		
								Inoculated	Recovered[f]	P[g]
1	100	5.8	3	+	5.2	6.9	+	55	≥99	
	101	5.1	4	+	5.6	7.4	+	55	≥99	
	104	5.5	5	−	ND	4.0	−	55	ND	
										<0.0001
2	110	5.7	2	+	5.5	6.8	+	53	≥99	
	111	5.6	3	+	4.1	5.9	+	53	≥99	
	112	5.4	4	+	5.1	6.3	+	53	≥99	

[a] Log_{10} CFU *N. gonorrhoeae* inoculated intraurethrally.
[b] Day postinoculation.
[c] Log_{10} CFU *N. gonorrhoeae*/ml urine sediment on day of treatment.
[d] Log_{10} white blood cells (WBC)/ml urine sediment on day of treatment.
[e] Greater than 5.8 log_{10} WBC/ml urine sediment (equivalent to >4 WBC/high-power field) on day of treatment.
[f] The limit of detection for recovered FA1090 Δ*lptA* was 1/96 colonies characterized.
[g] Single-sample *t* test for the percentage of wild-type FA1090 *lptA*+ recovered versus the mean percentage inoculated.
[h] +, positive; −, negative; ND, none detected.

studies with inocula containing only *N. gonorrhoeae* FA1090 or FA1090 Δ*lptA* are needed to confirm whether PEA-decorated lipid A plays dual immunostimulatory and protective roles during male urethral infection. The demonstrated impaired fitness of *N. gonorrhoeae* mutants lacking PEA in men and female mice validates gonococcal LptA as an important target for development of novel immunoprophylactic strategies or antimicrobial treatments for gonorrhea in males and females in the face of diminishing treatment options.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00892-13/-/DCSupplemental.

Figure S1, TIF file, 0.1 MB.

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Chapter 3: *Neisseria gonorrhoeae* modulates iron-limiting innate immune defenses in macrophages

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Neisseria gonorrhoeae Modulates Iron-Limiting Innate Immune Defenses in Macrophages

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Abstract

Neisseria gonorrhoeae is a strict human pathogen that causes the sexually transmitted infection termed gonorrhea. The gonococcus can survive extracellularly and intracellularly, but in both environments the bacteria must acquire iron from host proteins for survival. However, upon infection the host uses a defensive response by limiting the bioavailability of iron by a number of mechanisms including the enhanced expression of hepcidin, the master iron-regulating hormone, which reduces iron uptake from the gut and retains iron in macrophages. The host also secretes the antibacterial protein NGAL, which sequesters bacterial siderophores and therefore inhibits bacterial growth. To learn whether intracellular gonococci can subvert this defensive response, we examined expression of host genes that encode proteins involved in modulating levels of intracellular iron. We found that *N. gonorrhoeae* can survive in association (tightly adherent and intracellular) with monocytes and macrophages and upregulates a panel of its iron-responsive genes in this environment. We also found that gonococcal infection of human monocytes or murine macrophages resulted in the upregulation of hepcidin, NGAL, and NRAMP1 as well as downregulation of the expression of the gene encoding the short chain 3-hydroxybutyrate dehydrogenase (BDH2); BDH2 catalyzes the production of the mammalian siderophore 2,5-DHBA involved in chelating and detoxifying iron. Based on these findings, we propose that *N. gonorrhoeae* can subvert the iron-limiting innate immune defenses to facilitate iron acquisition and intracellular survival.

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Introduction

Neisseria gonorrhoeae is a strict human pathogen that causes the sexually transmitted disease gonorrhea with more than 100 million cases estimated yearly world-wide [1]. Gonococci can cause both symptomatic and asymptomatic infections in men and women, which is thought to be dictated by host and bacterial factors that determine the extent of stimulation of the pro-inflammatory response. Combined with the large number of infections worldwide and the medical complications associated with infection, particularly for women, the emergence of antibiotic-resistant strains of gonococci is now a major public health concern with worrisome predictions that gonorrhea may become an untreatable disease unless new antimicrobials are developed [2,3,4].

Gonococci express virulence factors that facilitate infection and promote survival within host phagocytic and epithelial cells [1]. Most work on such intracellular survival has concentrated on how the gonococcus subverts oxidative and nonoxidative killing systems of neutrophils [1,5]. Much less is known, however, regarding how it acquires nutrients for growth, especially iron, during intracellular residence. In this respect, most iron, whether in the extracellular or intracellular environments, is tightly complexed with iron-binding proteins and not readily available for microbes. To circumvent this problem, bacteria can either remove and transport iron to their surface through the action of siderophores or use surface-exposed proteins that bind host proteins complexed with iron (e.g., transferrin, ferritin, lactoferrin, and hemoglobin) [6,7,8]. The host can also influence the ability of bacteria to acquire iron by secreting NGAL (neutrophil gelatinase-associated lipocalin), which sequesters bacterial siderophores [9,10]. Additionally, the host increases production of hepcidin, the master iron-regulating hormone, to limit the bioavailability of iron [7]. This host defense strategy, called the iron-limiting innate immune defense [6], can influence iron availability and survival of intracellular bacteria in response to infection.

Macrophages play an important role in innate immunity and a central role in iron homeostasis. Since macrophages engulf senescent and damaged red blood cells (RBCs), they recycle iron daily in a process known as erythrophagocytosis [10]. Therefore, during infection, cellular iron metabolism is tightly regulated. A key cellular iron regulator involved in iron-limiting host defense is hepcidin [11], which is the master iron-regulating hormone that retains iron in macrophages [12] by binding to ferroportin (SLC40A1) [13], the only known iron exporter protein that exports iron to the extracellular milieu, leading to the internalization and subsequent degradation of ferroportin. Moreover, the action of NRAMP1 (Natural resistance-associated macrophage protein 1 or SLC11A1) [14] allows for transport of iron from the late endosome and phagolysosome to the cytosol where it can be safely stored in ferritin cages [15] or as part of iron-sulfur clusters.

As a consequence, during infection both hepcidin and NRAMP1 increase the cytosolic labile iron pool in macrophages. Since free labile iron is toxic, the cytosolic enzyme 3-hydroxybutyrate dehydrogenase type 2 (BDH2) [16] detoxifies cytosolic iron by catalyzing the synthesis of the mammalian siderophore 2,5-DHBA that binds free iron. Thus, BDH2 is also required for cellular iron homeostasis [17]. Further, NGAL, the iron carrier protein that shuttles and delivers liganded iron for cellular growth and differentiation [18] and scavenges bacterial siderophores [9], thereby exerts antibacterial function [19].

The aim of this work was to investigate whether macrophages sense the presence of intracellular *N. gonorrhoeae* and whether gonococci evade or modulate the host iron-limiting innate immune defenses. We now report that *N. gonorrhoeae* can survive intracellularly or in association with monocytes and macrophages, and that gonococcal infection of these cells upregulates expression of hepcidin, NGAL, and NRAMP1 and downregulates BDH2 and ferroportin expression. We hypothesize that such modulation facilitates gonococcal iron acquisition by increasing the cellular labile iron pool and its bioavailability.

Materials and Methods

Reagents

RPMI1640 medium, Dulbecco's modified Eagle medium (D-MEM), fetal bovine serum (FBS), penicillin/streptomycin, sodium pyruvate and nonessential amino acids were obtained from Cellgro Mediatech (Herdon, VA). Human NGAL, TNFα, IL-1β, IL-6 and CXCL10 (IP-10) ELISA kits were from R&D Systems (Minneapolis, MN). Phorbol myristate acetate and the iron chelator deferiprone (DFP) were purchased from Sigma Aldrich (St. Louis, MS). Purified synthetic hepcidin-25 and LL-37 peptides were a kind gift from Dr. Jan Pohl (CDC, Atlanta, GA).

Bacterial cultures

N. gonorrhoeae strain FA19 (GC-FA19) was grown as piliated, opacity-negative colony variants on GCB agar containing defined Supplements I and II under 3.8% (v/v) CO_2 at 37°C as described by Shafer et al. [20]. Broth cultures of gonococci were grown in GCB broth with supplements and 0.043% (w/v) sodium bicarbonate at 37°C in a shaking water bath. Viability of gonococcal cultures was determined using dilution plating onto GCB agar and colony forming units were enumerated after 24–48 hr of incubation at 37°C in a CO_2 incubator. Gonococci grown on GCB agar plates were resuspended in unsupplemented GCB broth and harvested by centrifugation at $5{,}000\times g$ for 10 minutes. The bacterial pellet was washed twice with PBS and resuspended in 10 ml of tissue culture medium D-MEM without antibiotics to prepare a live bacterial inoculum for the macrophage infection assay (see below). An aliquot of bacteria was used to prepare heat-killed bacteria by heating for 10 min at 95°C prior to use in the macrophage infection assay.

Cell cultures

THP-1 and MM6 human macrophage-like monocytic cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and grown in RPMI1640 containing L-glutamate and supplemented with 10% FBS (v/v), 50 IU/ml of penicillin, 50 μg/ml of streptomycin, 1% sodium pyruvate (w/v) and 1% non-essential amino acids (v/v). Culture flasks were incubated at 37°C with humidity and 5% CO_2. Murine macrophages (RAW264 from ATCC) were grown in D-MEM supplemented and incubated as noted above.

Macrophage infection assay

Freshly grown human THP-1 and MM6 monocytic cells as well as peripheral human monocytes were harvested and adjusted to one million cells/ml without antibiotics, transferred into 6-well tissue culture plates (3 ml/well), and infected with live or heat-killed GC-FA19 at a multiplicity of infection (MOI) of 10. Cells were then incubated at 37°C with 5% CO_2 for 5 hr or overnight. Uninfected cells in triplicate wells were also incubated simultaneously and used as a no-infection control. In some assays, monocytes were treated with the iron chelator deferiprone (DFP was added at 300 μM final concentration simultaneously upon infection with GC-FA19). Supernatants were harvested and saved at −20°C for determination of cytokine release and cells were used for RNA extraction.

Monocyte and macrophage bactericidal assay

To determine whether gonococci survive phagocytic killing by monocytes and macrophages, we employed THP-1 macrophage-like monocytic cells and RAW264 murine macrophages. Overnight plates of GC-FA19 (Pil^+/Opa^-) were adjusted to an OD_{600} of 1.0 ($\sim 1\times 10^8$ CFU/ml) in antibiotic-free RPMI1640 medium containing 10% heat-inactivated fetal bovine serum (FBS). To deactivate complement, FBS was heat-inactivated by incubation at 56°C for 30 min prior to use. Monocytes were freshly grown, washed, and adjusted to 1 million cells/ml in antibiotic-free RPMI1640 medium containing 10% heat-inactivated FBS as described above and were infected with GC-FA19 at an MOI of 50. Since THP-1 cells are not adherent, the infection process was initiated in 50 ml conical tubes containing 10 ml of THP-1 cell suspension. To facilitate phagocytosis of non-opsonized gonococci, the mixture of infected monocytes was centrifuged at 1300 rpm for 4 min, followed by a 10 min incubation at 37°C with 5% CO_2 and simple agitation every 5 min. The mixture was then fully resuspended using a 10 ml pipette and further incubated for another 50 min at 37°C with 5% CO_2 and simple agitation every 10 min. The cell mixture was then centrifuged at 1300 rpm for 4 min and washed three times with antibiotic-free medium containing 10% heat-inactivated FBS to remove extracellular gonococci. The viability of extracellular (nonphagocytosed) gonococci was assessed in the supernatants of infected cells after 1 hr of infection using the agar plate dilution method as described [21,22]. Infected monocytes were then resuspended in the original volume using fresh antibiotic-free medium containing 10% heat-inactivated FBS. Cell suspensions were then transferred into 12-well tissue culture plates (1 ml per well) and further incubated at 37°C for 2 and 5 hr. To quantify viable adherent and intracellular gonococci at 1, 2, and 5 hr post phagocytosis, a 1 ml aliquot of the cell suspension was pelleted by centrifugation at 4000 rpm for 4 min, washed once with antibiotic-free RPMI1640 medium, and harvested by another centrifugation. The cell pellet was thoroughly resuspended in 0.01% (v/v) Triton X-100 in sterile PBS and incubated for 5 min at 37°C, then vortexed thoroughly to permeate monocyte membranes (which facilitates the retrieval of viable intracellular gonococci). The lysed cell mixture was serially diluted in sterile PBS and cultured on GCB agar plates followed by 24–48 hr of incubation at 37°C with 5% CO_2, after which viable GC colonies were counted. In some experiments, the monocyte bactericidal assay was performed using the murine RAW264 macrophages. Since these RAW264 macrophages are adherent, cells were seeded in a 12-well tissue culture plate (1 million cells/well) and allowed to adhere overnight prior to infection with GC-FA19 at an MOI of 50 as described above. After 1 hour of the phagocytosis assay at 37°C, adherent RAW264 cells were washed three times with antibiotic-free medium containing 10% heat-

inactivated FBS to remove extracellular gonococci and all fluids were carefully removed without disturbing the adherent macrophages. One ml of fresh antibiotic-free medium containing 10% heat-inactivated FBS was added to each well and infected cells were further incubated for 2 and 5 hr. Viability of adherent and intracellular gonococci was assessed by serial plating of lysed macrophages in 0.01% (v/v) Triton X-100 in PBS as described above.

Staining of extracellular and intracellular gonococci

Viable and dead intracellular and extracellular gonococci were visualized using the LIVE/DEAD® BacLight^{TM} Bacterial Viability Kit (Invitrogen) as described by Criss et al. [22]. Briefly, freshly grown murine RAW264 macrophages were adjusted to 1 million cells/ml and seeded on pre-cleaned glass cover slips (24×24 #1 from Surgipath, Medical Industries INC.) placed in 8-well tissue culture plates and incubated overnight as above. Adherent macrophages were washed twice with and placed in antibiotic-free medium containing 10% heat-inactivated FBS prior to infection with GC-FA19 at an MOI of 50 followed by incubation at 37°C with 5% CO_2 for 1 hr and 5 hr. At the indicated time points, macrophages were washed three times with 0.1 M MOPS, 1 mM $MgCl_2$ (MOPS/$MgCl_2$) buffer, pH 7.2 to remove non-phagocytosed gonococci. Infected macrophages were then stained with BacLight^{TM} stain components (60 µM of propidium iodide [PI] and 2.5 µM of SYTO 9) prepared in MOPS/$MgCl_2$ containing 0.1% saponin to permeabilize macrophage membranes and were incubated at room temperature for 20 min in the dark with gentle rocking. Since PI is membrane impermeable, other infected macrophages in the same experiment were similarly stained with BacLight^{TM}, but without saponin, to visualize extracellular gonococci. Uninfected macrophages were also stained with BacLight^{TM} in MOPS/$MgCl_2$ with or without saponin as a control. Stained macrophages were washed twice with MOPS/$MgCl_2$ and glass cover slips were inverted on glass slides and sealed immediately. Adherent and intracellular viable gonococci were examined using laser scanning confocal microscopy (Olympus IX8S1F-3, Olympus Corporation, Japan) within 30 min of mounting as described [23]. Multiple fields were examined in each individual glass cover slip and images were captured and analyzed using FV10-ASW software from Olympus.

Minimum bactericidal concentration assay

A modified version of the minimum bactericidal concentration (MBC) assay of Shafer et al [24] was employed. Briefly, mid-logarithmic phase cultures of wild type strain FA19 and an isogenic mutant containing an insertional mutation in the *lptA* gene (*lptA::spc*), which renders gonococci hypersusceptible to cationic antimicrobial peptides due to loss of phosphoethanolamine (PEA) decoration at the 4′ lipid A phosphate [25], were normalized to an OD_{600} of 0.4 and diluted 1:100 in 0.2x strength unsupplemented GCB broth without sodium bicarbonate (pH 7.2 or pH 5.0). Ninety µl of the diluted cultures were added to sterile 96-well polypropylene microtiter wells (Costar cat# 3879) that contained 10 µl of peptide solution. Different concentrations of polymyxin B, hepcidin-25 or LL-37 were achieved by 2-fold serial dilution in antimicrobial peptide buffer [26] (0.01% acetic acid [v/v], 0.2% fatty acid- and endotoxin-free bovine serum albumin [w/v]), before addition of bacteria. In a control well, bacteria were also mixed with buffer alone to confirm that any killing was due only to peptide activity. Microtiter plates were incubated at 37°C under 3.8% (v/v) CO_2 for 1 hr. At the conclusion of the incubation period, 5 µl aliquots from each well were spotted onto GCB agar plates to determine the MBC of the peptides. The MBC was defined as the lowest tested peptide concentration at which no viable bacteria were recovered.

Differentiation of THP-1 macrophages

Human THP-1 monocytes are macrophage-like monocytic cells that grow in suspension and can be differentiated into an adherent macrophages using phorbol myristate acetate (PMA). Briefly, THP-1 monocytes were treated with PMA at 20 ng/million cells and incubated at 37°C with 5% CO_2 for one week with fresh RPMI1640 medium containing 10% FBS added every three days. Once cells became adherent, all medium was removed and replaced with fresh medium, then adherent macrophages were further incubated to form a confluent monolayer. To prepare for infection, adherent THP-1 macrophages were then harvested, counted and adjusted to 1 million cells/ml, seeded on glass cover slips placed in 8-well tissue culture plates, and allowed to adhere to glass cover slips overnight. The staining procedure was performed as described for RAW264 macrophages as mentioned above.

Isolation of peripheral monocytes

This study was deemed exempt from Institutional Review Board (IRB) at Emory University since peripheral monocytes were completely de-identified without any link to donors' identification. However, whole blood (15 ml with EDTA) was collected from healthy donors after obtaining written informed consent under Emory University IRB approval to collect healthy donors' plasma for other unrelated studies. Monocytes used in this study were obtained from the discarded and de-identified whole blood samples leftover after the removal of plasma fraction. Peripheral monocytes were isolated using Ficoll-density gradient centrifugation (Histopaque 1077, Sigma-Aldrich Co). Isolated mononuclear cells were then cultured in RPMI1640 medium at 37°C for 2 hr to remove the non-adherent cells, followed by overnight incubation in fresh medium as described [27]. Primary peripheral monocytes were isolated from four different healthy donors and infected with live GC-FA19 at an MOI of 10 and incubated overnight. Cytokine release was measured in the supernatants using ELISA. Monocytes were harvested for RNA isolation and gene expression was measured by quantitative RT-PCR.

Mammalian RNA isolation, quantitative Real-Time PCR and gene expression analysis

RNA was isolated using RNeasy Mini kits (Qiagen, Hilden, Germany) following the manufacturer's instructions. Briefly, infected and uninfected cells were harvested in RLT buffer containing 1% β-mercaptoethanol, passed over QiaShredder columns, and the resulting lysate was mixed in 70% ethanol and passed over RNeasy columns. Columns were washed then treated with 10 µl of RNase-free DNase (Qiagen) for 15 min at room temperature prior to RNA extraction, followed by additional washing and centrifugation. RNA was eluted with RNase-free water in 50 µl then was reverse transcribed to cDNA using the QuantiTect® Reverse Transcription kit (Qiagen) following the manufacturer's instructions. In brief, genomic DNA was eliminated using gDNA Wipeout on 1 µg of total RNA, which was then reverse transcribed in a 20 µl total volume containing reverse-transcription master mix. For reverse transcription, RNA mixture samples were incubated for 15 min at 42°C, and then 3 min at 95°C to inactivate the reverse transcriptase enzyme. The generated cDNA was diluted 1:10 in nuclease-free molecular grade sterile H_2O and stored at −20°C until further use. Relative gene expression was determined by quantitative RT-PCR using SYBR Green master mix (Promega, Madison, WI) and cDNA

from infected and uninfected cells (as template) following the manufacturer's instructions. Gene expression fold change was calculated in reference to uninfected controls using the $\Delta\Delta C_T$ method [28]. Results were normalized to those from uninfected cells, which were used as controls for basal gene expression levels.

The following primers were used for qRT-PCR reactions: human hepcidin 5′-GACCAGTGGCTCTGTTTTCC-3′ and 5′-CACATCCCACACTTTGATCG-3′; human BDH2 5′-CAG-CGTCAAAGGAGTTGTGA-3′ and 5′-TGGCGTATCAACT-GTTCCTG-3′; human MMP9 5′-CTGAAATGACGTCCC-TAAGT-3′ and 5′-AGGAGGTCTCACTATCTGGAT-3′; human NGAL 5′-ATGACATGAACCTGCTCGATA-3′ and 5′-TCATAGTCGTTCATTATCTTC-3′; human NRAMP1 5′-GCGAGGTCTGCCATCTCTAC-3′ and 5′-GTGTCCACGA-TGGTGATGAG-3′; human β-actin 5′-TCTTCCAGCCTTCC-TTCCT-3′ and 5′-AGCACTGTGTTGGCGTACAG-3′; murine HAMP1 5′-AGAAAGCAGGGCAGACATTG-3′ and 5′-CACTGGGAATTGTTACAGCATT-3′; murine LCN2 5′-TG-CCACTCCATCTTTCCTGTT-3′ and 5′-GGGAGTGCTGG-CCAAATAAG-3′; murine BDH2 5′-GAGAACAGATGTGTG-TACAGTGCTACC-3′ and 5′-CTAGGAAGGGCCTGTCT-TCCCAGC-3′; murine NRAMP1 5′-GACACAGCAGAGCA-ATTGGA-3′ and 5′-GGGAACTGGAGTCACCTTCA-3′; murine GAPDH 5′-ACCTGCCAAGTATGATGACATCA-3′ and 5′-GGTCCTCAGTGTAGCCCAAGAT-3′. Ferroportin QuantiTect primers (Hs_SLC40A1_1_SG) were purchased from Qiagen.

Bacterial RNA isolation and quantitative RT-PCR

Expression of iron-responsive genes in intracellular GC-FA19 living in monocytes was assessed by quantitative RT-PCR. Briefly, THP-1 cells were infected with GC-FA19 as mentioned above (see monocyte and macrophage bactericidal assay section) and GC-FA19 was harvested for RNA extraction. Intracellular GC-FA19 (phagocytosed gonococci) were harvested by lysing infected THP-1 cells using the Qiagen Shredder as mentioned above. Bacterial RNA was isolated using RNeasy columns (Qiagen) and genomic DNA was removed by DNase treatment and gDNA Wipeout (Qiagen) following the manufacturer's instructions. The absence of contaminating genomic DNA was confirmed by using purified RNA as template in a PCR reaction and visualization by gel electrophoresis. The extracted bacterial RNA was then reverse transcribed to cDNA using the QuantiTect Reverse Transcriptase kit (Qiagen) following the manufacturer's instructions. Quantitative RT-PCR was performed using the generated cDNA (undiluted) and results were normalized to 16S rRNA expression for each individual sample. The expression of iron-responsive genes *fetA*, *tbpA*, *tbpB*, *mpeR*, and non-iron-responsive genes *rmpM* and *serC* in monocyte-associated GC-FA19 after 5 hr of infection was compared to that after 1 hr of infection using the $\Delta\Delta C_T$ method [28]. GC-FA19 specific primers used in this study: *tbpA* forward: 5′-TTTCGACACGCGCGATATGA-3′, reverse: 5′-AGTCCGCCGTATTTGTGGTT-3′; *tbpB* forward: 5′-AGGG-CAAGGCGACAAATACA-3′, and reverse: 5′-CGAATCAGTT-TGCCCGTCAA-3′; *fetA* forward: 5′-AGAGTTTGCCGT-CAGCGAAA-3′, and reverse: 5′-TAGGCGTTGGCATC-CAGTTT-3′; *mpeR* forward: 5′-AAACAGCCCGGTTTG-CATCT-3′, and reverse: 5′-GCGCAGTTGTGGCTGAAATT-3′; *rmpM* forward: 5′-AAGCCAAGGTCGCGTAGAAT-3′, and reverse: 5′-GGCGCGCAATGAATCCTTAT-3′; *serC* forward: 5′-TGTTGCCTGAAGCTGTGTTG-3′, and reverse: 5′-TGT-TCCGCATGATGCAGGAT-3′; *16S* forward: 5′-CCATCGG-TATTCCTCCACATCTCT-3′, and reverse: 5′-CGTAGGG-TGCGAGCGTTAATC-3′.

Cytokine release quantification

The cytokines TNFα, IL-1β, IL-6, MCP-1, CXCL10 (IP-10), and NGAL released from THP-1 cells or from peripheral human monocytes were quantified by DuoSet ELISA (R&D Systems, Minneapolis, MN) as previously described [29,30].

Nitric oxide induction by murine macrophages

Freshly grown adherent RAW264 macrophages were harvested and adjusted to one million cells/ml without antibiotics, then transferred into 6-well tissue culture plates (3 ml/well) and infected with live GC-FA19 at an MOI of 10 and incubated at 37°C with 5% CO_2 overnight. Nitric oxide release was quantified in the supernatants using the Greiss chemical method as previously described [30].

Cytospin and cellular staining

To monitor intracellular gonococci residing in macrophages, 100 µl of infected and uninfected THP-1 and MM6 cells were subjected to cytospin centrifugation for 5 min at 500 rpm using Cytospin 4 (Thermo Scientific). Cells deposited on glass slides were allowed to dry for at least 5 min prior to staining with the modified Wright-Giemsa stain Diff-quick® (Dade Behring, Newark, DE, USA) following the manufacturer's instructions. Cellular morphology was examined by light microscopy and digital images of 10 different fields per sample were saved.

Measurement of iron retention in infected monocytic cells

Intracellular labile iron reflecting iron retention in infected and uninfected THP-1 and MM6 cells was determined using the well-established Calcein-AM method [31]. Infected and uninfected cells were washed and placed in RPMI1640 medium supplemented with 10% FBS, and 0.5 µM Calcein-AM was added to 1 million cells/ml at 37°C and incubated for 15 min. Calcein-AM-loaded cells were washed twice with PBS to remove extracellular Calcein-AM. Cells were resuspended in HBSS at 2 million cells/ml and 100 µl aliquots were transferred into quadruplicate wells in black 96-well plates. After 20 min of incubation, fluorescence was determined at 485 nm (excitation) and 535 nm (emission) wavelengths using a Bio-Tek Synergy 2 Instrument as described [31].

Western immunoblotting analysis

THP-1 cells were infected with GC-FA19 as above or treated with 1 ng/ml of exogenous hepcidin protein (RDG International Inc., USA) and incubated for 5 hrs. Untreated cells were used as a control. Treated and untreated cells were lysed with RIPA buffer containing protease inhibitors and 20 µg of cell protein extracts were loaded into a Tris-glycine 4–20% SDS polyacrylamide gel (Novex, Invitrogen, Carlsbad, CA) and subjected to electrophoresis. Resolved proteins were transferred onto a nitrocellulose membrane and blocked with 3% BSA overnight. Hepcidin immunoreactive bands were detected with the primary anti-hepcidin polyclonal antibody diluted 1:1000 (rabbit anti-human hepcidin polyclonal antibody from Ray Biotech Inc., Norcross, GA). The membranes were washed three times with TBS-T buffer, incubated with the goat anti-rabbit secondary antibody alkaline phosphatase conjugate (diluted 1:1000), washed an additional three times then developed using SigmaFast BCIP/NBT (Sigma Aldrich).

Statistical analysis

Mean values ± SD and *P* values (Student *t* test) of at least three independent determinations were calculated with Microsoft Excel software.

Results

N. gonorrhoeae survives in association with monocytes and macrophages and responds to an iron-limited environment

The strict human pathogen *N. gonorrhoeae* evades host innate defenses and survives in neutrophils [5,23], macrophages [32] and epithelial cells [33]. Macrophages play a central role in limiting iron bioavailability during infection to prevent bacterial growth [7]. Therefore, we examined whether gonococci can cope with the iron-limiting conditions within monocytes/macrophages and survive intracellularly. We determined the viability of gonococci during infection using a macrophage bactericidal assay adapted from previously described methods [22,32]. THP-1 monocytes were infected with unopsonized GC-FA19 at an MOI of 50 and the viability of extracellular and monocyte-associated gonococci was determined at 1, 2, and 5 hr post infection. We found that *N. gonorrhoeae* survived killing by human THP-1 monocytes at 1, 2, and 5 hr post infection (Figure 1A). In this respect, although 50% of the total gonococcal inoculum was killed within 1 hr of exposure to monocytes, 47% of this inoculum was viable extracellularly while approximately 3% was phagocytosed (data not presented). During the subsequent four hours, phagocytosed gonococci could be retrieved viable. Of the gonococci that were adherent or intracellular and viable at 1 hr, approximately 30% remained viable at the 2 hr time point, while at least 15% remained viable to the end (5 hr) of the phagocytic killing period (Figure 1A). The viability of gonococci (adherent and intracellular) in differentiated THP-1 macrophages (Figure 1B) was visualized using bacterial live/dead staining as described [22]. Further, gonococcal survival was also observed when murine RAW264 macrophages were infected with unopsonized GC-FA19 (see below). These data indicate that adherent and intracellular *N. gonorrhoeae* can survive phagocytic killing by monocytes and macrophages.

In order to determine the transcriptional response of gonococci to the reported iron-limiting conditions inside of phagocytic cells [7,12], we investigated the expression of gonococcal iron-responsive genes that encode the transferrin-binding protein complex (*tbpA* and *tbpB*), an enterobactin-like siderophore receptor (*fetA*) and a transcriptional activator (*mpeR*) of *fetA* that is negatively regulated by the gonococcal ferric uptake regulator (Fur) in the presence of iron; two non-iron-responsive genes that encode the gonococcal reduction modification protein (*rmpM*) and phosphoserine aminotransferase (*serC*) were also tested as controls [34,35,36,37]. We found that these iron-responsive genes were significantly upregulated in monocyte-associated gonococci at 5 hr compared with 1 hr of phagocytosis (Figure 1C). In contrast, the gene expression of *rmpM* and *serC* was largely unchanged. Taken together, the data suggest that *N. gonorrhoeae* survives in association with monocytes and macrophages and responds to iron limitation by upregulating iron-responsive genes to facilitate iron acquisition.

Innate immune recognition of *N. gonorrhoeae* in infected monocytes

Upon recognition of invading pathogens, macrophages secrete inflammatory mediators including cytokines and chemokines required for orchestrating innate immune defenses that limit or clear infection. In order to assess if we could detect classical macrophage responses to a bacterial infection, we first examined if their infection by gonococci influenced expression of cytokines and chemokines. As expected, human THP-1 monocytes recognized the presence of *N. gonorrhoeae* strain FA19 (GC-FA19) and released cytokines TNFα, IL-6 and IL-1β [38] as well as chemokines CXCL10 (also known as IP-10) and MCP-1 (Figure 2A). The presence of intracellular gonococci and morphological changes such as the appearance of vacuoles associated with macrophage activation were visualized by microscopic examination of Wright-Giemsa stained smears of infected and uninfected THP-1 and MM6 cells (Figure 2B). These data indicated that monocytes can sense the presence of intracellular gonococci and respond accordingly. We next examined if viability of GC-FA19 influences pathogen sensing and cytokine release from monocytes. We found that infection with live GC-FA19 induced significantly more TNFα and IL-1β release from MM6 (Figure 3A) and THP-1 cells (Figure 3B) when compared to a heat-killed (H-K) preparation of GC-FA19, indicating that monocytes recognized the presence of both live and dead GC-FA19 infection, but responded more robustly to infection by live GC-FA19, possibly due to the growth or biologic activities of viable gonococci. Having established that we could detect classical macrophage responses due to infection by gonococci, we next evaluated if this infection also influenced expression of host genes involved in modulating levels of bioavailable iron.

N. gonorrhoeae modulates monocyte iron-limiting innate immune defenses

During bacterial infection the host limits the bioavailability of iron by inducing hepcidin, which retains iron in macrophages and as a consequence, limits bacterial growth due to sequestration of this important nutrient. Although *N. gonorrhoeae* survives intracellularly in neutrophils, macrophages, and epithelial cells [5,32,33], it is not known how or if it modulates the iron-limiting innate immune defenses in macrophages. To test this possibility, we investigated hepcidin expression by quantitative RT-PCR in human monocytic cell lines MM6 and THP-1 infected with GC-FA19 for 5 or 18 hr. We found that hepcidin gene expression was highly upregulated in MM6 cells infected with live or heat-killed gonococci when assessed at 5 hr and 18 hr post infection, with the highest expression seen at 5 hr (Figure 4A), but live gonococci seemed more proficient in this capacity. This suggests that monocytes can sense and respond more robustly to infection by live *N. gonorrhoeae* and supports our results in Figure 3. Since hepcidin expression is modulated by iron levels, we next examined the effect of iron chelation on hepcidin expression in monocytes. To this end, THP-1 monocytes were treated with 300 μM of the iron chelator deferiprone (DFP) at the time of infection with live gonococci. The results show that iron chelation upregulated basal levels of hepcidin gene expression in uninfected THP-1 cells and further increased hepcidin gene expression in infected THP-1 cells at 5 and 18 hr post infection, with the highest hepcidin gene expression seen at 5 hr (Figure 4B). Hepcidin protein induction in GC-FA19 infected, but not uninfected, THP-1 monocytes was confirmed by immunoblotting with anti-hepcidin antibody (Figure 4C). Based on this observed induction of hepcidin production, we tested whether purified hepcidin-25 antimicrobial peptide might exert anti-gonococcal activity. Although it reportedly lacks strong antibacterial action against clinical strains of *Escherichia coli* and *Klebsiella pneumoniae* [39], hepcidin-25 seems to have some activity against *Mycobacterium tuberculosis* [40]. In order to learn if hepcidin-25 could kill gonococci, we compared its activity to a model cationic antimicrobial peptide (polymyxin B;

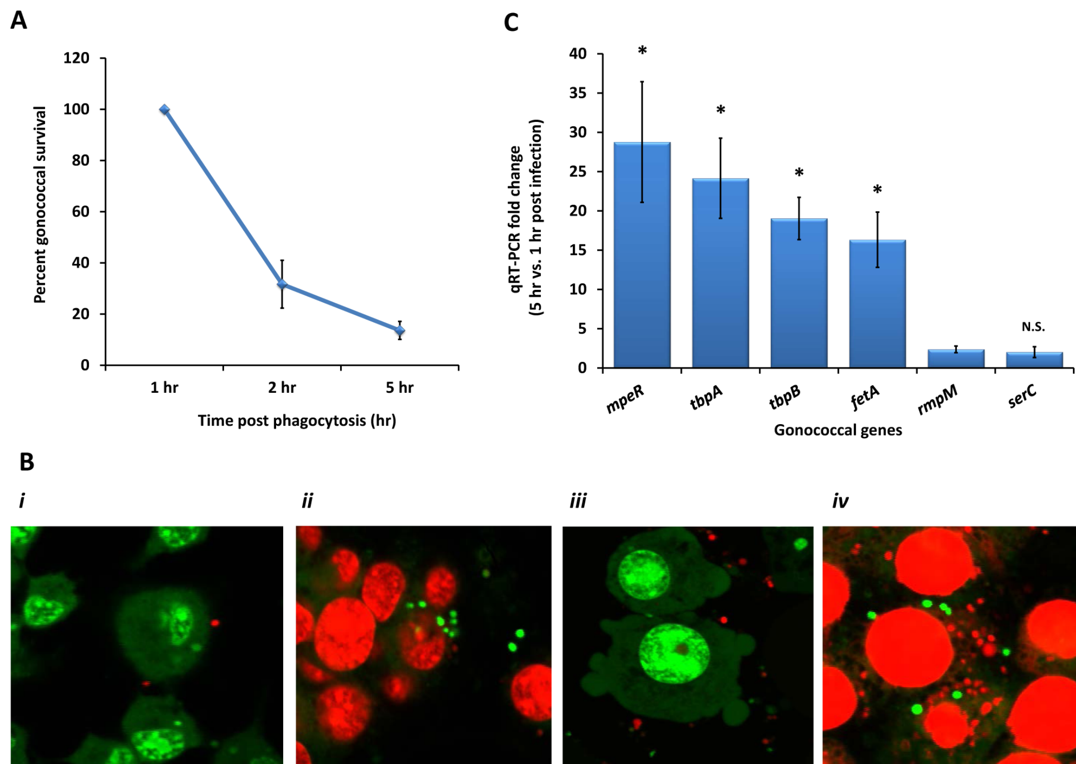

Figure 1. *N. gonorrhoeae* survives in association with monocytes. A. Survival of gonococci with human THP-1 monocytes at 2 and 5 hr post infection. Percent monocyte-associated gonococcal survival is calculated in reference to the number of viable gonococci cultured from infected THP-1 cells after 1 hr of phagocytosis. Error bars are ± standard deviation from the mean of 4 independent experiments. **B**: Viability of adherent and intracellular and extracellular GC-FA19 in differentiated THP-1 cells, i.e. adherent macrophages, were visualized with BacLight staining where red is dead and green is live gonococci. Adherent THP-1 cell membranes were permeabilized with 0.1% saponin to stain phagocytosed gonococci. ***i***: 1 hr post infection stained without saponin. ***ii***: 1 hr post infection stained in the presence of 0.1% saponin. ***iii***: 5 hr post infection stained without saponin. ***iv***: 5 hr post infection stained in the presence of 0.1% saponin. These data are representative of two independent experiments. **C**: Quantitative RT-PCR of iron-responsive and -unresponsive genes in monocyte-associated GC-FA19 at 5 hrs post infection compared to 1 hr post infection. Error bars represent SD from the mean of three independent experiments. * *P* values (<0.005) were calculated in comparison to *rmpM* gene expression. N.S.: not significant.
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PMB) that has potent anti-gonococcal activity *in vitro* [25]. For this purpose, we used wild-type gonococcal strain FA19 and an isogenic mutant, FA19 *lptA::spc* (the latter is hypersusceptible to cationic antimicrobial peptides due to loss of PEA decoration of lipid A [25], which also renders gonococci less fit *in vivo* as assessed using experimental female mouse and human male models of genital tract infection [41]). We found that in contrast to PMB, hepcidin-25 lacked anti-gonococcal activity *in vitro* (the minimum bactericidal concentration [MBC] of PMB against wild-type FA19 was 12.5 µg/ml, while that of hepcidin-25 was >100 µg/ml) even against the cationic antimicrobial peptide-hypersusceptible strain FA19 *lptA::spc* (Figure 4D). In additional contrast to hepcidin-25, we found (data not presented) that the human cathelicidin LL-37 exerted potent anti-gonococcal activity *in vitro* (MBC = 6.25 µg/ml), which is consistent with previous studies [24]. Since the antibacterial activity of hepcidin-25 has been reported to increase at acidic pH [39,42], we also tested whether hepcidin-25 would exert anti-gonococcal activity in a pH-dependent manner. We found (data not presented) that hepcidin-25 lacked anti-gonococcal activity when tested against FA19 WT at pH 7.2 (MBC>100 µg/ml) and at pH 5.0 (MBC>100 µg/ml).

Hepcidin induction causes iron retention in macrophages, i.e. increases the cytosolic labile iron pool (LIP), by binding ferroportin leading to its internalization and degradation thereby inhibiting iron export [6,13]. To confirm that upregulation of hepcidin in GC-FA19 infected monocytes causes iron retention, we measured the labile iron pool in infected THP-1 monocytes (Figure 5) using the Calcein-AM fluorescent probe [31]. Upon binding labile (free) iron, Calcein-AM fluorescence is quenched and is therefore inversely correlated with labile intracellular iron accumulation. We found that Calcein-AM fluorescence was significantly quenched in THP-1 monocytes infected with GC-FA19 when compared to uninfected monocytes (Figure 5). Similar results were seen when MM6 monocytes were infected with gonococci (data not shown), suggesting that the LIP is increased upon infection with gonococci. To further confirm that hepcidin upregulation caused iron retention in infected monocytes, we investigated the

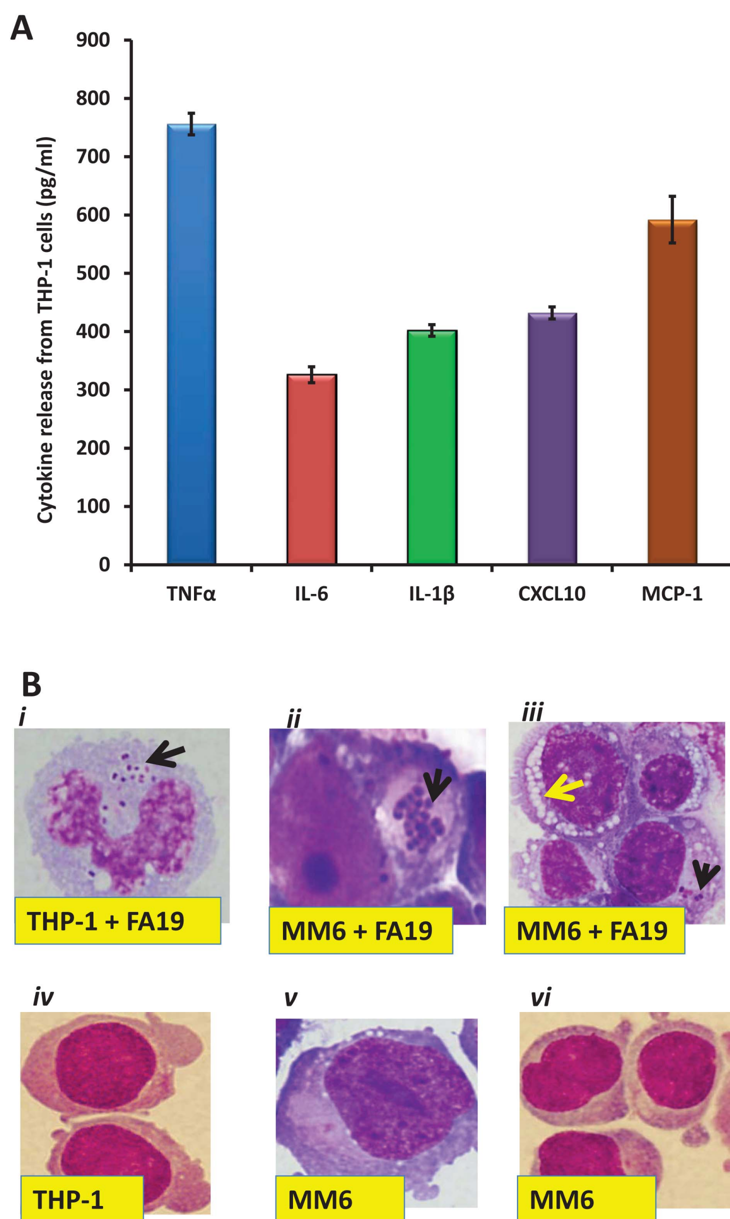

Figure 2. Cytokine release from THP-1 cells infected with *N. gonorrhoeae* strain FA19. A. Human macrophage-like monocytic THP-1 cells were infected with GC-FA19 at an MOI of 10 and incubated overnight. Released cytokines TNFα, IL-6, IL-1β and chemokines CXCL10 (IP-10) and MCP-1 were quantified in the supernatants of infected THP-1 cells using ELISA. Cytokine release was not detectable in the supernatants of uninfected THP-1 cells. Error bars represent the SD from the mean of triplicate readings and data are representative of three independent experiments. **B.** Intracellular gonococci (black arrows) were visualized by differential staining of infected THP-1 (***i***) and MM6 (***ii***) cells showing cytoplasmic vacuoles (yellow arrow) (***iii***) indicating monocyte activation and differentiation into macrophage-like cells, shown in the upper panel. Uninfected THP-1 (***iv***) and MM6 (***v, vi***) cells are shown in the lower panel.
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expression of the only known iron exporter, ferroportin (also known as SLC40A1). We found that ferroportin gene expression was significantly downregulated in THP-1 monocytes infected with gonococci (Figure 6). Taken together, these data suggest that *N. gonorrhoeae* infection of monocytes, especially with live gonococci, upregulates hepcidin and downregulates ferroportin to retain iron

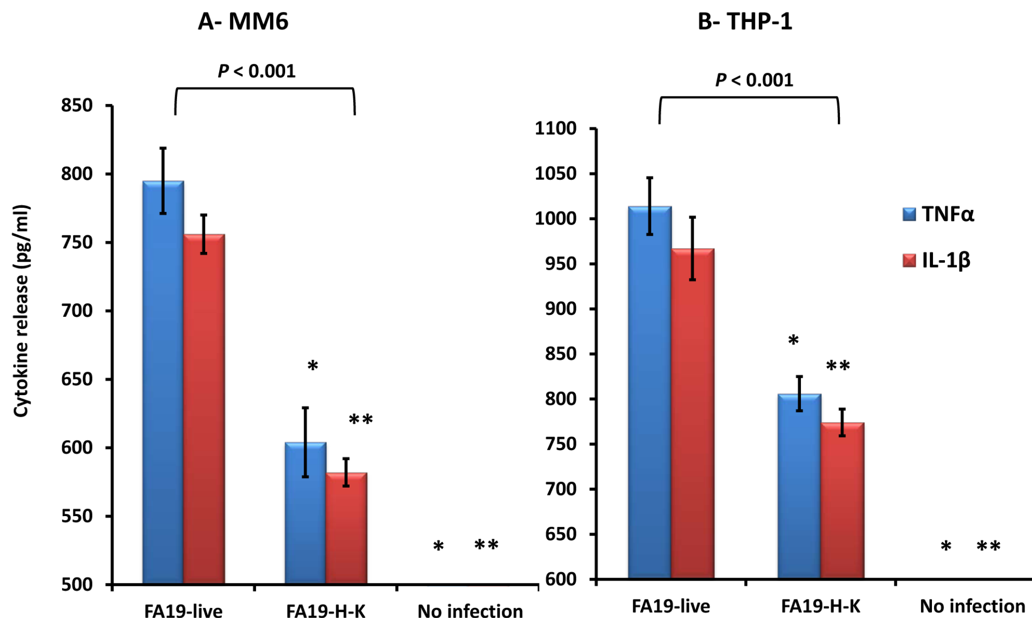

Figure 3. Infection with live gonococci induces more cytokine release than infection with heat-killed gonococci. Human macrophage-like monocytic MM6 (**A**) and THP-1 (**B**) cells were infected with live or heat-killed (H-K) GC-FA19 at an MOI of 10 and incubated overnight. Released cytokines TNFα and IL-1β were quantified in the supernatants of infected cells by ELISA. Cytokine release was not detectable in the supernatants of uninfected cells. Error bars represent the SD from the mean of triplicate readings and data are representative of two independent experiments. *P* values ($<$0.001) were calculated for values of infections with live GC-FA19 compared to heat-killed and * TNFα, ** IL-1β in reference to no infection values.

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in monocytes, which would increase the LIP and facilitate intracellular iron acquisition.

Since cellular iron homeostasis is also tightly controlled by NRAMP1 and NGAL, we evaluated if their expression is influenced by gonococcal infection. NRAMP1, the intracellular iron transporter, is important in cellular iron recycling since it transports iron from the late endosome and phagolysosome. Herein, iron-containing proteins such as ferritin and transferrin are degraded and recycled to the cytosol, which increases labile cytosolic iron. NGAL is an iron carrier protein that shuttles and delivers liganded iron needed for cellular growth and differentiation. NGAL has been shown to shuttle the mammalian siderophore 2,5-DHBA [17] and also plays an important role in iron-limiting innate immune defenses since it exerts antibacterial function by sequestering bacterial siderophores, hence limiting bacterial growth [9]. We investigated NRAMP1 expression by quantitative RT-PCR in human macrophage-like monocytic cell lines MM6 and THP-1 infected with GC-FA19 for 18 hr. We found that NRAMP1 gene expression was highly upregulated in human MM6 (Figure 7) and THP-1 (data not shown) monocytes infected with GC-FA19. Similarly, we found that expression of NGAL was highly upregulated in MM6 (Figure 7) and THP-1 (data not shown) cells upon infection with GC-FA19.

N. gonorrhoeae downregulates the gene encoding the labile iron-detoxifying enzyme BDH2

To maintain homeostatic iron regulation, the host detoxifies free iron by multiple mechanisms [10]. Thus, iron retention increases the LIP which in turn increases the toxicity of ROS generation through the iron-dependent Fenton reaction [43]. Recently, the enzyme termed BDH2 was discovered to play a crucial role in intracellular iron homeostasis since its transcript possesses an iron responsive element (IRE) [16,17]. BDH2 is an EntA homologue and EntA is the protein responsible for producing the bacterial siderophore 2,3-dihydroxybenzoic acid (2,3-DHBA) known as enterobactin [44]. Similarly, BDH2 was discovered to mediate the synthesis of the mammalian siderophore 2,5-DHBA that binds free iron [17]. BDH2-depleted cells were found to accumulate free iron and have increased oxidative stress [17]; therefore, BDH2 plays a very important role in detoxifying labile iron and maintaining intracellular iron homeostasis. We hypothesized that *N. gonorrhoeae* infection of monocytes would downregulate BDH2 expression to sustain the LIP and thus facilitate iron acquisition. We found that gonococcal infection downregulated BDH2 gene expression in human THP-1 monocytes as assessed by qRT-PCR (Figure 8). Since iron chelation induces BDH2 expression [16], we treated THP-1 monocytes with 300 μM of DFP at the time of infection with GC-FA19. We found that even with iron chelation, BDH2 gene expression was still downregulated by *N. gonorrhoeae* infection (Figure 8). These data suggest that gonococcal infection of monocytes likely results in reduced synthesis of the mammalian siderophore 2,5-DHBA. Thus, downregulation of the BDH2 gene would increase the labile iron pool and, consequently, facilitate intracellular iron acquisition by gonococci. To our knowledge, this is the first report to demonstrate that bacterial infection of a host cell can decrease BDH2 gene expression.

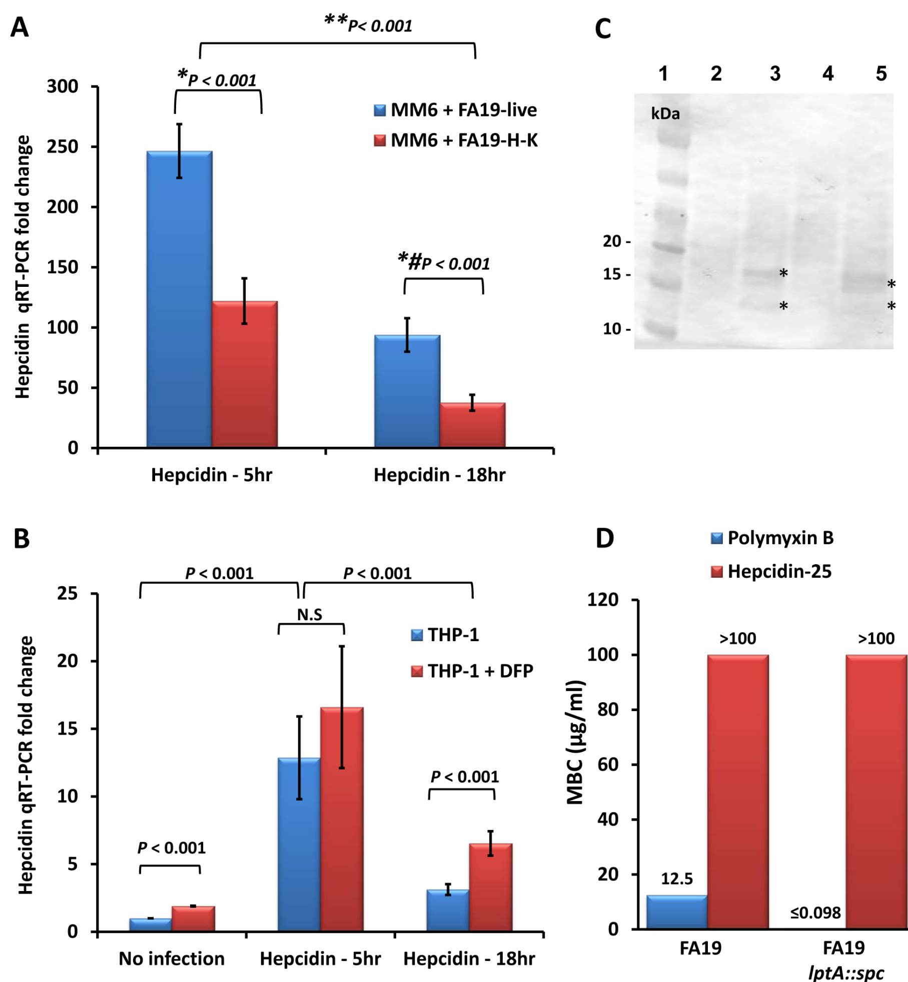

Figure 4. Hepcidin expression in monocytes is highly upregulated upon infection with live gonococci. A: Human macrophage-like monocytic MM6 cells were infected with live or heat-killed (H-K) GC-FA19 and incubated for 5 hr or 18 hr. **B:** THP-1 cells were infected with live GC-FA19 in the presence or absence of 300 μM of the iron chelator deferiprone (DFP) and incubated for 5 hr or 18 hr. RNA was extracted from infected cells and from controls (uninfected cells) and hepcidin gene expression was assessed by quantitative RT-PCR and normalized to that of β-actin. The fold change in hepcidin gene expression was calculated in reference to uninfected controls using the ΔΔCT method. Error bars represent the SD from the mean of quadruplicate wells and data are representative of three independent experiments. ***P* values ($<$0.001) were calculated for values of hepcidin expression at 5 hr or 18 hr post infection. **P* and *#*P* values ($<$0.001) were calculated for values of hepcidin expression upon infection with live GC-FA19 compared to heat-killed (H-K) at 5 hr and 18 hr post infection, respectively. N.S.: not significant. **C:** Western blot analysis of hepcidin protein expression in THP-1 cell extracts detected with polyclonal anti-hepcidin antibody. Lane 1: molecular weight marker; Lane 2: Untreated THP-1 cells; Lane 3: THP-1 cells treated with exogenous hepcidin (1 ng/ml); Lane 4: Uninfected THP-1 cells; Lane 5: THP-1 infected with GC-FA19. *Anti-hepcidin immunoreactive bands might be dimers of prohepcidin protein (~16–18 kDa) or processed hepcidin (~12 kDa). **D:** Activity of synthetic hepcidin-25 antimicrobial peptide against *N. gonorrhoeae* FA19 WT and the FA19 *lptA::spc* isogenic mutant as determined by minimum bactericidal concentration (MBC) assay. The model antimicrobial peptide, polymyxin B, is active against FA19 and highly active against the FA19 *lptA::spc* mutant, and was used as a control. Data are representative of four independent experiments. For the FA19 *lptA::spc* mutant, experiments were performed twice.
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N. gonorrhoeae modulates iron-limiting innate immune defenses in primary peripheral human monocytes

In order to confirm the significance of our experimental findings generated using human monocytic cell lines THP-1 and MM6, we extended our work to primary peripheral human monocytes freshly obtained from healthy donors. We found that peripheral monocytes recognized the presence of live GC-FA19 infection and robustly responded by releasing cytokines IL-6 and TNFα as well as the antibacterial lipocalin NGAL (Figure 9A), and by upregulating hepcidin gene expression (Figure 9B). Further, we found that gonococcal infection in primary monocytes led to significant downregulation of ferroportin (Figure 9C) and BDH2

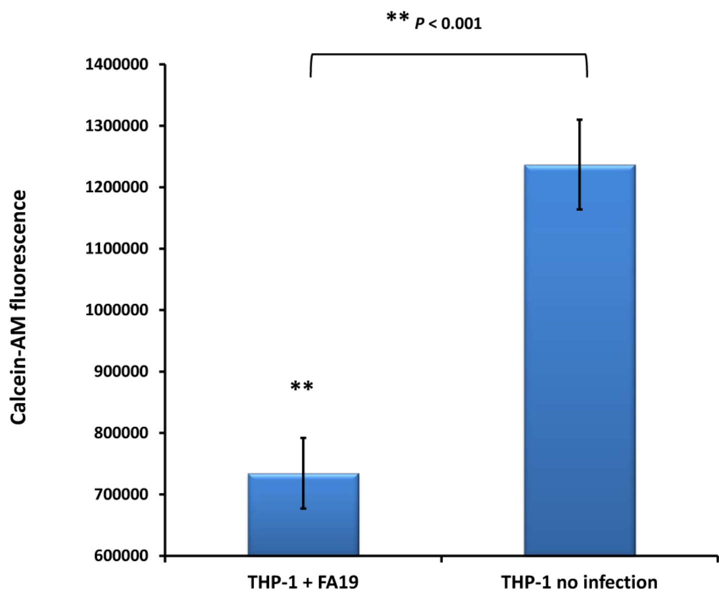

Figure 5. Infection with gonococci induces iron retention in monocytes. THP-1 macrophage-like monocytic cells were infected with GC-FA19 at an MOI of 10 overnight and iron retention in monocytes was determined using the Calcein-AM fluorescent probe method. Uninfected cells were incubated simultaneously and used as controls. Calcein-AM fluorescence was measured by excitation at 488 nm and emission at 528 nm wavelength (see methods). Calcein-AM fluorescence is quenched upon binding iron and is therefore inversely correlated with intracellular iron accumulation. Error bars represent the SD from the mean of quadruplicate readings and data are representative of three independent experiments. **P values (<0.001) were calculated in reference to no infection values.
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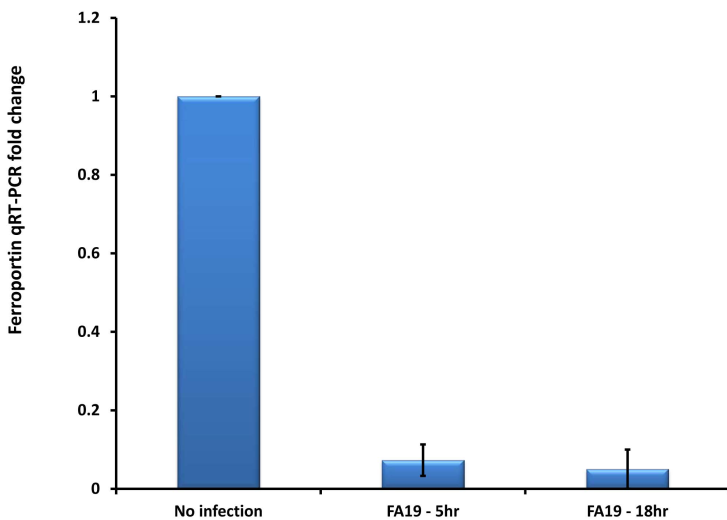

Figure 6. Gonococcal infection downregulates ferroportin gene expression in human THP-1 monocytes. THP-1 cells were infected at an MOI of 10 with live GC-FA19 and incubated for 5 or 18 hr. RNA was extracted from infected and uninfected cells. Ferroportin gene expression was assessed by quantitative RT-PCR and normalized to that of β-actin. Ferroportin gene expression fold change was calculated in reference to uninfected controls. Error bars represent the SD from the mean of at least quadruplicate wells and data are representative of two independent experiments.
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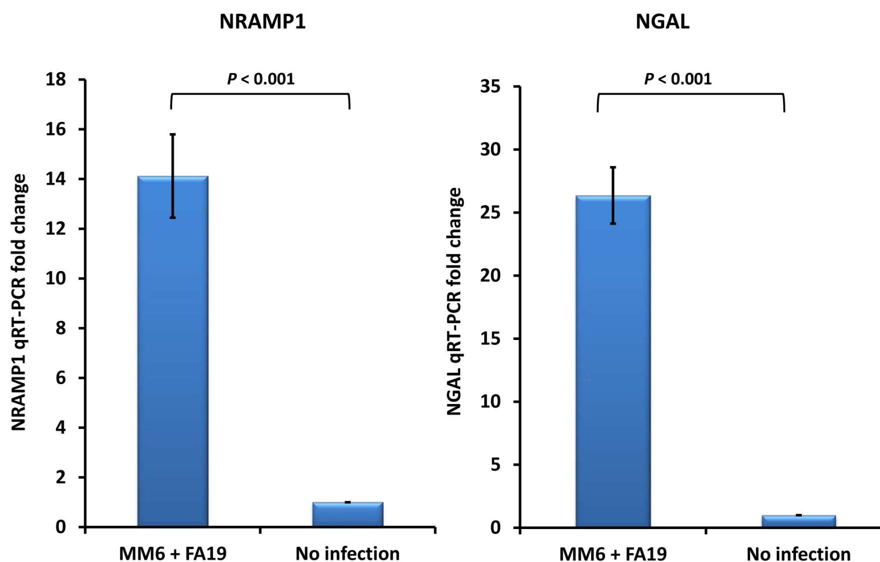

Figure 7. Gonococcal infection upregulates expression of the cytosolic iron transporter NRAMP1 gene and the antibacterial iron carrier NGAL gene in monocytes. Human macrophage-like monocytic MM6 cells were infected with live GC-FA19 at an MOI of 10 and incubated for 18 hr. RNA was extracted from infected cells and from controls (uninfected cells). NRAMP1 and NGAL gene expression was assessed by quantitative RT-PCR and normalized to that of β-actin. NRAMP1 and NGAL gene expression fold change was calculated in reference to uninfected controls. Error bars represent the SD from the mean of at least quadruplicate wells and data are representative of three independent experiments. *P* values (<0.001) were calculated in reference to no infection values.
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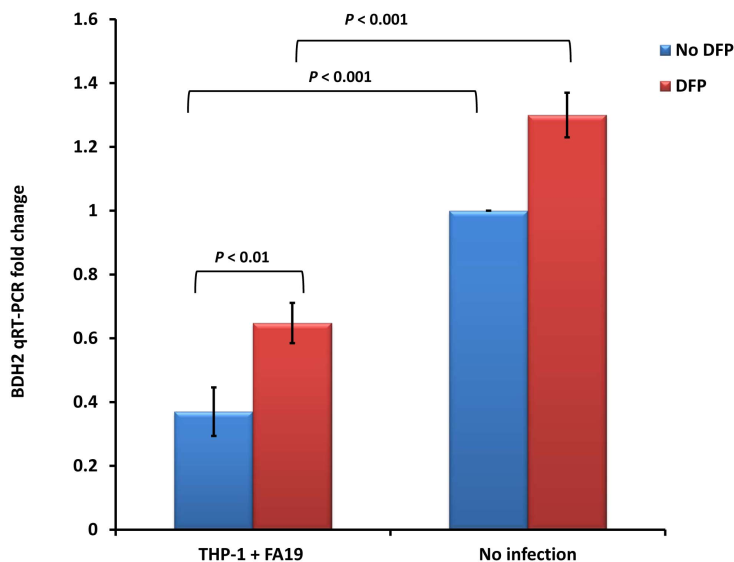

Figure 8. Gonococcal infection downregulates expression of the labile iron-detoxifying enzyme BDH2-encoding gene in monocytes. Human macrophage-like monocytic THP-1 cells were infected with GC-FA19 at an MOI of 10 in the presence or absence of 300 μM of deferiprone (DFP) (an iron chelator) and incubated for 18 hr. RNA was extracted from infected cells and from controls (uninfected cells) and BDH2 gene expression was assessed by quantitative RT-PCR and normalized to that of β-actin. BDH2 gene expression fold change was calculated in reference to uninfected controls. Error bars represent the SD from the mean of at least quadruplicate wells and data are representative of three independent experiments. *P* values (<0.01) were calculated in reference to no infection values.
doi:10.1371/journal.pone.0087688.g008

gene expression (Figure 9D). Since *in vivo* experimental models for GC are performed using primarily murine animal models [45,46], we also investigated whether *N. gonorrhoeae* infection modulates iron-limiting innate immune defenses in murine RAW264 macrophages. Results similar to those in monocyte experiments were obtained; GC-FA19 infection induced nitric oxide release and upregulated murine HAMP1 (the hepcidin homologue), LCN2 (the NGAL homolog) and NRAMP1 expression from infected macrophages, while it downregulated expression of the murine BDH2 gene (Figure 10). The viability of adherent and intracellular GC-FA19 in RAW264 macrophages after 1 and 5 hr post infection was confirmed using bacterial live-dead staining (Figure 11). Notably, few macrophages were heavily infected with GC-FA19 after 5 hr of infection, which resembles the pattern of infected neutrophils observed in urethral exudates (Figure 11-*v*).

Discussion

Human macrophages constitute the second wave of phagocyte influx that follows an often intense entry of polymorphonuclear leukocytes (PMNs) at sites of infection. While there is now compelling evidence that many gonococci survive this PMN influx and can exist within phagolysosomes, less is known about the fate of gonococci within macrophages. We evaluated the survival of gonococci in monocytes and macrophages and found that *N. gonorrhoeae* survived in association with these phagocytes and

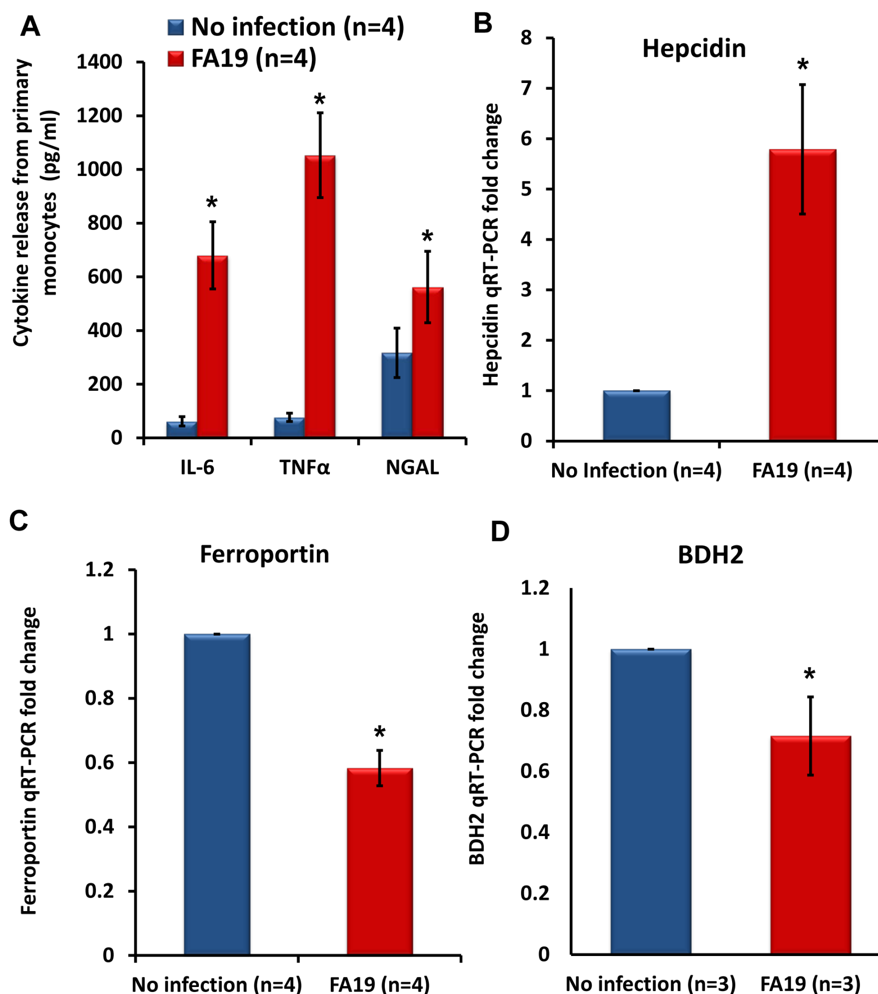

Figure 9. Gonococcal infection of peripheral monocytes from healthy human donors induces cytokine release, upregulates hepcidin and downregulates ferroportin and BDH2 gene expression. A: Peripheral monocytes derived from healthy donors were infected with GC-FA19 at an MOI of 10 and incubated overnight. Released cytokines IL-6 and TNFα and the antibacterial protein NGAL were quantified in the supernatants of infected monocytes by ELISA. Error bars represent the SD from the mean of four different healthy donors, each assayed in duplicate readings. B, C and D: Hepcidin, ferroportin, and BDH2 gene expression in healthy donor monocytes infected with GC-FA19 used in panel A above was determined by quantitative RT-PCR and normalized to uninfected monocytes. Error bars represent the SD from the mean fold change in gene expression from different healthy donors, each assayed in triplicate. *P value <0.01.
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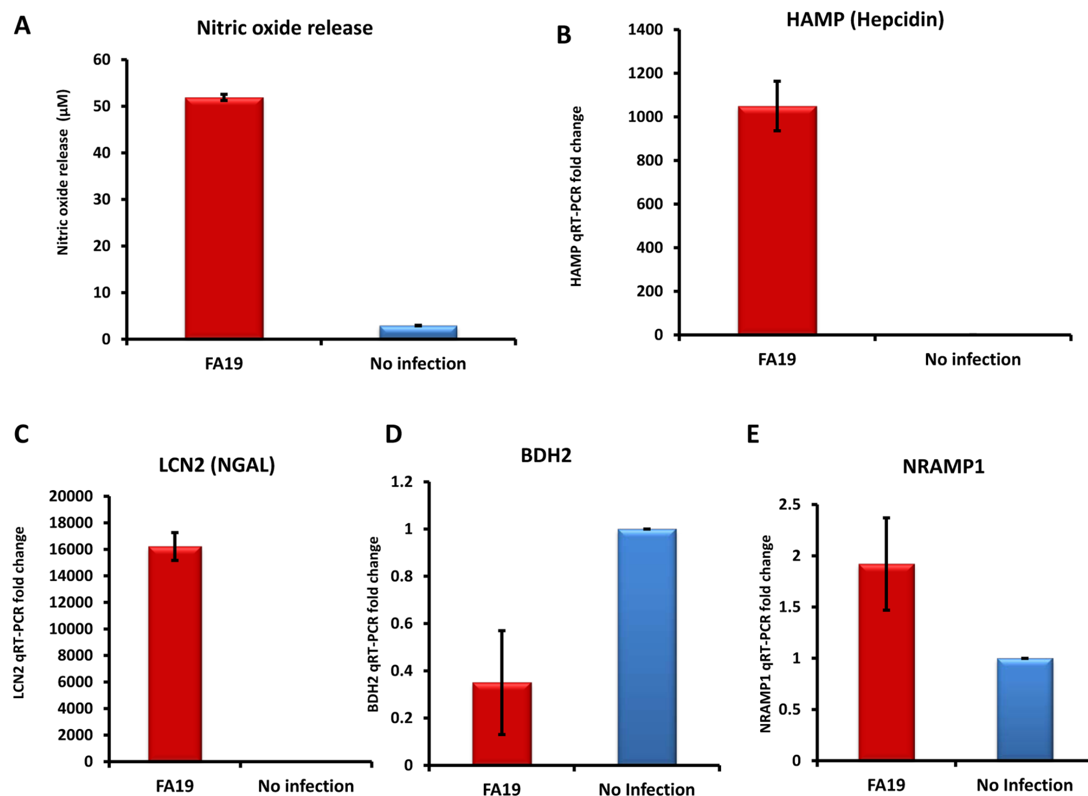

Figure 10. Gonococcal infection modulates iron-limiting innate immune defenses in murine RAW264 macrophages. A: Nitric oxide release from RAW264 cells infected with GC-FA19 was measured after 18 hr of incubation. Iron-regulated gene expression was determined by qRT-PCR from RNA extracted from the RAW264 infected cells used in the experiment shown in panel **A**: Hepcidin (**B**); LCN2 (**C**); BDH2 (**D**); NRAMP1 (**E**). Error bars represent the SD from the mean fold change of qRT-PCR, each assayed in triplicate. This result is representative of two independent experiments.
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responded under the conditions employed to monitor phagocytic killing by upregulating a panel of its iron-responsive genes (*fetA, tbpA, tbpB, mpeR*) [34,35,36,37]. Further, our findings indicate that some gonococci can survive within both human and murine macrophages. Importantly, our work revealed that such infected phagocytes could sense the presence of gonococci and respond robustly by secreting pro-inflammatory mediators. Hence, gonococcal stimulation of macrophages could have a profound influence on the continued expression of a pro-inflammatory, often damaging, response during natural infection.

Since gonococci can survive intraleukocytically [1,5,23], it is important to understand how they adapt to the intracellular environment and acquire nutrients within phagocytes for survival and growth. We asked if gonococci could manipulate the bioavailability of iron that is normally tightly regulated by macrophages. The capacity of gonococci to use TonB-dependent and -independent mechanisms of iron acquisition, either constitutively or induced upon infection, is considered essential for both intracellular and extracellular growth. Herein, we show that gonococci could facilitate iron acquisition by upregulating expression of some of its own iron-responsive genes and by modulating the host cellular iron metabolism, leading to increased intracellular labile iron pools in macrophages. In this respect, we found that infection of macrophages by live gonococci led to increased hepcidin gene and protein expression that would cause iron retention in the overall macrophage population. It is important to emphasize that this cationic peptide, unlike LL-37 (24 and data not presented), does not exert anti-gonococcal activity as assessed under the conditions of our *in vitro* assay used for other cationic peptides, which is consistent with hepcidin behaving more as a hormone as opposed to an antibacterial peptide [47]. Furthermore, while increased hepcidin-25 activity against other bacteria has been shown to be acid pH-dependent [39,42], our data indicate that in the context of gonococci acidic pH does not increase the bactericidal activity of hepcidin-25. Therefore, when hepcidin concentration is increased in response to gonococcal infection in the acidic phagosome or the neutral pH cytosol of macrophages, gonococci would likely be able to survive this challenge. Others reported that hepcidin is induced in myeloid cells in response to bacterial pathogens via TLR4 [48] and bacterial cell wall components [49]. Further, gonococcal infection of macrophages also modulated the expression of other genes encoding factors involved in cellular iron metabolism. Specifically, its infection of macrophages reduced gene expression for ferroportin and BDH2 while increasing that for NRAMP1 and NGAL. Collectively, these changes would enhance the cytosolic

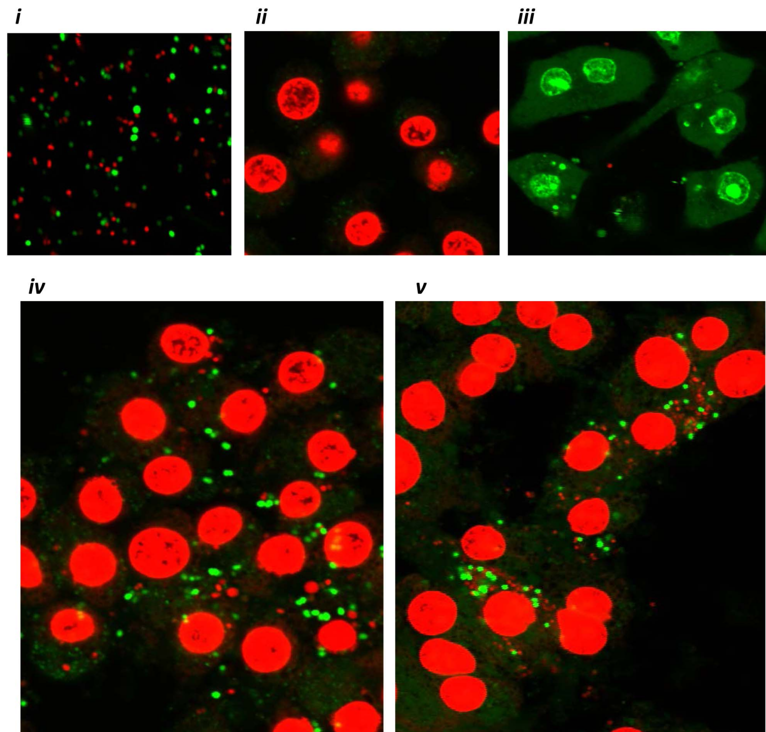

Figure 11. *N. gonorrhoeae* survives in association with murine RAW264 macrophages. Viability of GC-FA19 in RAW264 macrophages was visualized with BacLight staining. ***i***: GC-FA19 in medium alone incubated for 1 hr at room temperature prior to staining where red is dead and green is live GC-FA19. ***ii***: Uninfected RAW264 macrophages incubated at 37°C for 1 hr prior to staining in the presence of 0.1% saponin. ***iii***: RAW264 macrophages infected with GC-FA19 for 1 hr and stained without saponin. ***iv***: RAW264 macrophages infected with GC-FA19 for 1 hr and stained in the presence of 0.1% saponin. ***v***: RAW264 macrophages infected with GC-FA19 for 5 hr and stained in the presence of 0.1% saponin. These data are representative of three independent experiments.
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iron pool in macrophages. Taken together, our data suggest that *N. gonorrhoeae* modulates the iron-limiting innate immune defenses in macrophages, which we propose would facilitate its ability to acquire iron and survive intracellularly.

Iron retention in mammalian cells has been reported to be associated with depletion of BDH2 [16,17]. This enzyme is a homologue of EntA in *E. coli*, which mediates the synthesis of the bacterial siderophore 2,3-DHBA known as enterobactin [17]. Similarly, BDH2 mediates the synthesis of the mammalian siderophore 2,5-DHBA that binds and traffics labile cellular iron. BDH2 mRNA transcript contains an iron responsive element (IRE) that controls its expression in an iron-dependent manner, and has been shown to associate with cellular iron regulatory proteins (IRPs). Thus, BDH2 plays a very important role in cellular iron homeostasis [16]. To date, only the physiological role of BDH2 in cellular iron metabolism has been described. BDH2 depletion led to increased labile iron, which impacts mitochondrial iron-sulfur cluster biogenesis, heme synthesis and ROS redox balance [17]. Our study here is the first to report the role of BDH2 in innate immunity. Our findings suggest that BDH2 may be a new key player in the iron-limiting innate immune defenses against gonococcal infection in macrophages. We show that *N. gonorrhoeae* infection in macrophages resulted in significant downregulation of BDH2 expression. It is not known whether BDH2 downregulation is consequent to increased hepcidin expression since BDH2 harbors an IRE, and hence can be modulated by cellular iron retention [16]. The direct downregulation of BDH2 by gonococci cannot be ruled out and remains to be investigated. Nevertheless, the capacity of *N. gonorrhoeae* to modulate intracellular iron bioavailability by increasing hepcidin and reducing BDH2 expression is novel and may serve to facilitate gonococcal iron acquisition.

Other key components of cellular iron metabolism were also investigated in this study including NRAMP1, the endosomal iron transporter, and NGAL the iron carrier lipocalin. Interestingly, NRAMP1 plays an important role in host defense against intracellular bacterial infections such as tuberculosis by depleting iron from the phagolysosome where *Mycobacterium tuberculosis* (MTB) usually resides [50]. Genetic mutations/SNPs in NRAMP1 are shown to genetically predispose the host to infection with MTB and other intracellular pathogens [50]. Therefore, NRAMP1 is a key participant in iron metabolism and in host defense by exporting iron from the late endosome and phagolysosome, consequently increasing the transient labile cytosolic iron pool [51]. Importantly, we found that gonococcal infection of human monocytes or murine macrophages resulted in increased NRAMP1 gene expression, which is consistent with it being an integral part of the host defense to deplete iron in the

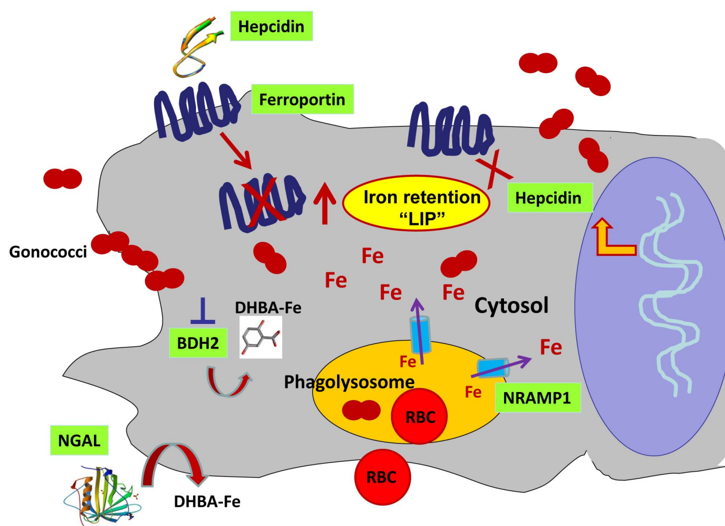

Figure 12. Model for how gonococcal infection in macrophages alters cellular iron homeostasis to facilitate iron acquisition. Macrophages play an essential role in iron homeostasis by engulfing senescent red blood cells (RBC) and recycling iron. Macrophages also play a very important role in host defense. GC infection in macrophages induces the expression of hepcidin (which then degrades the iron exporter ferroportin) and downregulates ferroportin gene expression, causing iron retention in macrophages. GC infection also induces expression of NRAMP1 (the cytosolic iron transporter) and NGAL (an iron carrier protein), and downregulates expression of BDH2, the enzyme that catalyzes the synthesis of the mammalian siderophore 2,5-DHBA which helps detoxify the labile iron pool (LIP). Collectively, these alterations in cellular iron homeostasis lead to increased iron bioavailability that facilitates iron acquisition and promotes gonococcal intracellular survival.
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phagolysosomal compartment. We also found that NGAL gene expression is highly upregulated in macrophages infected with gonococci. NGAL plays a role in both cellular iron homeostasis (since it shuttles liganded iron complexes such as the mammalian siderophore 2,5-DHBA [17,52]) and in host defense (due to its ability to scavenge bacterial siderophores [19,53]). NGAL is rapidly upregulated in response to infection and to other cellular stresses or perturbations. It has been shown that NGAL is highly upregulated during oxidative stress as an antioxidant cellular response [54]. NGAL also has adipokine function and therefore, exerts immunomodulatory effects on immune cells [55]. Gonococci are known to suppress adaptive and humoral immune responses [56]; therefore, the upregulation of NGAL may be an important part of the overall ability of this human pathogen to evade immune responses.

In summary, we report a novel mechanism by which *N. gonorrhoeae* modulates host cellular iron metabolism and innate immune defenses (Figure 12). We propose that this system facilitates gonococcal acquisition of iron so that it can survive within a macrophage. Given the longevity of macrophages and their capacity to migrate across epithelial surfaces, this survival strategy would potentiate the exacerbation and spread of infection.

Acknowledgments

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Author Contributions

Conceived and designed the experiments: SMZ WMS. Performed the experiments: SMZ JLK. Analyzed the data: SMZ JLK WMS. Contributed reagents/materials/analysis tools: SMZ WMS. Wrote the paper: SMZ WMS.

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Chapter 4: Phase-variable expression of *lptA* modulates the resistance of *Neisseria gonorrhoeae* to cationic antimicrobial peptides

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Phase-Variable Expression of *lptA* Modulates the Resistance of *Neisseria gonorrhoeae* to Cationic Antimicrobial Peptides

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Phosphoethanolamine (PEA) decoration of lipid A produced by *Neisseria gonorrhoeae* has been linked to bacterial resistance to cationic antimicrobial peptides/proteins (CAMPs) and *in vivo* fitness during experimental infection. We now report that the *lptA* gene, which encodes the PEA transferase responsible for this decoration, is in an operon and that high-frequency mutation in a polynucleotide repeat within *lptA* can influence gonococcal resistance to CAMPs.

Neisseria gonorrhoeae is a strict human pathogen that has caused the sexually transmitted infection termed gonorrhea for thousands of years. Over the millennia, *N. gonorrhoeae* has developed multiple mechanisms to resist innate host defenses, including cationic antimicrobial peptides/proteins (CAMPs) produced by phagocytes and epithelial cells (1). Phosphoethanolamine (PEA) decoration of the lipid A possessed by *N. gonorrhoeae* and *N. meningitidis* has been shown to contribute to their resistance to CAMPs by a mechanism that likely involves a reduction in ionic interactions of CAMPs with the bacterial surface (1–6), resistance of *N. gonorrhoeae* to complement-mediated killing by normal human serum (3, 4), *N. gonorrhoeae* fitness during experimental infection in mice and humans (5, 7), and the proinflammatory potential of *N. gonorrhoeae* (7, 8). Most commensal *Neisseria* do not encode *lptA* (8), but *N. gonorrhoeae* and *N. meningitidis* (2, 3, 8) typically contain *lptA* and produce multiple isoforms of lipid A that differ in PEA decoration at the 4′ and/or 1 position, though the basis of these isoforms has not been fully defined. We now provide evidence that gonococcal *lptA* is within an operon and that *N. gonorrhoeae* resistance to a model CAMP (polymyxin B; PMB) is modulated by high-frequency mutation due to a phase-variable (PV) polynucleotide stretch in the *lptA* coding sequence.

Organization and expression of the *lptA* locus in *N. gonorrhoeae*. Bioinformatic analysis of the DNA sequence of the *N. gonorrhoeae* FA 1090 chromosome (http://www.genome.ou.edu/gono.html) suggested that *lptA* is transcriptionally linked to two upstream genes (*serC* and a hypothetical gene annotated as NGO1282) and a downstream gene (*nfnB*) (Fig. 1). This hypothesis was confirmed by results from reverse transcription-PCR (RT-PCR) experiments (Fig. 2A) that demonstrated transcriptional linkage of *lptA* with the *serC*, hypothetical, and *nfnB* genes; details of the experimental procedures and a list of oligonucleotide primers are provided in the legends of Fig. 1 and 2 and in Table 1, respectively. However, primer extension analysis of total *N. gonorrhoeae* RNA performed as described previously (9) identified a transcriptional start point (TSP) positioned 61 nucleotides (nt) upstream of the *lptA* translational start codon and four nt downstream of near-consensus −10 and −35 elements (Fig. 1 and 2B). Thus, we tentatively conclude that *lptA* expression in *N. gonorrhoeae* can be initiated by two promoters upstream of *serC* and *lptA*, respectively. The mechanisms that control use of these promoters are now under investigation.

Analysis of the online FA 1090 genome sequence indicated that the *lptA* coding sequence contains a polynucleotide tract consist-

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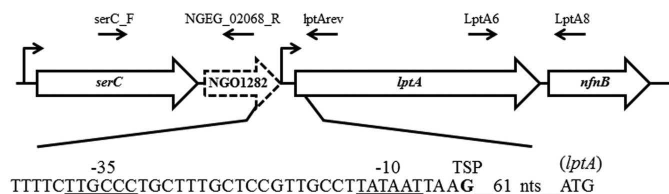

FIG 1 Genetic context of *lptA* in *N. gonorrhoeae* FA19. The 3.8-kb region of the FA19 genome shown corresponds to nucleotides 1236150 to 1232381 in *N. gonorrhoeae* FA 1090 (http://www.genome.ou.edu/gono.html and GenBank accession number AE004969.1). *serC* encodes a putative phosphoserine aminotransferase, NGO1282 encodes a hypothetical gene, *lptA* encodes a lipid A phosphoethanolamine transferase, and *nfnB* encodes a putative nitroreductase. The locations of the *serC* (undefined) and *lptA* (defined in the Fig. 2B legend) promoters are depicted with bent arrows. The *lptA* transcriptional start point, −10, and −35 promoter elements are shown below the illustration. The approximate sites of annealing for oligonucleotides used in RT-PCR experiments (Table 1) are shown with arrows.

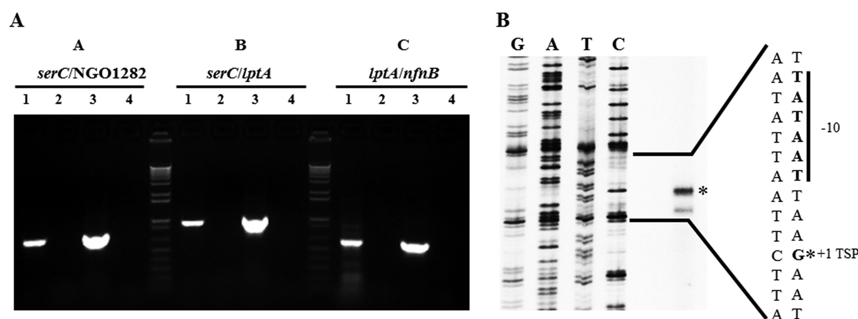

FIG 2 Transcription of the *lptA* coding sequence. (A) Transcriptional linkage between *serC*, NGO1282, *lptA*, and *nfnB*. All RT-PCRs were performed on purified RNA harvested (RNeasy minikit; Qiagen) from a log-phase culture of strain FA19 grown as described previously (9). First-strand cDNA was generated using SuperScript II reverse transcriptase (Invitrogen) and a gene-specific reverse primer (LptA8) that binds in the *nfnB* gene and primes elongation of a single-stranded cDNA toward *serC*. PCR was then performed to confirm transcriptional linkage between pairs of genes. Sections A, B, and C of the gel are grouped by forward- and reverse-primer locations and separated by 1-kb PLUS DNA ladders (Invitrogen). Lane 1, FA19 cDNA; lane 2, −RT negative control (RT omitted); lane 3, FA19 genomic DNA positive control; lane 4, no-template negative control. Section A, "*serC*" = serC_F; "NGO1282" = NGEG_02068_R. Section B, "*serC*" = serC_F; "*lptA*" = lptArev. Section C, "*lptA*" = LptA6; "*nfnB*" = LptA8. (B) Primer extension of the *lptA* transcript. Primer extension analysis was performed as described previously (9) using 20 μg of FA19 total RNA as the template and a radioactively ([γ-^{32}P]ATP) labeled reverse primer (LptA7_R) that anneals 67 bp downstream of the *lptA* start codon. RNA was purified and cDNA generated as described for panel A. Radioactive single-stranded cDNA products were separated on a polyacrylamide gel alongside sequencing reactions that used the same reverse primer (LptA11 was the forward primer used for generation of the sequenced *lptA* promoter template). The TSP corresponds to the band labeled with an asterisk and is 4 bp downstream of a consensus σ^{70}-type −10 element. A second band appeared running approximately 4 to 5 nucleotides shorter than the proposed +1 TSP band and could be due to a degraded mRNA transcript.

ing of seven Ts (T-7), which would result in production of a truncated LptA enzyme due to a new translational stop codon (Fig. 3). However, our independent sequencing of a PCR product containing the *lptA* gene from FA 1090 as well from strain FA19 showed the presence of a T-8 tract (data not presented and Fig. 3A), which would result in production of a full-length LptA enzyme (Fig. 3B). Moreover, analysis of the online (http://www.broadinstitute.org/annotation/genome/neisseria_gonorrhoeae/GenomesIndex.html) whole-genome sequences of 13 other gonococcal strains indicated that their *lptA* gene contains the T-8 tract (data not presented). In addition, the genome sequence for 73 *N. gonorrhoeae* clinical isolates from patients with symptomatic gonorrhea was determined using Illumina technology; the details of this genome shotgun sequencing effort will be published separately. The nucleotide sequence of the FA19 *lptA* gene was searched against a BLAST database of all the whole-genome *de novo*-assembled contigs of these clinical isolates using BLASTN in WUBLAST, in order to identify the genome location of the gene within each of the strains. We used the default blastn parameters and specified hspsepSmax (maximum separation allowed between HSPs [high-scoring segment pair] along subject) to be 100 bp. Sequences of *lptA* genes were then extracted and screened for the presence of a T-8 tract on both the forward and reverse strands of the gene using pattern matching. The results showed that all strains contained a T-8 tract and a full-length *lptA* sequence with 100% nucleotide identity to FA 1090 (data not presented). Accordingly, we propose that possession of an in-frame *lptA* gene is a common feature of *N. gonorrhoeae* isolates.

***lptA* behaves as a PV gene in *N. gonorrhoeae*, and phase-off variants are hypersusceptible to PMB.** The presence of the T-8 tract in the 5′ end of the *lptA* coding sequence suggested to us that it is a member of the PV gene family possessed by *N. gonorrhoeae* (10). If so, production of a full-length LptA, PEA decoration of lipid A, and CAMP resistance could differ within a population of gonococci. To test this possibility, we employed a PMB screen/selection procedure since loss of *lptA* expression renders *N. gonorrhoeae* hypersusceptible to this model CAMP (3, 5, 7). After replica plating approximately 3,000 colonies of strain FA 1090 (T-8 tract and PMB MIC of 100 μg/ml) onto gonococcal base (GCB) agar plates with or without PMB selection, we identified (approximate frequency of 3.3 × 10^{-4}) a colony (strain BB22) that was unable to grow on GCB agar plates containing 10 μg/ml

TABLE 1 Oligonucleotide primers used in this study

Primer name	Primer sequence	Purpose
LptA6	5′-CGGTTTTGTATGTGGATCAGTT-3′	Transcriptional linkage
LptA7	5′-GCCTTTCTTTCCCTGTATTCTT-3′	Sequencing of the poly-T tract
LptA7_R	5′-AAGAATACAGGGAAAGAAAGGC-3′	Primer extension
LptA8	5′-ACGTTGCAATCCTACCTCGC-3′	Transcriptional linkage
LptA11	5′-CCGGTTCGAATTTTGCTTACG-3′	Primer extension
LptAdelL	5′-TGCAGGTACATCATGAAATTAGAC-3′	Sequencing of the poly-T tract
lptAJK4	5′-TAAGAATCTTTTTCAATAATCCGGAT-3′	Sequencing of the poly-T tract
lptArev	5′-GCCTCAGGTTCGGTTTTATC-3′	Transcriptional linkage
LptAstart	5′-TCTAGAAAGCTTCATCGACTTGT-3′	Sequencing of the poly-T tract
NGEG_02068_R	5′-GCGGGCAAAGCATTTCATAT-3′	Transcriptional linkage
serC_F	5′-CGACTACGGACTGATTTACG-3′	Transcriptional linkage

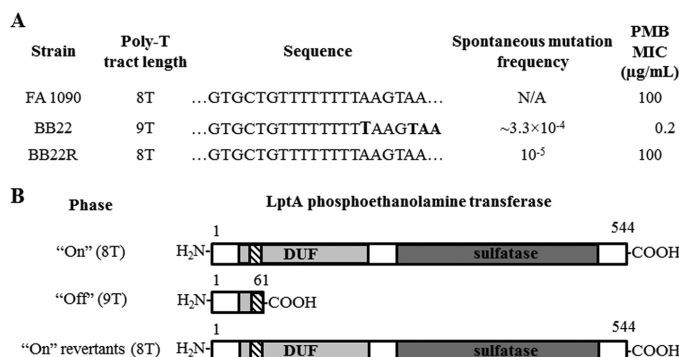

FIG 3 The *lptA* PV poly-T tract impacts LptA protein length and function. (A) Summary of the *lptA* PV poly-T tract. The PV poly-T tract comprises nucleotides 172 to 179 of the phase-on *lptA* open reading frame. Analysis of phase-on and phase-off *lptA* coding sequences using the ExPASy Translate tool (http://web.expasy.org/translate/) revealed that insertion of a ninth T nucleotide (bolded) in the poly-T tract of strain BB22 results in a frameshift mutation, which would generate a "UAA" stop codon shortly after this PV tract in the *lptA* mRNA transcript. (B) Impact of the PV poly-T tract on LptA protein length. When in the phase-on state, the *lptA* poly-T tract (hatched boxes) has 8 nucleotides and *lptA* encodes a protein 544 amino acids long. Frameshift of the *lptA* open reading frame (ORF) due to an insertion of a single T nucleotide within this poly-T tract would result in a premature stop codon and subsequent truncation of the LptA nascent polypeptide at just 61 amino acids. Phase-off variants are not predicted to translate the C-terminal sulfatase domain of the LptA protein (13). DUF, domain of unknown function.

of PMB. The PMB MIC against BB22 was 0.2 µg/ml (Fig. 3A), which is similar to the PMB MIC against an *lptA* deletion mutant of FA 1090 described previously (5). DNA sequence analysis of the *lptA* sequence of BB22 revealed that it possessed a T-9 tract that would result in premature truncation of LptA (Fig. 3). We then selected for spontaneous variants of BB22 that would grow on GCB agar containing 10 µg/ml of PMB. In four separate experiments, spontaneous PMB-resistant variants arose at a frequency of approximately 10^{-5}; in contrast, spontaneous erythromycin-resistant mutants (selected at 1 µg/ml) were recovered at a frequency of 10^{-8} (data not presented). The PMB MIC against PMB-resistant mutants of BB22 was, like that seen with parental strain FA 1090, 100 µg/ml (see strain BB22R data in Fig. 3A). DNA sequencing of the *lptA* PV tract from sixteen randomly picked PMB-resistant revertants of BB22 showed that all possessed a wild-type T-8 tract (see BB22R in Fig. 3A) and would produce a full-length LptA (Fig. 3B). Based on this reversion frequency, we estimate that the poly-T tract in *lptA* phase varies at an approximate frequency of 10^{-5}. This frequency is 2 to 3 orders of magnitude lower than that seen with other PV genes of *N. gonorrhoeae*, which may be due to its shorter tract (8 nt) and A/T characteristics that reduce slipped-strand mispairing events compared to the results seen with longer, G/C-rich repeats (10–12).

Conclusions. Production of PEA-decorated lipid A by *N. gonorrhoeae* has been linked with bacterial resistance to mediators of innate host defense, the capacity of *N. gonorrhoeae* to elicit a proinflammatory response, and *in vivo* fitness (3–5, 7, 8). The structurally variable lipooligosaccharide (LOS) chemotypes produced by gonococci have been linked to PV genes that encode enzymes responsible for adding carbohydrates within the branched-chain oligosaccharide region (11, 12). Our work now extends this PV expression property of LOS to the lipid A isoforms and emphasizes the complexity of LOS structures that can be presented by *N. gonorrhoeae*. Importantly, to our knowledge, this is the first direct evidence that gonococcal resistance to CAMPs can be modulated by a PV process.

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We declare that we have no conflicts of interest.

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Chapter 5: Identification of regulatory elements that control expression of the *tbpBA* operon in *Neisseria gonorrhoeae*

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Identification of Regulatory Elements That Control Expression of the *tbpBA* Operon in *Neisseria gonorrhoeae*

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Iron is an essential nutrient for survival and establishment of infection by *Neisseria gonorrhoeae*. The neisserial transferrin binding proteins (Tbps) comprise a bipartite system for iron acquisition from human transferrin. TbpA is the TonB-dependent transporter that accomplishes iron internalization. TbpB is a surface-exposed lipoprotein that makes the iron uptake process more efficient. Previous studies have shown that the genes encoding these proteins are arranged in a bicistronic operon, with the *tbpB* gene located upstream of *tbpA* and separated from it by an inverted repeat. The operon is under the control of the ferric uptake regulator (Fur); however, promoter elements necessary for regulated expression of the genes have not been experimentally defined. In this study, putative regulatory motifs were identified and confirmed by mutagenesis. Further examination of the sequence upstream of these promoter/operator motifs led to the identification of several novel repeats. We hypothesized that these repeats are involved in additional regulation of the operon. Insertional mutagenesis of regions upstream of the characterized promoter region resulted in decreased *tbpB* and *tbpA* transcript levels but increased protein levels for both TbpA and TbpB. Using RNA sequencing (RNA-Seq) technology, we determined that a long RNA was produced from the region upstream of *tbpB*. We localized the 5′ endpoint of this transcript to between the two upstream insertions by qualitative RT-PCR. We propose that expression of this upstream RNA leads to optimized expression of the gene products from within the *tbpBA* operon.

Neisseria gonorrhoeae is the etiological agent of the sexually transmitted infection (STI) gonorrhea. This disease is the second most commonly reported infectious disease in the United States, with 334,826 cases reported in 2012 (http://www.cdc.gov/nchhstp/newsroom/docs/STD-Trends-508.pdf); however, the CDC estimates that the actual number of gonorrhea cases is over 800,000 per year (http://www.cdc.gov/std/stats/sti-estimates-fact-sheet-feb-2013.pdf). Worldwide, the WHO estimated that there were 106.1 million new cases of gonorrhea in adults in 2008 (1). Infection manifests primarily as urethritis in men and as cervicitis in women; however, asymptomatic infections are common, especially in women. Due to a rapid rise in resistance to previously effective antibiotics (2, http://www.cdc.gov/nchhstp/newsroom/docs/STD-Trends-508.pdf, and http://www.cdc.gov/std/stats/sti-estimates-fact-sheet-feb-2013.pdf), the CDC currently recommends combination therapy with the extended-spectrum cephalosporin ceftriaxone, plus doxycycline or azithromycin, for treatment (3). Thus, the need for new treatments or ideally preventative methods is evident.

Thus far, vaccine development efforts against gonorrhea have been unsuccessful. Previous attempts focused on surface antigens, such as porin (4) or pilin (5, 6), and have been ineffective, in part because of high-frequency antigenic and/or phase variation of most surface-exposed antigens. The transferrin binding proteins (Tbps) are attractive vaccine targets because they are expressed by all gonococcal isolates tested to date (7), are surface accessible, have conserved sequences, and have been shown to be necessary for the establishment of infection in an experimental human male infection model (8). We demonstrated that recombinant Tbp proteins conjugated to the cholera toxin B subunit are capable of inducing antibody responses in the serum and the genital tract of female mice (9, 10), suggesting that these antigens can be components of an efficacious vaccine.

Most pathogenic bacteria require iron in order to sustain essential metabolic processes and to establish successful infections (11). In a mammalian host, bacteria are presented with the challenge of having to compete for iron with the host; thus, they need to be armed with mechanisms that will allow them to appropriate iron or take advantage of the iron transport and storage mechanisms of the host. Many bacteria, like *Escherichia coli*, are capable of secreting siderophores as their primary mechanism for iron acquisition (12–14). Gonococci do not produce siderophores (15, 16) but are able to obtain iron from human transferrin by expressing transferrin-binding proteins (17, 18).

The gonococcal transferrin iron acquisition system is comprised of two transferrin-binding proteins, TbpA and TbpB. TbpA is an integral outer membrane protein which shares sequence similarity with TonB-dependent outer membrane transporters (18). TbpA is capable of transporting iron across the outer membrane of gonococci, making it essential in the process of iron uptake from transferrin (19, 20). TbpB is a surface-exposed lipoprotein (19, 20) that has the ability to discriminate between holo- and apotransferrin (21, 22). Expression of TbpA is essential for uptake

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TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Description	Reference
N. gonorrhoeae		
FA19	Wild type (TbpB$^+$/TbpA$^+$)	7
FA1090	Wild type (TbpB$^+$/TbpA$^+$)	52
F62	Wild type (TbpB$^+$/TbpA$^+$)	53
MS11	Wild type (TbpB$^+$/TbpA$^+$)	54
FA6815	*tbpB*::Ω (TbpB$^-$/TbpA$^-$)	17
MCV108	*tbpA-lacZ* fusion (TbpB$^+$/TbpA$^-$)	24
MCV109	*tbpB-lacZ* fusion (TbpB$^-$/TbpA$^+$)	24
MCV113	FA19 *tbpB*$\Omega-_{447}$	This study
MCV114	FA19 *tbpB*$\Omega-_{82}$	This study
MCV117	−10 Promoter element replaced by SmaI site	This study
MCV118	−35 Promoter element replaced by BamHI site	This study
MCV119	Fur box sequence replaced by SmaI site	This study
MCV120	*tbpA-lacZ* fusion in MCV113	This study
MCV121	*tbpB-lacZ* fusion in MCV113	This study
MCV122	*tbpA-lacZ* fusion in MCV114	This study
MCV123	*tbpB-lacZ* fusion in MCV114	This study
Plasmids		
pCRII-TOPO	Amp^r Kan^r	Invitrogen
pCR2.1-TOPO	Amp^r Kan^r	Invitrogen
pHSS6-GCU (Kan^r)	Vector containing gonococcal uptake sequence	27
pVCU108	*lacZ-ermC′* insertion in the MluI site of *tbpA*	24
pVCU109	*lacZ-ermC′* insertion in the PmlI site of *tbpB*	24
pVCU122	pCRII-TOPO containing a 609-bp segment of *tbpBA* upstream region with novel SmaI site at position −447 relative to TbpB start codon	This study
pVCU123	pCRII-TOPO containing a 609-bp segment of *tbpBA* upstream region with novel SmaI site at position −82 relative to TbpB start codon	This study
pVCU124	pCRII-TOPO containing a 609-bp segment of *tbpBA* upstream region with an Ω insertion at position −447 relative to TbpB start codon	This study
pVCU125	pCRII-TOPO containing a 609-bp segment of *tbpBA* upstream region with an Ω insertion at position −82 relative to TbpB start codon	This study
pVCU126	pHSS6-GCU containing EcoRI fragment of VCU124	This study
pVCU127	pHSS6-GCU containing EcoRI fragment of VCU125	This study

of iron (18), and although expression of TbpB is not essential, its presence makes the iron uptake process more efficient (17).

The *tbpA* and *tbpB* genes are arranged in a bicistronic operon, with the *tbpB* gene located upstream of *tbpA* (17, 23, 24). The genes are separated by an 86-bp region that contains an inverted repeat. The genes are cotranscribed (24) and the operon is under the control of the Fur protein, resulting in preferential expression of Tbps under iron-limited conditions. However, the genes are differentially expressed. Using a variety of reverse transcription-PCR (RT-PCR) and fusion techniques, we previously demonstrated that *tbpB*-specific transcripts are approximately 2-fold more prevalent than *tbpA*-specific transcripts under iron-stressed conditions (24). Given that TbpB binds specifically to holotransferrin, it may be advantageous to the gonococcus to produce more TbpB and present it on the cell surface as an iron-sequestering mechanism.

In this study, we sought to characterize the mechanisms that coordinately control expression of the two genes within the *tbpBA* operon. We identified the promoter elements required for *tbpB* and *tbpA* expression and further identified a novel repeat-rich region that influences expression of these genes. A long RNA species apparently is located upstream of *tbpB* in a polarity opposite that of the genes encoding the Tbps. A mutant in which this RNA is insertionally interrupted showed dysregulation of the *tbp* genes. Therefore, we propose that this long RNA is critical to the optimized expression of the components of the transferrin receptor complex. A complete understanding of the conditions under which this system is expressed is imperative in order to exploit their potential as vaccine candidates.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains used in this study are listed in Table 1. *Neisseria gonorrhoeae* strains were routinely maintained on GC medium base (Difco) agar with Kellogg's supplement I (25) and 12 μM Fe $(NO_3)_3$ at 37°C in a 5% CO_2 atmosphere. When required, iron stress was imposed by overnight growth of strains on GC agar plates containing Kellogg's supplement I and 10 μM deferoxamine mesylate (DFO) (Desferal; Sigma) with no additional iron.

PCR amplification, sequencing, and DNA sequence alignment. Wild-type gonococcal strains FA19, MS11, and F62 were propagated on GC agar plates as described above. Single colonies were resuspended in 100 μl of distilled water. Samples were boiled at 100°C for 5 min. PCR amplification was conducted using primers oVCU151 and oVCU153 (see Table S1 in the supplemental material) and Platinum *Taq* DNA polymerase (Invitrogen). The size of the PCR amplicon was confirmed by gel electrophoresis visualization. The PCR product was cloned into pCR2.1-TOPO (Invitrogen) and sequenced by the Nucleic Acids Research Facility at Virginia Commonwealth University. DNA sequence alignments were produced and visualized with ClustalW.

Construction of promoter region mutants. Promoter region mutants were generated by gene splicing using overlap extension PCR (26). The putative −10 region was replaced by an SmaI site using primers oVCU258 and oVCU259. The putative −35 region was replaced by a BamHI site using primers oVCU256 and oVCU257. Six residues in the putative Fur box were replaced by an SmaI site using primers oVCU260 and oVCU261. Mutagenized PCR products were subcloned into pHSS6-GCU (27) to provide the gonococcal uptake sequence, which is necessary for transformation. The resulting plasmids were then used to transform wild-type gonococcal strain FA19. Successful recombination into the chromosome was confirmed by PCR amplification followed by digestion with either SmaI or BamHI, as necessary. PCR products derived from amplification of chromosomal DNA from recombinant strains were sequenced by the Nucleic Acids Research Facility at Virginia Commonwealth University. The resulting mutant strains were named MCV117 (−10 mutant), MCV118 (−35 mutant), and MCV119 (Fur box mutant), as described in Table 1.

Construction of Ω cassette insertion mutants. The Ω cassette insertion mutations upstream of *tbpB* were generated by gene splicing using overlap extension PCR (26). Novel SmaI sites were generated at positions −447 and −82 relative to the *tbpB* transcriptional start site. The mutated upstream regions were cloned into pCRII-TOPO (Invitrogen) and designated pVCU122 and pVCU123 (corresponding to positions −447 and −82, respectively). The resulting plasmids were digested with SmaI and then ligated to the Ω fragment, which encodes streptomycin resistance (28). The ligation mixture was transformed into *E. coli*, generating pVCU124 and pVCU125, and the sequences were confirmed by PCR and DNA sequencing. The *tbpBA* upstream regions containing the Ω fragments from pVCU124 and pVCU125 were subcloned into pHSS6-GCU, generating plasmids pVCU126 and pVCU127, respectively. The plasmids were linearized and then transformed into gonococcal strain FA19. Transformants were selected on GC agar containing streptomycin, confirmed by PCR, and designated MCV113 and MCV114, respectively.

Generation of iron stress conditions for whole-cell lysate preparation. *N. gonorrhoeae* strains were cultivated on plates as described above. For iron-depleted cultures, colonies were picked from plates containing GC medium base with supplement I and DFO and used to inoculate GC broth containing Kellogg's supplement I only. Cultures were grown at 37°C at 5% CO_2 with vigorous shaking. Culture densities were monitored, and after one doubling, the cultures were supplemented with 250 μM DFO. Cultures were then allowed to grow for 4 h, after which the final cell densities were measured and samples standardized to the same density. Samples were centrifuged for 10 min at 13,000 rpm. Pellets were resuspended in Laemmli solubilizing buffer (29), and lysates were stored at −20°C.

SDS-PAGE and Western blot analysis. Whole-cell lysates, prepared as described above, were treated with 5% β-mercaptoethanol and boiled for 2 min. Solubilized samples were subjected to SDS-PAGE, and proteins were transferred to nitrocellulose membranes (GE Healthcare Life Sciences) in 20 mM Tris base, 150 mM glycine, and 20% methanol in a submerged transfer apparatus (Bio-Rad). For TbpA detection, membranes were blocked with 5% nonfat dry milk in high-salt Tris-buffered saline (TBS) and 0.05% Tween 20 (Sigma). Membranes were probed with primary anti-TbpA polyclonal antibodies (16), washed with high-salt TBS and 0.05% Tween 20, and incubated with a goat-anti-rabbit alkaline phosphate-conjugated (Bio-Rad) secondary antibody. For TbpB detection, membranes were blocked with 5% nonfat dry milk in low-salt Tris-buffered saline. Membranes were probed with primary anti-TbpB polyclonal antibodies (30) and washed with low-salt TBS and 0.05% Tween 20, followed by goat-anti-rabbit alkaline phosphatase-conjugated secondary antibody. All blots were developed using the nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate colorimetric system (Sigma). Blots were scanned with Adobe Photoshop, and band intensity was quantified using NIH Image J software (31). Each TbpA and TbpB signal was normalized to the loading control from the same sample. The normalized values were then averaged.

Transferrin-iron utilization assays. The promoter mutants were tested for transferrin utilization by inoculating strains onto chelexed defined media (CDM) (32) supplemented with 30% iron-saturated human transferrin (Sigma) as the sole iron source. Plates were incubated at 37°C and 5% CO_2, and bacterial growth was monitored after 24 to 48 h.

Construction of transcriptional *lacZ* fusion strains. Upstream insertion mutants MCV113 and MCV114 were transformed by plasmids pVCU109 and pVCU108 (24), which contain promoterless *lacZ* genes fused to either *tbpB* or *tbpA*, respectively. Allelic replacement mutants in which the *lacZ* gene was located downstream of the polar Ω mutations were selected for by growth on erythromycin, resistance to which is encoded by the promoterless *lacZ* cassette. The resulting strains were named as described in Table 1.

β-Galactosidase assays. At each time point, aliquots were removed and mixed with Z-buffer (33). Cells were lysed with SDS and chloroform, and the β-galactosidase assay was performed according to the method of Miller (33). All strains were analyzed in triplicate at every time point. Values shown represent the means from at least four independent experiments conducted on different days.

RNA isolation. Total RNA was isolated from gonococcal cultures grown for 2 h under iron-depleted or iron-replete conditions using an RNeasy maxi kit as directed by the manufacturer (Qiagen). For qualitative reverse transcription-PCR (RT-PCR) experiments, gonococcal cultures were treated with RNAprotect bacterial reagent (Qiagen) immediately prior to RNA isolation. Purified RNA was treated twice with RQ1 RNase-free DNase (Promega) at 37°C for 1 h prior to storage at −80°C.

Primer extension analysis. Primer oVCU151 (see Table S1 in the supplemental material), located 37 bases downstream of the TbpB start codon, was end labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The radioactive primer was hybridized with 100 μg total RNA, isolated as described above, and treated with avian myeloblastosis virus (AMV) reverse transcriptase (GE Healthcare). The resulting primer extension products were analyzed on a sequencing gel containing 8% polyacrylamide and 8 M urea. Sequencing reactions using the same primer were loaded alongside the primer extension products to serve as a marker.

Relative RT-PCR. RNA was isolated as described above and treated with DNase. The Thermoscript RT-PCR system (Invitrogen) was utilized in combination with a specific primer designed to prime reverse transcription of an upstream RNA species (oVCU735C; see Table S1 in the supplemental material) or with random hexamers. Following reverse transcription, cDNAs were amplified with gene-specific primers (see Table S1 in the supplemental material) using Platinum *Taq* polymerase (Invitrogen). PCR products were separated on 2.5% agarose gels and visualized by ethidium bromide staining.

Quantitative, real-time RT-PCR. Total RNA was isolated from iron-depleted gonococcal cultures as described above. cDNA was generated by reverse transcription of 100 ng of total RNA using the primers listed in Table S1 in the supplemental material and the Accuscript high-fidelity 1st strand cDNA synthesis kit (Agilent Technologies). cDNA was amplified with the CFX96 real-time system (Bio-Rad) utilizing the SensiMix SYBR No-ROX kit (Bioline). The expression level of the target genes were normalized to *porB1A* expression as an internal control. All oligonucleotides used are listed in Table S1 in the supplemental material. Control wells without template were conducted for every reaction in every experiment. Each assay was performed at least in triplicate, and cDNA from 3 independent RNA preparations was analyzed. Relative expression values for each gene were calculated using the $2^{-\Delta\Delta CT}$ method (34, 35).

RNA-Seq analysis. Recently, we (J. L. Kandler and W. M. Shafer, unpublished) performed a transcriptional profiling comparison by RNA sequencing (RNA-Seq) using enriched mRNA prepared from wild-type strain FA19 and an isogenic transformant bearing an insertionally inactivated copy of the *misR* gene (annotated NGO0177 in strain FA1090 [www.genome.ou.edu/gono.html]), which is part of a two-component regula-

tory system in pathogenic *Neisseria* species (36–38). The details of this comparison will be published elsewhere. However, from analysis of the transcripts produced by these strains, we detected equal levels of a 1.8-kb RNA species that would be located upstream of the *tbpBA* operon (see Results). The procedure for RNA-Seq relevant to the work presented here is described below. Bioinformatic analysis used the transcriptional profiling data from the RNA-Seq work performed on strain FA19 *misR*::*kan*. This strain was grown in liquid culture in triplicate to late-log phase in GC broth containing Kellogg's supplement I and iron, 0.043% (wt/vol) sodium bicarbonate, and 10 mM $MgCl_2$ in a 37°C water bath with shaking at 150 rpm. At late-log phase, 8 ml was harvested from each of the three cultures and processed in RNAprotect bacterial reagent (Qiagen) according to the manufacturer's instructions. Total RNA was harvested from culture pellets using an RNeasy minikit (Qiagen) according to the manufacturer's instructions. Removal of any contaminating DNA was accomplished by treatment with TURBO DNA-free (Ambion) according to the manufacturer's instructions. rRNA was depleted using two rounds of treatment with the MICROBExpress bacterial mRNA enrichment kit (Ambion) according to the manufacturer's instructions. RNA purity was confirmed by analysis on an Agilent 2100 Bioanalyzer (Agilent Technologies) after each step. cDNA libraries were prepared from enriched FA19 mRNA transcriptomes using the SuperScript double-stranded cDNA synthesis kit (Invitrogen) and random hexameric primers (Invitrogen) according to the manufacturer's instructions. Because the RNase H provided in the SuperScript kit digests RNA only in DNA-RNA hybrids, samples were treated with 10 μg of RNase A (Novagen) for 10 min at 37°C to ensure complete removal of any remaining, unhybridized RNA. cDNA was purified using phenol extraction and ethanol precipitation. cDNA libraries were amplified by 15 cycles of PCR for the addition of adaptors with TruSeq indexes using the Illumina TruSeq sample preparation kit according to the manufacturer's instructions. After quantitation and dilution, the cDNA libraries representing the FA19 and FA19 *misR*::*kan* mutant late-log transcriptomes were clustered in a single lane and sequenced on an Illumina Genome Analyzer IIx instrument with 50-bp paired-end (PE) reads. The raw reads were trimmed by removing adapter sequences and ambiguous nucleotides. Reads with quality scores of less than 20 and a length below 30 bp all were trimmed. The resulting high-quality reads from each of the 3 replicate samples were mapped onto the *N. gonorrhoeae* FA1090 reference genome (GenBank accession no. AE004969.1) using CLC Genomics Workbench software. For the reference mapping, at least 95% of the bases were required to align to the reference genome, and a maximum of 2 mismatches were allowed. The total number of reads mapped for each transcript was determined and then normalized to detect the reads-per-kilobase-per-million-reads (RPKM) measure. Transcribed regions of the FA19 *misR*::*kan* genome were visualized using SeqMan software (DNASTAR).

Statistical analysis. Statistical significance was evaluated using a two-tailed, unpaired Student's *t* test. Statistical significance is noted when $P \leq 0.05$.

Accession numbers. The new sequence information presented in this report has been submitted to GenBank under the following accession numbers: F62 sequence upstream of *tbpB*, KJ579423; MS11 sequence upstream of *tbpB*, KJ579424; and FA19 sequence upstream of *tbpB*, KJ579425. The complete data set of the sequence reads can be accessed through GEO accession number GSE50184 and SRA accession number SRP029218.

RESULTS

***tbpBA* promoter mapping.** While the genetic arrangement and cotranscription of the *tbpBA* operon has been described (17, 24), little is known about *cis*- or *trans*-acting factors that control expression of the operon. We first sought to identify the transcriptional start site of *tbpB* using primer extension analysis. A primer extension product was observed using RNA isolated from gonococcal strain FA19 grown under iron-restricted growth conditions (Fig. 1A). This primer extension product was not detectable from gonococcal cultures grown under iron-replete conditions. The primer extension product detected under iron-depleted growth conditions corresponded to the C residue located 31 bp upstream of the TbpB start codon (Fig. 1A and B).

To identify putative promoter elements, the upstream region of *N. gonorrhoeae* wild-type strain FA19 (Fig. 1B) was compared to consensus *E. coli* sequences (39). A putative −10 region was identified 7 bp upstream of the identified transcriptional start site and matched the *E. coli* consensus sequence (5′-TATAAT-3′) at five of six positions. A putative −35 region matched the *E. coli* consensus sequence (5′-TTGACA-3′) at four of six positions. These two motifs were separated by 16 nucleotides. We also identified a predicted Fur binding site (Fig. 1B), which overlaps the −10 sequence and matched the *E. coli* consensus sequence (5′-GATAATGATA ATCATTATC-3′) at 13 of 19 positions. The identified site is a perfect match with the consensus Fur binding site identified for *Neisseria* species (40).

To confirm that the putative −10, −35, and Fur box elements were indeed responsible for iron-regulated promoter activity, mutational analysis was conducted. Using PCR and the mutagenic primers listed in Table S1 in the supplemental material, the putative promoter elements were changed as shown in Fig. 1B. We evaluated TbpA and TbpB protein expression in the mutants by Western blotting. As expected, TbpA and TbpB were detected in the wild-type strain under iron-depleted conditions. Mutagenesis of the −10 region (strain MCV117) abolished expression of the Tbps even under low-iron conditions (Fig. 1C). Mutagenesis of the −35 region (strain MCV118) almost completely abrogated expression of both Tbps, although TbpA was weakly detectable in the blot probed with the α-TbpA antibody. Mutation of the putative Fur binding site (strain MCV119) resulted in expression of both Tbps regardless of iron availability during growth. This phenotype is consistent with that observed using a *fur* point mutant (41).

The promoter region mutants were also tested for growth on human transferrin as a sole iron source (Fig. 1D). The wild-type strain FA19 was able to grow on the plates and served as the positive control. Strain FA6815 (Table 1) was the negative control, as this strain is TbpA and TbpB deficient (17). The −10 region mutant (MCV117) was incapable of growth on transferrin even after 48 h, and the Fur box mutant (MCV119) grew much like the wild-type strain on transferrin. The −35 region mutant (MCV118) also grew on transferrin (Fig. 1D), demonstrating that even a very low level of TbpA expression apparently was sufficient to support transferrin-dependent growth. Taken together, these data confirm that the predicted *tbpBA* promoter elements and Fur box do indeed control the production of TbpA and TbpB.

Characterization of an extended intergenic region upstream of *tbpB*. The validated promoter extends to 66 bp upstream of the TbpB start codon (Fig. 1B). However, in the genome sequence of gonococcal strain FA1090 (accession number NC_002946), there is an extremely long (approximately 1.9 kb) intergenic region that separates the *tbp* operon from the next proximal upstream gene (NGO1499) (Fig. 2). This region contains two short, annotated hypothetical open reading frames (ORFs) (NGO1497 and NGO1498), but it is unclear whether either is actually translated into protein. A third, unannotated ORF could be identified further upstream of NGO1498, as shown in Fig. 2. Because the ORFs are within repeated regions, they share sequence identity and are

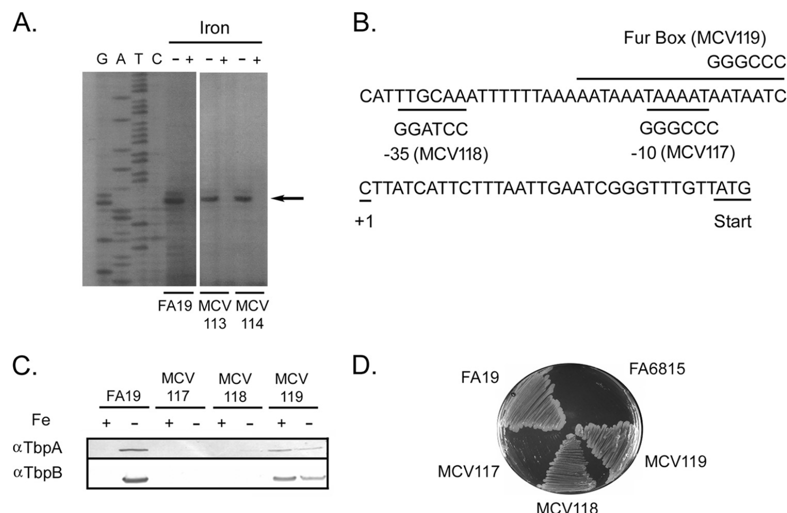

FIG 1 Identification of promoter elements controlling expression of the *tbpBA* operon. (A) Primer extension analysis demonstrating the transcriptional start site for *tbpB* in the wild-type and upstream insertion mutants. Total RNA was isolated from gonococcal cultures grown under iron-depleted (−) and iron-replete (+) conditions as indicated at the top. A sequencing ladder generated with the same primer is shown on the left. The arrow marks the location of the primer extension product. This analysis compared the transcriptional start sites of the wild-type strain (FA19) to two Ω insertion mutants (MCV113 and MCV114; see the text). (B) Mutagenesis of promoter elements. The *tbpB* transcriptional start site (+1) is shown. The TbpB initiation codon is underlined. The mutagenized promoter elements (−10, −35, and Fur box) are labeled; the new nucleotides in the mutants are shown above or below the wild-type sequence. (C) Expression of Tbps in the promoter region mutants. Gonococci were grown under iron-replete (+) or iron-depleted (−) conditions and then lysed. The whole-cell lysates were subjected to SDS-PAGE, and proteins were transferred to nitrocellulose. Blots were probed with α-TbpA or α-TbpB antibodies as labeled on the left. MCV117 is the −10 mutant, MCV118 is the −35 mutant, and MCV119 is the Fur box mutant. (D) Gonococcal mutants were tested for growth in human transferrin by plating on CDM containing 30% saturated transferrin as the sole iron source. FA19 is the positive control and FA6815 (*tbpB*Ω) is the negative control. MCV117 is the −10 mutant, MCV118 is the −35 mutant, and MCV119 is the Fur box mutant.

paralogous. A similar distance separates the *tbp* locus from the next upstream open reading frame in *Neisseria meningitidis* (42, 43). Several dRS3 repeats (43), which have the consensus sequence of ATTCCC(N_8)GGGAAT, are located within this intergenic region (Fig. 2). These repeats, which are also located upstream of the meningococcal *tbpB* gene, have been proposed to promote recombination with exogenously acquired DNA (42). Within 1 kb of the *tbpB* promoter region, we identified three direct repeats in the FA1090 DNA sequence, each of which could encode a hypothetical, short open reading frame (Fig. 2). Repeat 1 (R1) is 366 nt in length, R2 is 365 nt in length, and R3 is 237 nt in length. These tandem repeats share extensive sequence identity; R1 is 97.8% identical to R2, and R1 is 84.2% identical to R3 (Fig. 3). Each of the repeats is also flanked by at least one inverted repeat sequence of CTCTCTCCC(N_3)GGGAGAGAG (Fig. 2). Figure 4 shows the intergenic DNA sequence in the FA1090 genome between *tbpB* and the NGO1499 open reading frame with relevant repeats and primer sequences highlighted. The particular arrangement of tandem, direct, and short inverted repeats downstream of the dRS3 units (Fig. 2 and 4) is unique to the *tbpB* upstream region in the gonococcal chromosome, as we were unable to find similar arrangements within the genomes of the other *Neisseria* species. Moreover, the arrangement of direct repeat units (R1, R2, and R3) located upstream of *tbpB* is unique to this locus and not found elsewhere in the gonococcal genome. However, partial matches for R1 can be found near *pilC1* (NGO0055) and *carA* (NGO0053). We next explored the possibility that these sequences form part of an extended regulatory region for the *tbpBA* operon.

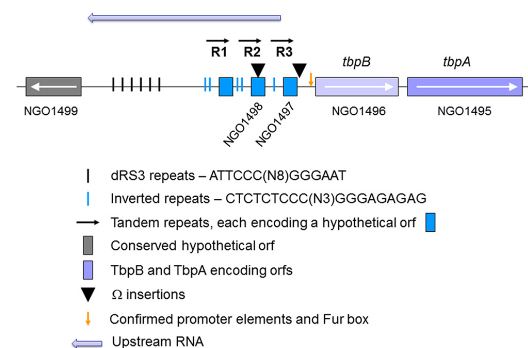

FIG 2 Representation of the region (approximately 1.9 kb) between *tbpB* and NGO1499 from the FA1090 genome database (www.genome.ou.edu/gono.html). Six dRS3 repeats are located 5′ of *tbpB* (short, black lines). Three tandem, direct repeats (horizontal arrows) are located between the dRS3 repeats and the *tbpB* start codon. Each repeat unit contains a short hypothetical ORF (blue boxes) and is flanked at the 5′ end by at least one inverted repeat (short blue lines). ▼, Locations of Ω cassette insertions. Genes and intergenic regions are not drawn to scale.

We rationalized that if the repeats were involved in regulation of the operon, they would be highly conserved among gonococcal

```
R1    GTCCTCTCTCCCCGTGGGAGAGAGTTAGAGAGAGGGCAGCAAGCCGTATTAGTTTGCATA 60
R2    GTCCTCTCTCCCTGCGGGAGAGAGTTAGAGAGAGGGCAGCAAGCCGTATTAGTTTGCATA 60
R3    ------------------------------------------------------------


R1    AACCTTAACCCCAACAGCCACCCCGTCCTCTCTCCCTGCGGGAGAGAGTTAGAGAGAGGG 120
R2    AACCTTAACCCCAACAGCCACCCCGTCCTCTCTCCCTGCGGGAGAGAGTTAGAGAGAGGG 120
R3    ------------------------GTCCTCTCTCCCTGCGGGAGAAAGCTAGAGAGAGGG 36
                              ********************* ** ***********

R1    CAACAAGCCGCAAGGTTTGTATTTTAGAAGACTAAGGGAGTTTGGGAAAGATTGCCGCAA 180
R2    CAACAAGCCGCAAGGTTTGTATTTTAGAAGACTAAGGGAGTTTGGGAAAGATTGCCGCAA 180
R3    CAACAAGCCGCAAGGCTTGTGTTTTAGAAGACTAAGGGAGTTTGGGAAAGATTGCCGCAA 96
      *************** **** ***************************************

R1    CTCGGAGAATGCCCTCTCCCCGGCCCTCCCCCACG--GGGGAGGGAGCGGATTGCGGCAG 238
R2    CTCGGAGAATGCCCTCTCCCCGGCCCTCCCCCACG--GGGGAGGGAGCGGATTGCGGCAG 238
R3    CTTGGAAAGTGCCCTCTCCCCAGCCCTCCGCCGCGCAGGGGAGGGAGCGGATTGCAGCAG 156
      ** *** * ************ ******* ** **  ****************** ****

R1    ATTTTGCGGTTGCAGGCGGTTTGAAAAGCAACTTGGATTTACCGTTGATTTCAGGTCGTA 298
R2    ATTTTGCGGTTGCAGGCGGTTTGAAAAGCAACTTGGATTTACCGTTGATTTCAGGTCGTA 298
R3    GTTTGGCGGTTGCAGGCGGTTTTAAAAGCAGCGCGGGAATGACGGT-ATTTTGGTTTATT 215
       *** ***************** ******* *  **   *  ** * ****  * *  *

R1    TG--AAAAATAAAAAACAGCCTGCACAAGCCGTATTAGTTTGCATAAACCTTAACCCCAA 356
R2    TG--AAAAATAAAAAACAGCCTGCACAAGCTG-ATTAGTTTACAGAAACCTTAACCCCAA 355
R3    TACTAAAAATATAAAAAAGCAT-------------------------------------- 237
      *   ******* **** *** *

R1    CAGCCACCCC 366
R2    CAGCCGCACC 365
R3    ----------
```

FIG 3 Comparison of repeat sequences upstream of the *tbpBA* operon. Repeats are between 237 and 366 nucleotides in length. Repeat 1 (R1) shares 97.8% identity with repeat 2 (R2). R1 shares 84.2% identity with repeat 3 (R3). Repeat 2 shares 84.5% identity with repeat 3. Multiple-sequence alignment of R1, R2, and R3 demonstrates that the greatest level of sequence identity is centrally located. Asterisks mark positions of identity between the aligned sequences.

strains. Accordingly, based upon the DNA sequence from strain FA1090, we designed primers to amplify the upstream intergenic region from other gonococcal strains, including our type strain, FA19. We were only able to amplify and clone a product of approximately 600 bp from gonococcal strain FA19. Repeated attempts to amplify and clone regions longer than 600 bp failed. The amplicon that we were able to clone and sequence (Fig. 5) was flanked by primers oVCU151 and oVCU153 (see Table S1 in the supplemental material) and included only two of the repeat units identified in the genome sequence of FA1090 (Fig. 2 and 4). We subsequently utilized these same oligonucleotides to amplify the homologous regions from gonococcal strains MS11 and F62. Alignment of the resulting sequences revealed that one repeated region was well conserved in all strains examined, while the other repeat was not as well conserved and was largely absent from strain F62 (Fig. 5). The sequences shown in Fig. 5 are not present in the partially completed genome sequences of strains FA19, MS11, and F62 (Broad Institute). These areas represent gaps in the partial genome sequences, which is perhaps consistent with our difficulties in cloning DNA from this repeat-rich intergenic region.

Disruption of the region upstream of the *tbpB* promoter affects transcript levels. To elucidate whether the repeat units impacted the expression of the *tbp* operon, the upstream region was disrupted by insertion of Ω cassettes. An Ω cassette was inserted 447 bp upstream of the *tbpB* transcriptional start site (strain MCV113) (Table 1). A second Ω cassette was inserted 82 bp upstream of the *tbpB* transcriptional start site (strain MCV114) (Table 1). The Ω cassette in MCV113 disrupts R2, and the cassette in MCV114 is just 3′ of the end of R3 (Fig. 4). Insertional mutagenesis did not alter the transcriptional start site for the operon (Fig. 1A).

Transcriptional *lacZ* fusions were created for each *tbp* gene in the Ω_{-447} insertion background (MCV120 and MCV121) as well as for the Ω_{-82} background (MCV122 and MCV123) (Table 1) in order to assay transcript levels. Strains MCV108 and MCV109 were previously generated (24) and contain *lacZ* insertions into the *tbpA* and *tbpB* genes, respectively. These fusions have wild-type upstream regions; thus, they were used as positive controls for *tbp* transcript levels. All strains were grown in GC base media supplemented with Kellogg's supplement I. DFO was added to all cultures in order to generate iron-depleted conditions. β-Galactosidase assays showed that *tbpB* transcript levels decreased in the Ω_{-447} insertion mutant compared to the positive-control strain (Fig. 6A). *tbpA* transcript levels were also decreased relative to wild-type *tbpA* levels. Similarly, *tbpB* transcript levels appeared decreased in the Ω_{-82} insertion mutant compared to the positive control (Fig. 6B). However, the modest decrease in *tbpA* transcript levels in the Ω_{-82} insertion mutant did not reach the level of statistical significance in this assay. Overall, these results indicated that the Ω cassette inserted 447 bp upstream of the *tbpB* transcriptional start site had a dramatic, negative effect on *tbpB* and *tbpA*

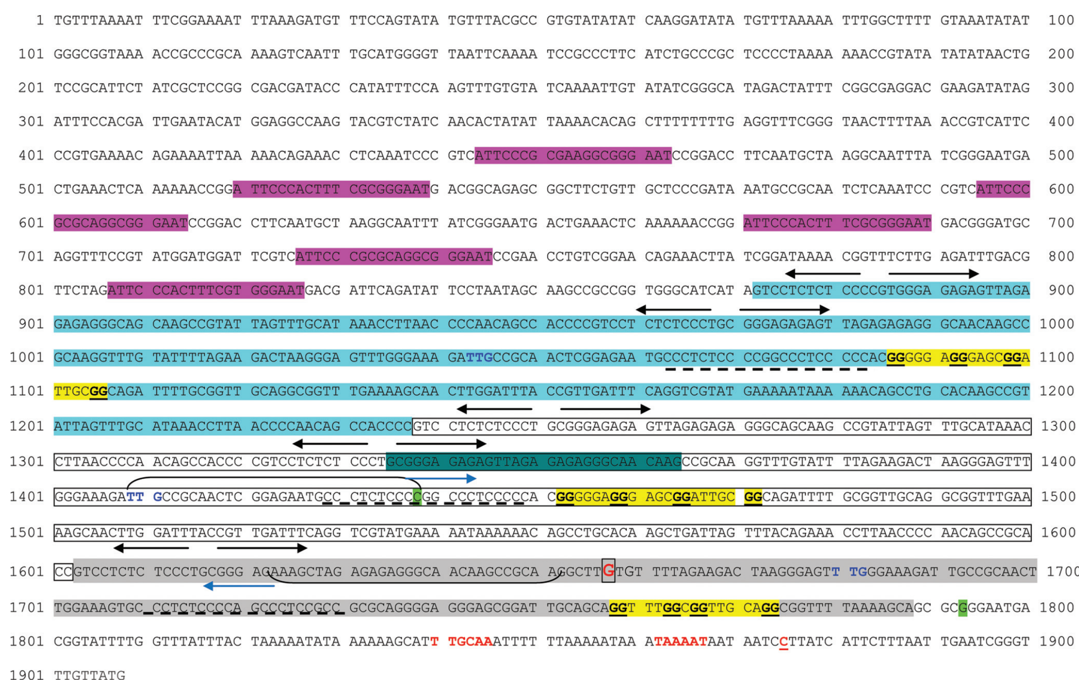

FIG 4 Sequence upstream of *tbpB* gene in FA1090 genome with repeats and positions of Ω insertions highlighted. Yellow highlighting shows predicted G4 sequences (see the text). Dotted lines below sequences indicate C-rich domains that are complementary to G4 sequences. Dark green shows the position of oligonucleotide oVCU153, used for cloning. Light green highlighting indicates the nucleotide immediately 3′ of Ω insertion positions. Cyan highlighting, repeat 1 sequence; open box, repeat 2 sequence; gray highlighting, repeat 3 sequence; inverted arrows, inverted repeats; blue boldfaced text, predicted start codons for hypothetical ORFs; pink highlighting, dRS3 repeats. The boxed, red G residue indicates the start of long RNA predicted from the RNA-Seq result. Upstream RNA extends 1.8 kb in the orientation opposite that of *tbpB*. Red text shows the −35, −10, and transcriptional start sites for the *tbpBA* operon. Brackets with blue arrows show positions of primers used for relative RT-PCR.

transcript levels. In contrast, the Ω insertion located closer to the start of *tbpB* (−82) had less of an impact on *tbp* transcript levels.

We confirmed that these results were not an artifact due to the orientation of the polar Ω cassette and its internal termination signals by evaluating the cassette in the opposite direction, which resulted in the same outcomes (data not shown). Additionally, we confirmed our findings by means of qRT-PCR. RNA extracted from iron-stressed cultures was utilized to detect changes in *tbp* gene expression. Our results confirmed that the Ω cassette insertion mutants did indeed demonstrate decreased *tbp* gene expression compared to the wild-type strain (Table 2). Similar to the results of the *lacZ* fusion analysis, transcript levels of *tbpB* and *tbpA* in MCV113 were more profoundly affected than in MCV114. Indeed, *tbpB* transcript levels were 6.3-fold lower in the MCV113 mutant than in the wild-type strain. *tbpB* transcript levels were only 2-fold lower in the MCV114 strain than in the wild-type strain. *tbpA* transcript levels were 6-fold lower in MCV113 and 2-fold lower in MCV114 than in the wild type.

Upstream Ω insertions result in increased TbpB and TbpA protein levels. We analyzed TbpB and TbpA protein levels in the upstream Ω insertion mutants (MCV113 and MCV114) by Western blotting. As shown in Fig. 7, TbpB and TbpA protein levels were increased in the Ω_{-447} mutant (MCV113) relative to the parent, FA19. Protein levels in MCV114 appeared to be between wild-type and MCV113 levels. When Western blots were scanned and imaged, we determined that TbpB protein levels increased 240% in MCV113 and 220% in MCV114. Similarly, TbpA levels increased 290% in MCV113 and 150% in MCV114. All of the increases in protein levels in the mutants reached statistical significance ($P < 0.01$) relative to the wild type, except for the TbpA levels in MCV114 ($P > 0.05$). This result is similar to that with the *lacZ* transcriptional fusion activity (Fig. 6), in which the change in *tbpA* levels did not reach the level of statistical significance when comparing the wild-type strain to MCV114. Cumulatively, the results from the insertional mutagenesis studies indicated that an Ω insertion 447 bp upstream of the *tbpB* start site resulted in decreased transcript levels for both *tbp* genes but increased levels of both proteins. The impact of the insertion closer to the *tbpB* start site (−82) was similar in effect but of diminished magnitude.

A long RNA is synthesized from within the region impacted by the Ω insertions upstream of *tbpB*. The region upstream of *tbpB* potentially carried three short, hypothetical open reading frames, all oriented in the same direction as *tbpB* transcription (Fig. 2 and 4). However, visual analysis of RNA-Seq data obtained from FA19 *misR*::*kan* demonstrated that the nucleotides comprising NGO1497 (Fig. 2) were not transcribed under our *in vitro*

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MS11      ------------------------------CCGCAAGGTTTGTATTTTAGAAGACTAAGG 60
FA1090    GCGGGAGAGAGTTAGAGAGAGGGCAACAAGCCGCAAGGTTTGTATTTTAGAAGACTAAGG 60
F62       ------------------------------CCGCAAGGTTTGTATTTTAGAAGACTAAGG 60
FA19      ------------------------------CCGCAAGGTTTGTATTTTAGAAGACTAAGG 60

MS11      GAGTTTGGGAAAGATTGCCGCAACTCGGAGAATGCCCTCTCCCCGGCCCTCCCCCACGGG 120
FA1090    GAGTTTGGGAAAGATTGCCGCAACTCGGAGAATGCCCTCTCCCCGGCCCTCCCCCACGGG 120
F62       GAGTTTGGGAAAGATTGCCGCAACTCGGAGAATGCCCTCTCCCCGGCCCTCCCCCACGGG 120
FA19      GAGTTTGGGAAAGATTGCCGCAACTCGGAGAATGCCCTCTCCCCGGCCCTCCCCCACGGG 120

MS11      GGAGGGAGCGGATTGCGGCAGATTTTGCGGTTGCAGGCGGTTTGAAAAGCAACTTGGATT 180
FA1090    GGAGGGAGCGGATTGCGGCAGATTTTGCGGTTGCAGGCGGTTTGAAAAGCAACTTGGATT 180
F62       GGAGGGAGCGGATTGCGGCAG--------------------------------------- 141
FA19      GGAGGGAGCGGATTGCGGCAGATTTTGCGGTTGCAGGCGGTTTGAAAAGCAACTTGGATT 180

MS11      TACCGTTGATTTCAGGTCGTATGAAAAATAAAAAACAGCCTGCACAAGCTGATTAGTTTG 240
FA1090    TACCGTTGATTTCAGGTCGTATGAAAAATAAAAAACAGCCTGCACAAGCTGATTAGTTTA 240
F62       ------------------------------------------------------------
FA19      TACCGTTGATTTCAGGTCGTATGAAAAATAAAAAACAGCCTGCACAAGCTGATTAGTTTA 240

MS11      CATAAACCTTAACCCCAACAGCCGCACCGTCCTCTCTCCCTGCGGGAGAGAGTTAGAGAG 300
FA1090    CAGAAACCTTAACCCCAACAGCCGCACCGTCCTCTCTCCCTGCGGGAGAAAGCTAGAGAG 300
F62       ------------------------------------------------------------
FA19      CAGAAACCTTAACCCCAACAGCCGCACCGTCCTCTCTCCCTGTGGGAGAAAGCTAGAGAG 300

MS11      AGGGCAGTAAGCCGCAAGGCTTGTGTTTTAGAAGACTAAGGGA-TTTGGGAGAGGTTGCC 359
FA1090    AGGGCAACAAGCCGCAAGGCTTGTGTTTTAGAAGACTAAGGGAGTTTGGGAAAGATTGCC 360
F62       ------------------------------------------------------------
FA19      AGGGCAGTAAGCCGCAAGGTTTGTATTTTAGAAGACTAAGGGAGTTTGGGAAAGATTGCC 360

MS11      GCAACTTGGAAAGTGCCCTCTCCCCAGCCCTCCCCCGCGCAGGGGAGGGAGCGGATTGCA 419
FA1090    GCAACTTGGAAAGTGCCCTCTCCCCAGCCCTCCGCCGCGCAGGGGAGGGAGCGGATTGCA 420
F62       ------------------------------------------------------------
FA19      GCAACTCGGAGAATGCCCTCTCCCCGGCCCTCCCCCACG--GGGGAGGGATCGGATTGCG 418

MS11      GCAGGTTTGGCGGTTGCAGGCGGTTTTAAAAGCAGCGCGGGAATGACGGTATTTTGGTTT 479
FA1090    GCAGGTTTGGCGGTTGCAGGCGGTTTTAAAAGCAGCGCGGGAATGACGGTATTTTGGTTT 480
F62       ----GTTTGGCGGTTGCAGGCGGTTTTAAAAGCAGCGCGGGAATGACGGTATTTTGGTTT 197
FA19      GCAGATTTTGCGGTTGCAGGCGGTTTTAAAAGCAGCGCGGGAATGACGGTATTTTGGTTT 478

MS11      ATTTACTAAAAATATAAAAAAGCATTTGCAAATTTTTTAAAAATAAATAAAATAATAATC 539
FA1090    ATTTACTAAAAATATAAAAAAGCATTTGCAAATTTTTTAAAAATAAATAAAATAATAATC 540
F62       ATTTACTAAAAATATAAAAAAGCATTTGCAAATTTTTTAAAAATAAATAAAATAATAATC 257
FA19      ATTTACTAAAAATATAAAAAAGCATTTGCAAATTTTTTAAAAATAAATAAAATAATAATC 538

MS11      CTTATCATTCTTTAATTGAATCGGGTTTGTT 570
FA1090    CTTATCATTCTTTAATTGAATCGGGTTTGTT 571
F62       CTTATCATTCTTTAATTGAATCGGGTTCGTT 288
FA19      CTTATCATTCTTTAATTGAATCGGGTTTGTT 569
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FIG 5 Alignment of sequence upstream of the *tbpBA* operon from four wild-type *N. gonorrhoeae* strains. Regions of approximately 600 bp upstream of *tbpB* were cloned and sequenced from wild-type strains FA19, MS11, and F62. The resulting sequences were aligned with the homologous region from strain FA1090. Dashes represent spaces introduced into the alignment. The *tbpB* transcriptional start site is highlighted in yellow. The conserved promoter elements are highlighted in blue. The Fur box is shown in red text. Part of R2, highlighted by the single horizontal line, is included in the aligned sequence. All of R3, which is highlighted by the double horizontal line, is included in the aligned sequence. The blue text (TTG) designates the predicted start codons for two hypothetical ORFs upstream of *tbpB*. The green highlighting shows the nucleotides in FA19 that are immediately 3′ of the Ω insertions created in this study. The gray shading shows the predicted G4 sequences (see the text).

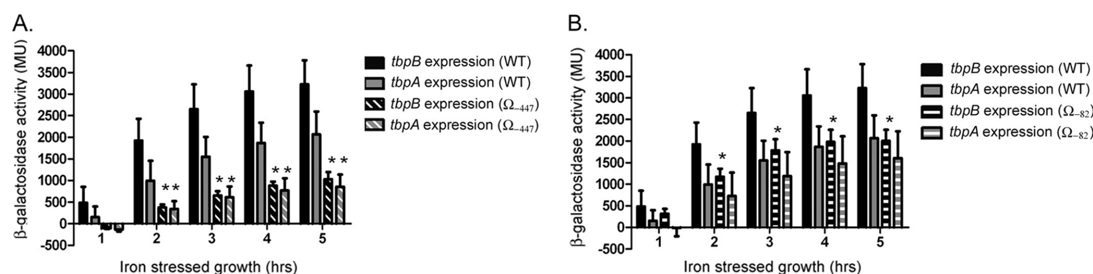

FIG 6 Disruption of the upstream region decreases transcription of *tbpB* and *tbpA*. In strain MCV113, repeat 2 of wild-type strain FA19 was disrupted by insertion of an Ω cassette 447 bp upstream of the *tbpB* transcriptional start site. In strain MCV114, an Ω cassette was inserted 82 bp upstream of the *tbpB* transcriptional start site. *tbpB* and *tbpA* transcript levels in upstream insertion mutants were analyzed by β-galactosidase assay after insertion of a transcriptional *lacZ* fusion into *tbpB* or *tbpA* in the MCV113 and MCV114 backgrounds. (A) *tbpB* and *tbpA* transcription levels when the upstream sequence is wild type (solid bars) compared to when the Ω cassette is inserted 447 bp upstream of the *tbpB* transcriptional start site (striped). (B) *tbpB* and *tbpA* transcription levels when the upstream sequence is wild type (solid bars) compared to when the Ω cassette is inserted 82 bp upstream of the *tbpB* transcriptional start site (striped). For both panels, each bar represents the means from at least four assays conducted on different days; error bars represent standard deviation. For both panels, MU represents Miller units. Asterisks denote $P \leq 0.01$ compared to the wild-type transcript levels for the corresponding gene.

conditions (iron-replete GC medium). In contrast, we did observe transcription of a 1.8-kb RNA (mapping to FA1090 nucleotides 1465413 to 1467214; see File S1 in the supplemental material) that encompasses NGO1498 and the undesignated ORF region (Fig. 8). Coverage was quite high near the *tbpB* gene but waned as the distance increased. This suggested that the observed long RNA was transcribed in the orientation opposite that required for translation of the hypothetical proteins and the *tbpBA* operon, as areas of high coverage in promoter regions are usually followed by declining coverage moving toward the 3′ end of the RNA. This RNA species is not anticipated to encode any proteins, as no conserved protein domains were detectable in either orientation upon analysis with the Conserved Domain Database search engine (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Although a few short, hypothetical proteins are predicted to be encoded by sequence in the FA1090 genome database between *tbpB* and NGO1499, the vast majority of the 1.8-kb transcript is not anticipated to be translated.

The 5′ end of the upstream 1.8-kb RNA species is located between the positions into which the Ω cassettes were inserted. We next used qualitative RT-PCR to discern whether the upstream Ω insertions impacted expression of the 1.8-kb RNA species. We predicted that Ω_{-82} (in strain MCV114) would not interrupt expression of the regulatory RNA species, whereas Ω_{-447} (in strain MCV113) would, based upon the predicted 5′ end of the RNA. When RT-PCR was conducted on RNA isolated from strains FA19, MCV113, and MCV114 using primers oVCU199C and oVCU735C (Fig. 4; also see Table S1 in the supplemental material), we detected an amplification product only in FA19 and MCV114 (Fig. 9). The predicted-sized product (238 bp; arrow in Fig. 9) was not detected when RNA from strain MCV113 was subjected to RT-PCR. The RT-PCR product detected in FA19 and MCV114 was not sensitive to iron concentrations, unlike the control *tbpA* gene (Fig. 9). Other controls shown in Fig. 9 include no reverse transcriptase (negative control) and 16S rRNA (positive control). The results shown in Fig. 9 confirm that an RNA species is located upstream and in the orientation opposite that of *tbpB* in gonococcal strain FA19. Furthermore, this analysis maps the 5′ endpoint of the RNA to between −82 and −447 upstream of the *tbpB* start site.

TABLE 2 Fold changes in *tbp* expression in the Ω cassette insertion mutants compared to the wild-type strain determined by qRT-PCR

	porBIA normalization of[a]:	
Strain	*tbpB*	*tbpA*
WT/MCV113	**6.30** (4.20–9.58)	**6.07** (3.94–9.32)
WT/MCV114	**1.98** (1.07–3.20)	**1.86** (1.04–2.48)

[a] Numbers in boldface are the means from three experimental replicates from each of three biological replicates conducted on different days (total of nine samples). Numbers in parentheses indicate the range of values obtained by analysis with the $2^{-\Delta\Delta CT}$ method (34, 35). All fold differences comparing the wild-type strain to the mutants for both *tbpB* and *tbpA* were statistically significant ($P < 0.05$).

DISCUSSION

Studies on the gonococcal transferrin receptor have focused mainly on deciphering the structure-function relationships of its components (19, 20), and little is known about the precise mechanisms that coordinately control expression of these proteins. A

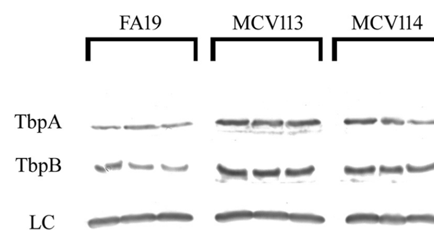

FIG 7 Western blot demonstrating that TbpA and TbpB are produced at higher levels in the Ω insertion mutants. Expression of TbpA and TbpB in wild-type (FA19) and upstream Ω insertion mutants, MCV113 (Ω_{-447}) and MCV114 (Ω_{-82}). The three lanes per strain represent biological replicates grown under iron-stressed conditions on different days. Lanes were loaded equivalently as assessed by the standardized culture density of the whole-cell lysates and Ponceau S staining of the nitrocellulose membrane, and the loading control (LC) is shown. The loading control is a cross-reactive band that is detected when probing whole-cell lysates with the polyclonal anti-TbpA antibody used in this assay. The loading control recapitulates the equivalent staining in each lane detected by Ponceau S.

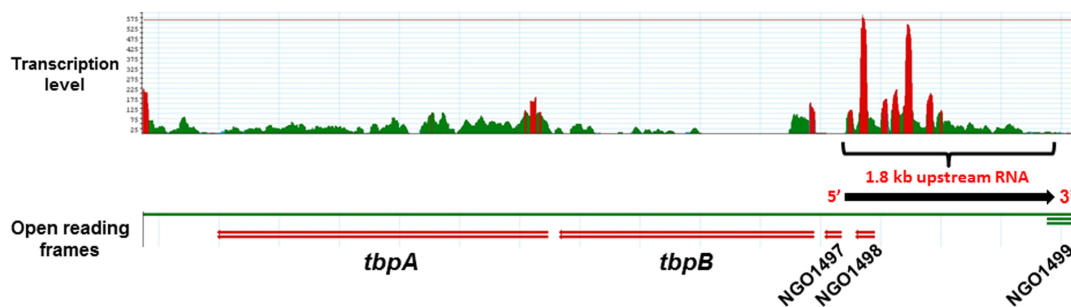

FIG 8 An RNA species (1.8 kb) is transcribed upstream and in the opposite orientation to that of the *tbpBA* operon. RNA-Seq analysis, which maps and quantifies transcribed regions of a genome, revealed that a long RNA is transcribed from a region mapping to nucleotides 1465413 to 1467214 of strain FA1090. The transcription level (top) represents the number of sequencing reads at each base pair (green, transcribed areas that do not exceed 100 sequencing reads; red, transcribed areas that exceed 100 sequencing reads at the corresponding sequence). The genomic position (bottom) of the annotated open reading frames for *tbpB*, *tbpA*, NGO1497, NGO1498, and NGO1499 can be seen in relation to the 1.8-kb RNA (black arrow). Context of the 1.8-kb RNA was visualized using SeqMan Pro software (DNASTAR).

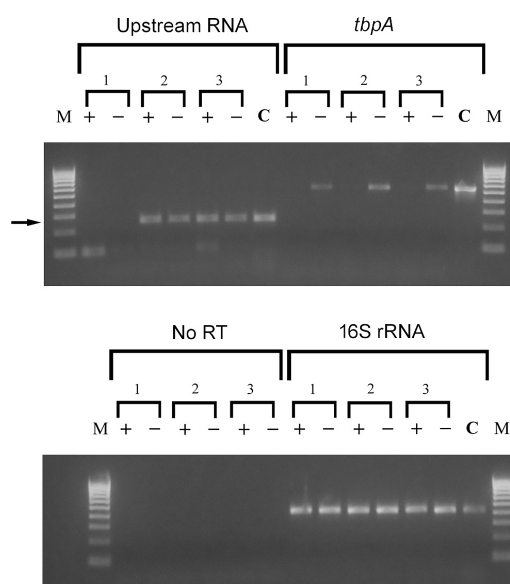

FIG 9 RT-PCR experiment demonstrating that Ω insertion into R2 (−447 relative to the transcriptional start site) prevents expression of the upstream RNA species. Cultures of MCV113 (1), MCV114 (2), and wild-type FA19 (3) were grown under iron-replete (+) and iron-depleted (−) conditions. RNA was isolated from each culture and subjected to reverse transcription. oVCU735C was used to reverse transcribe the upstream RNA, and random hexamers were used to reverse transcribe *tbpA* and 16S rRNA. A negative control in which no reverse transcriptase was added, along with random hexamers, was also conducted (no RT). For PCRs, the following oligonucleotides were utilized: oVCU735C and oVCU199C to amplify the upstream RNA, oVCU186 and oVCU187 to amplify *tbpA*, and oVCU110 and oVCU111 to amplify 16S rRNA and no-RT reactions. Chromosomal DNA from FA19 (C) also was amplified with the same primer sets to demonstrate the wild-type size of each amplicon. Molecular mass markers are shown on the left and right of each ethidium bromide-stained gel. Markers (M) range from 100 bp to 1,000 bp and increase in increments of 100 bp. The arrow indicates the position of the amplicon that was amplified with primers oVCU735C and oVCU199C, corresponding to the upstream RNA.

putative promoter for the operon had previously been proposed (17) but never experimentally defined. The purpose of this study was to characterize the extent of the functional promoter for the operon. Using primer extension analysis, we mapped the start site of *tbp* transcription at a position 31 bp upstream of the TbpB start codon. The C residue, representing the transcriptional start site for the *tbpBA* operon, was preceded by a C residue at position −1 and followed by a T residue at position +2. These nucleotides are most commonly found at positions −1 and +2 in *E. coli* promoters (39, 44). Tbp protein expression was abolished or severely decreased when promoter elements were mutagenized, confirming that the sequences of these regions are necessary for transcription of the operon. Although mutagenesis of the −35 promoter element did not completely abrogate expression of the Tbps, it did lead to a severe decrease in the detectable levels of the proteins. In transcription, the −35 motif serves as a point of initial contact between the RNA polymerase and the DNA, but it is the −10 region where the DNA starts to unwind to initiate transcription. It is possible that the mutagenesis of the −35 element was enough to disturb gene expression but not eliminate it entirely, since the −10 region was still intact. Additionally, the Fur binding site motif was similarly identified by homology and mutagenized. The resulting mutant demonstrated Tbp expression regardless of the iron availability during growth. This is the first report to experimentally define the promoter for the gonococcal *tbpBA* operon. Thus, our results confirm and extend our findings that the genes are cotranscribed from a common upstream promoter and that the operon is under the control of the Fur protein (24).

Analysis of the region upstream of the *tbpBA* operon revealed approximately 1.9 kb of sequence with several repeat regions and three short, hypothetical open reading frames. In the 600 bp directly upstream of the *tbpB* transcriptional start site, we identified two direct repeats. A third repeat is located immediately upstream of this region in the complete FA1090 genome database (www.genome.ou.edu/gono.html). The other gonococcal genomes that have been sequenced by the Broad Institute have not been closed or completely annotated, and each is lacking sequence data in the region immediately upstream of the *tbp* genes. Thus, the sequences presented here for strains FA19, MS11, and F62 are new and not currently in any other database. The repeats within this

upstream region resulted in our cloning difficulties and also likely contributed to the failure of genome sequencing in this area of the chromosome.

The repeat-rich region immediately upstream of *tbpB* is unique to this locus and is not found in the analogous position in *N. meningitidis* or the commensal *Neisseria* genomes. This suggests that the regulatory region described here is specific to the gonococcal *tbp* locus and important for appropriate, coordinated expression of the gonococcal Tbp proteins. We have previously reported that the ratio of *tbp* transcripts is 2 *tbpB* transcripts to 1 *tbpA* transcript in wild-type FA19, even though the genes are cotranscribed as a bicistronic operon. The mechanism by which this ratio is achieved is not completely clear, but we proposed that the intergenic region between *tbpB* and *tbpA*, which includes a putative secondary structure, contributes to increased *tbpB* transcripts relative to *tbpA* transcripts. In the current study, we also found that insertionally interrupting expression of an upstream RNA resulted in a ratio of nearly 1 *tbpB* transcript to 1 *tbpA* transcript (Fig. 6A), suggesting that preventing expression of this long RNA alters the relative amounts of *tbpB* and *tbpA* transcripts that are detectable.

Regulatory RNAs have been detected widely in bacteria, utilizing RNA-Seq and other transcriptomics approaches (for reviews, see references 45–47). While the best-characterized examples of regulatory RNAs in bacteria are relatively short (between 50 and 300 nucleotides [48]), it has become clear that bacteria also produce relatively long RNAs with the capability to alter gene expression by affecting both transcript and protein levels. *cis*-encoded regulatory RNAs generally overlap the genes they regulate and share a great deal of sequence complementarity (45, 48). On the other hand, *trans*-encoded regulatory RNAs are transcribed from loci far removed from those genes they regulate and share less sequence complementarity (46). The RNA species transcribed upstream of *tbpB*, in the opposite orientation, appears to be distinct from either of the two well-characterized paradigms of how regulatory RNAs impact gene expression. The RNA transcribed upstream of *tbpB* is predicted to be large, at 1.8 kb, and is produced from a locus adjacent to the genes it regulates. However, this *cis*-encoded RNA species does not share extended regions of complementarity with *tbpB* or *tbpA* and does not overlap the 5′ end of the *tbpB* transcript, which is typical of classical *cis*-encoded regulatory RNAs. Thus, the RNA upstream of the *tbpBA* operon, first described in this study, has features that distinguish it from regulatory RNAs previously characterized in other bacteria.

G4 sequences, or G-quadruplexes, are guanine-rich sequences that have been identified in many genomes and have wide-ranging impacts on DNA recombination and gene expression, among others (for a review, see references 49 and 50). Cahoon and Seifert (51) have recently shown that pilin antigenic variation in *N. gonorrhoeae* is affected by a proximal G4 sequence, which directs the expression of a noncoding RNA upstream of *pilE*. Several predicted G4 sequences are located upstream of the *tbpB* gene (Fig. 4) and could have effects on transcription of the long RNA detected in the current study. In addition to the guanine-rich repeats, we identified complementary cytosine-rich sequences (Fig. 4) that could base pair with the G4 sequences, potentially altering the folding of the planar G-quadruplexes and their effects on the surrounding genes. While we did not directly demonstrate folding of these G4 sequences or their impacts on expression of the Tbps, their presence in this complicated, repeat-rich regulatory region is intriguing and worthy of further study.

We found that the repeats upstream of *tbpB* were well conserved in several *N. gonorrhoeae* strains. In strain F62, the region between two putative G4 sequences was deleted, consistent with these repeats contributing to recombination and impacting the number and length of repeats upstream of *tbpB*. Deletion of this region in F62 would be expected to impact the expression and potentially the length of the upstream regulatory RNA. The impact of this alteration in strain F62 is unclear but would be expected to result in changes in expression of the transferrin-binding proteins. While sequence upstream of *tbpB* in FA19, FA1090, and MS11 all have the potential to encode the short, atypical ORFs (Fig. 5; TTG start codons are highlighted in blue), it is anticipated that these short peptides, which would start with leucine rather than methionine, are not actually produced, since the long RNA detected by RNA-Seq appeared to be in the orientation opposite that in which *tbpB* is expressed. The orientation of the only RNA detectable upstream of *tbpB* is opposite that of the upstream ORFs, and the 5′ endpoint of the RNA does not encompass NGO1497, precluding its production under the *in vitro* conditions used in this study.

Insertions in and around repeats 2 and 3 upstream of *tbpB* decreased both *tbpB* and *tbpA* transcript levels but, conversely, increased Tbp protein levels relative to the wild-type strain. However, the insertion at −447 clearly had the greatest impact, both on transcript and protein levels. The Ω insertion at position −447 also clearly interrupts the expression of the upstream RNA, whereas the Ω insertion at −82 was not anticipated to insertionally inactivate the upstream RNA. The RT-PCR results from this study map the 5′ end of the upstream RNA species to between −447 and −82 nucleotides upstream of the *tbpB* transcriptional start site, consistent with the RNA-Seq data. The Ω insertion at −447 results in decreased *tbpBA* transcript levels, as assessed by qRT-PCR and *lacZ* fusion analysis. This result suggests that expression of the RNA upstream of *tbpB* results in enhanced expression or stability of the *tbpBA* cotranscript. Furthermore, the Ω insertion at −447 resulted in increased production of the Tbp proteins. Thus, expression of the upstream RNA appears to negatively influence translation of the Tbps. These results are both potentially achievable if the regulatory RNA located upstream of *tbpB* enhances transcription or mRNA stability but negatively affects the translatability of this message into protein. Using both Target RNA (http://snowwhite.wellesley.edu/targetRNA/index_2.html) and RNA Predator (http://rna.tbi.univie.ac.at/RNApredator2/target_search.cgi), modest regions of alignment between the sequence of the upstream RNA (see File S1 in the supplemental material) and *tbpB* and *tbpA* genes can be detected (data not shown). Our working hypothesis is that hybridization between the long RNA and the *tbp* locus influences both transcript and protein levels. Clearly, further studies are necessary to extend and validate this proposed model.

Overall, the results of this study provide insight into the coordinated regulation of the *tbpBA* operon. We identified the promoter elements required for iron-regulated expression of both TbpA and TbpB. Our data suggest that both the quantity and stoichiometry of the *tbp* gene products are affected by interruption of an extended, repeat-filled regulatory region, which is located immediately upstream of *tbpB*. While this region generally is well conserved within the gonococcal genome sequences ana-

lyzed, some heterogeneity was identified, particularly in proximity to predicted G4 sequences. This repeat-filled region is unique to this location in gonococcal genomes. We detected the expression of a regulatory RNA upstream of *tbpB* in the orientation opposite that of *tbpB*. This RNA species possesses several unique features, including its relatively long length and the fact that it is *cis* encoded but does not share significant complementarity with the genes it apparently regulates, the *tbpBA* locus. Wild-type gonococcal strain FA19 expresses this RNA regardless of iron status. Wild-type expression of this regulatory RNA species appears to enhance transcription or transcript levels of *tbpB* and *tbpA*; however, in contrast, wild-type expression of the regulatory RNA also results in decreased translatability of the *tbpBA* mRNA. Although the precise mechanisms by which these phenomena are achieved as a consequence of expression of this regulatory RNA are currently unclear, our results indicate that wild-type expression of this novel RNA species is important for coordinated, optimized expression of the gonococcal transferrin binding proteins. Further study of this system and the conditions that influence their expression is warranted, as the transferrin binding proteins are potential vaccine candidates for the prevention of gonococcal infections.

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Chapter 6: Mechanisms and significance of bacterial resistance to human cationic antimicrobial peptides

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Abstract

Cationic antimicrobial peptides (CAMPs) are essential compounds of the innate immunity system possessed by humans. CAMPs protect the host by exerting bactericidal activity, molecular signaling, modulating the immune response, and facilitating the communication between innate and acquired immunity. Over the millennia, bacteria have developed mechanisms to circumvent the antimicrobial activity of CAMPs, thereby promoting their survival during infection. In this chapter, we focus on the mechanisms used by various bacterial pathogens to resist the antibiotic-like action of CAMPs and the consequences of such resistance.

1 Introduction

Regardless of the host, signs of infection caused by a bacterial pathogen are typically noticed after damage to the host has occurred and symptoms are manifested. These symptoms of infection can arise from toxins produced by the pathogen or host inflammatory processes triggered when the host recognizes pathogenic bacteria or their associated virulence factors. Early during infection, mediators of innate immunity are brought to the front line of defense to combat the invader and protect the host. The efficacy of this response can determine the duration, spread and severity of disease. Cationic antimicrobial peptides (CAMPs), also appropriately called “host defense peptides” (1), are important in this response as they can directly or indirectly exert antibacterial activity. Any successful pathogen must find ways to evade the direct action of CAMPs or risk having their numbers severely reduced or even eliminated.

Although thousands of CAMPs exist in nature, humans confront bacteria with three main classes: the α- and β-defensins, the sole human cathelicidin termed LL-37 and peptides derived from protease digestion of proteins that perform important roles in other host response processes (e.g. cathepsin G). Unless otherwise stated, we focus this review on the mechanisms employed by bacterial pathogens to escape the action of gene-encoded CAMPs. It is important to note that CAMPs are, in essence, antibiotics. It is therefore not surprising that many of the general mechanisms developed by bacteria to resist classical antibiotics are in concept also used by bacteria to resist CAMPs. These mechanisms are summarized in Figure 1.

Studies on mechanisms of bacterial resistance to CAMPs have been facilitated by the ability to construct and use isogenic strains that differ by a single, defined mutation, or the presence of a gene that impacts levels of bacterial susceptibility to CAMPs. To assess the significance of such differences, it is first important to have reproducible antibacterial assays, which are discussed below. It is difficult to use purified human CAMPs or synthetic versions, due to availability of materials and cost constraints, to select genetic variants, but these problems can be circumvented by the use of recombinantly produced CAMPs or commercially available compounds that mimic the bactericidal action of CAMPs; the cationic antimicrobial peptide polymyxin B has been a reliable CAMP substitute used by many investigators.

Understanding how bacteria can develop CAMP resistance requires the use of *in vitro* antimicrobial assays that are reproducible and relatively easy to use. Typically, these assays involve an assessment of direct colony forming unit reduction when bacteria are incubated in liquid media with purified CAMPs. Other standardized assays for

determining minimal inhibitory concentrations (MIC) or minimal bactericidal concentrations (MBC) of CAMPs are also routinely employed (2). Typically, actively growing bacteria are diluted and incubated with CAMPs in broth or phosphate buffer-based solutions. These liquid media can be altered in pH and ionic strength to assess the impact of changes in these conditions on the killing efficacy of CAMPs. The radial diffusion agar overlay/underlay assay developed in the Lehrer laboratory is also particularly useful and can provide quantitative results as well as allowing one to test how changes in ionic strength, presence of divalent cations and pH impact CAMP activity. While these assays can collectively provide important information, laboratory growth media have little resemblance to the natural environments in which CAMPs must function *in vivo*. For instance, it is rare that the presence of other host compounds are taken into account, but these can either have positive or negative actions on the susceptibility to CAMPs. The presence of lysozyme or phospholipase A2 can enhance bacterial killing in CAMP assays. In our own studies with *Neisseria gonorrhoeae*, which typically (and often) infects the human genital tract, it was found that physiologically relevant levels of polyamines (e.g., spermine and spermidine) can decrease bacterial susceptibility to LL-37 (3). Additionally, Dorschner and colleagues deduced that physiologically relevant levels of carbonate (CO_3^{2-}) in laboratory media greatly increase the killing potential of numerous and varied CAMPs, but not of the anionic skin-derived antimicrobial peptide (AMP) termed dermcidin (4). They further found that *Staphylococcus aureus* responds to CO_3^{2-} through a dynamic transcriptional response, thinning its peptidoglycan via repression of the alternative sigma factor, *sigB*, which may explain the increased susceptibility of this pathogen to the tested peptides. Interestingly,

others have shown that LL-37 adopts an α-helical (active) conformation in the presence of carbonate (5), which may have played some part in the enhanced killing activity seen in the Dorschner study. Thus, CAMP behavior in the lab setting may not always reflect CAMP-bacteria interactions *in vivo*.

Environmental conditions (e.g., limitation of iron, anaerobiosis, local pH, ionic strength and presence of divalent cations) can also significantly impact AMP activity against bacteria. The recent report (6) that anaerobic conditions can potentiate the antibacterial action of human β-defensin 1 (hBD-1) against commensal gut bacteria nicely illustrates this point. In this instance, reduction of the intramolecular disulfide bonds by the thioredoxin system significantly enhanced HBD-1 activity against anaerobic commensal bacteria and this was proposed by the authors as a means used by the host to prevent their overgrowth. Interestingly, Nuding *et al.* found that similar anaerobic conditions can actually weaken the antibacterial action of the related peptide human β-defensin 3 (HBD-3) (7). Such diversity of CAMP function may be a way for the body to fine-tune its gut microbiota population. Alternatively, conditions of hypoxia may induce, in the absence of a surrogate electron acceptor, a state of bacteriostasis for some bacteria. This has been observed in *N. gonorrhoeae*, resulting in gonococcal resistance to antimicrobial proteins and CAMPs (8). Bacteria also face iron-starvation conditions imposed by host iron-binding proteins and have a variety of response mechanisms to acquire iron (9). As an example, this environmental stress can influence the level of susceptibility of *Streptococcus pyogenes* to LL-37 (10).

Ex vivo and *in vivo* infection models have also been employed to gain insights regarding the significance of mechanisms of bacterial resistance to CAMPs. Perhaps the

most widely used *ex vivo* model is that of polymorphonuclear leukocytes (PMNs). These professional phagocytic cells are used in monolayers or in suspension to evaluate changes in the intraleukocytic microbicidal activity that may be due to AMP resistance mechanisms. CAMPs (e.g., neutrophil defensins termed HNP 1-4 and LL-37) are, along with antimicrobial proteins such as the bactericidal/permeability increasing (BPI) protein (11), lactoferrin, cathepsin G, CAP37 and lysozyme, important mediators of non-oxidative killing by PMNs (12, 13). These antimicrobial compounds are stored within the cytoplasmic specific and azurophilic granules and are delivered into the phagocytic vacuole after granule fusion and degranulation. Bacteria that inhibit phagosome-lysosome fusion can resist CAMPs and antimicrobial proteins. For instance, *Salmonella* presents a well-studied example where a protein (SipC) belonging to the *Salmonella* Pathogenicity Island-2 (SPI-2) is essential to prevent the fusion of *Salmonella*-containing vacuoles with host cell lysosomes. A recent study suggests that *Salmonella* recruits host proteins to the vacuole to inhibit the fusion. However, once CAMPs are delivered into the developing phagolysosome, they rapidly coat the bacterial surface and can achieve milligram per mL concentrations, making it remarkable that any bacteria can survive inside the phagolysosome at all (14). With PMN models, it is possible to test inferences made regarding the significance of bacterial mutations that alter the susceptibility to isolated, PMN-derived CAMPs; specific examples are described below.

To test the idea that CAMP-resistance mechanisms can enhance bacterial survival during infection, numerous studies have employed whole animal models. Mouse models of infection have been particularly useful in this respect, allowing investigators to readily test inferences they have drawn from *in vitro* and *ex vivo* tests. In this regard, mouse

strains bearing knockout mutations in genes similar to human genes [e.g., CRAMP encoding the murine version of LL-37 (15)] or knocked-in human CAMP genes [e.g., the HBD-5 used by N. Salzman and colleagues in their studies dealing with *Salmonella typhimurium* (16, 17)] have been used to test both the significance of CAMPs in host defense, and if bacterial resistance mechanisms are important in promoting microbial survival during infection. Briefly, CRAMP knock-out mice were more susceptible to invasive group A streptococcal infection than their CRAMP$^{+/+}$ counterparts, while expression of HBD-5 provided mice with increased resistance to a lethal *S. typhimurium* infection.

To date, only a single report has appeared in the literature describing a human bacterial infection model for the purpose of studying the significance of a CAMP-resistance mechanism. In this instance, Bauer *et al.* (18, 19) utilized the forearm skin puncture model (20) to study host responses to infection by *Haemophilus ducreyi*, the causative agent of the sexually transmitted infection chancroid. *H. ducreyi* is intrinsically resistant to CAMPs and uses two transport systems, the Mtr efflux and Sap importer systems, for this purpose; these systems are described in greater detail below. Loss of these systems significantly decreased the survival of *H. ducreyi* and lesion pathology in this model (21, 22), indicating that CAMP resistance is important for its ability to cause disease.

2 Mechanisms of bacterial resistance to CAMPs

How do we define CAMP-resistance? This is not an easy question to answer, as "breakpoints" typically used to differentiate antibiotic-sensitive from antibiotic-resistant strains are seldom considered for CAMP studies undertaken by research laboratories. This matter is complicated by a number of issues: CAMPs can achieve very different concentrations depending on their location; local environmental conditions can be antagonistic or agonistic; inducible resistance can be displayed in the presence of sub-lethal levels of CAMPs, yet lost under normal conditions; CAMPs can exert multiple mechanisms of killing which, for a given peptide, might differ depending on the target bacteria. The precise mechanism by which CAMPs kill bacteria is a matter of some controversy and no unifying mechanism has been readily accepted by the CAMP research community. Certainly, CAMPs must first bind to the microbial surface and traverse the cell envelope. The events occurring post-binding that result in bacterial death is where controversy exists and it has not always been easy to separate direct killing from post-mortem events. For instance, changes in membrane integrity and potential, inhibition of cell wall biosynthesis and interaction of CAMPs with nucleic acids have been invoked as bactericidal events for certain CAMPs acting on a given bacterial target. Our purpose below is not to review how CAMPs kill bacteria, but rather describe how bacteria use constitutive and inducible mechanisms to circumvent their action.

In order for CAMPs to efficiently kill bacteria, they must reach their target in extracellular fluids or within intracellular compartments avoiding the action of peptidases/proteases, navigate past hydrophilic surface structures such as capsules and O-antigen chains of lipopolysaccharide (LPS), interact with negatively charged surface structures, insert into the cell envelope, reach the cytoplasmic membrane and, in some

instances, enter the cytosol. All of these steps provide opportunities for bacterial interference, which can decrease the susceptibility of the target microbe to CAMPs. Briefly, pathogens have evolved several strategies to circumvent the attack by CAMPs: 1) modulate CAMP gene expression, 2) degrade CAMPs by extracellular or intracellular peptidases/proteases, 3) trap CAMPs, 4) reduce binding of CAMPs to the cell surface, 5) export CAMPs by efflux pumps, and 6) alter intracellular targets. CAMP resistance mechanisms are typically expressed constitutively, but many are also under control of regulators of gene expression that respond to environmental cues. Below, we review examples of these strategies from several medically relevant pathogens. Table 1 summarizes specific examples from various pathogens, while Figure 1 summarizes the different strategies described in this section. We review this subject by beginning with examples of downregulation of CAMP production and then follow CAMPs as they bind and enter target bacteria, providing descriptions of the various systems employed by different bacteria to avoid the killing action of these important peptides.

2.1 Bacterial modulation of CAMP gene expression

Bacteria have developed novel and diverse strategies to modulate the availability of CAMPs in extracellular fluids and within phagolysosomes of phagocytes; the latter subject has been extensively reviewed elsewhere and we will concentrate on studies dealing with bacterial modulation of CAMP production. It is important to note that modulation of CAMP production can have profound downstream effects on the overall host immune system, which can facilitate bacterial growth and dissemination during infection. Bacterial products can directly or indirectly modulate CAMP expression and activation of immune responses. For instance, *E. coli* lipopolysaccharide (LPS) can

increase mRNA production of human β-defensin 2 (HBD-2) via CD14-activation of neutrophils (23). Tada *et al.* showed that proteases from *Porphyromonas gingivalis* can cleave the macrophage CD14 outer membrane receptor. CD14 recognizes pathogen associated molecular patterns (PAMPs), and cleavage of this protein rendered macrophages unresponsive to the presence of this pathogen and prevented CAMP production (24). Additionally, uropathogenic *E. coli* (UPEC), collected from patients with urinary tract infections (UTI), express the so-called "curli" fimbriae that modulate the immune system of the host and provide resistance to LL-37. Curli, an amyloid-like fiber expressed in biofilms, promotes cell adherence, increases induction of IL-8 (a human proinflammatory cytokine), binds to LL-37 inhibiting its killing activity and increases bacterial virulence in a mouse model (25). Futhermore, Islam *et al.* showed that *Shigella flexneri* and *S. dysenteriae* downregulate and prevent expression of CAMPs such as LL-37 and HBD-1 by the host. Even though the molecular mechanism was not completely elucidated, the authors suggested a role for plasmid DNA from the bacteria (26). Finally, *N. gonorrhoeae* can impair expression of LL-37 in cervical epithelial cell line ME180 (27). This effect was observed with live bacteria, but not with dead gonococci nor with live commensal *Neisseria* species considered avirulent in a normal host. They concluded that a specific interaction took place between *N. gonorrhoeae* and the ME180 epithelial cell that suppressed expression of LL-37. The gonococcal structures responsible for this suppression of LL-37 production remain to be discovered.

2.2 Degradation of CAMPs

Bacteria can degrade CAMPs by proteolytic cleavage before they reach or pass the bacterial surface. CAMP degradation can occur extracellularly, by secreted and

membrane-associated proteases, or intracellularly; the latter is facilitated by importers that deliver CAMPs to the bacterial cytosol, where they are degraded by peptidases and cytosolic proteases.

Studies with *Salmonella enterica* and *Staphylococcus aureus* have contributed significantly to our understanding of the role of bacterial peptidases/proteases in CAMP-resistance. *S. enterica* expresses an outer-membrane protease, PgtE, which cleaves LL-37 and other linear CAMPs, and results in increased resistance to LL-37 *in vitro* and *in vivo* (28). PgtE is similar to the OmpT protein produced by *E. coli*, which cleaves protamine. *E. coli ompT* mutants are more susceptible to human protamine (29). *S. aureus* produces many proteases and evidence has been presented that the action of aureolysin, a metalloprotease, and V8, a serine endopeptidase, can enhance its resistance to CAMPs. V8 cleaves and inactivates LL-37 (30) as well as complement proteins C3a and C4a, which have antimicrobial action (31). Aureolysin cleaves complement protein C3 at a non-physiological site, rendering its cleavage products inactive (32). Aureolysin-cleaved C3 protein is further degraded by host mechanisms, thus blocking the complement cascade, inactivating the antimicrobial activity of C3a, and preventing the targeting of *S. aureus* by the host immune response (32). Schmidtchen *et al.* have published a series of elegant papers that collectively emphasize the role of proteases produced by medically important pathogens (and other relevant microorganisms), highlighting their importance for resistance to CAMPs and antimicrobial proteins (33, 34). Briefly, these studies showed that elastase and alkaline protease from *Pseudomonas aeruginosa*, gelatinase from *Enterococcus faecalis*, a secreted cysteine proteinase (SpeB) from *Streptococcus pyogenes* and a 50kDa proteinase from *Proteus mirabilis*, possibly

ZapA can degrade LL-37 (33). As a secondary effect, these enzymes also degrade proteoglycans from the host's extracellular matrix, releasing negatively-charged dermatan and/or heparan sulfate, which significantly inhibits the antimicrobial activity of the α-defensin human neutrophil peptide 1 (HNP-1) (33), and of bactenecin-5 and -7 (35). An additional mechanism developed by *S. pyogenes* involves the bacterial membrane-associated protein GRAB, which binds α-2-Macroglobulin (α2M), a host-derived protease inhibitor. α2M, in turn, binds the secreted bacterial protease SpeB, which maintains its proteolytic activity against CAMPs, preventing the action of CAMPs at their target site on the bacterial surface. Thus, *Salmonella* binding of host α2M appears to serve two purposes: (i) facilitate cleavage of CAMPs before they reach their target, and (ii) promote immunological mimicry by presenting self-antigens at the bacterial surface.

Bacteria can also degrade CAMPs intracellularly through the combined action of an importer, which delivers CAMPs to the cytosol, and intracellular peptidases normally used to cleave bacterial peptides and increase the available pool of amino acids. For example, non-typeable *H. influenzae* (NTHi), a commensal Gram-negative bacterium that can cause conjunctivitis, sinusitis, acute and chronic otitis media, and bronchitis (36), expresses the Sap (sensitivity to antimicrobial peptides) ABC transporter. This transporter, first identified and characterized in *Salmonella* (37, 38), can increase bacterial resistance to CAMPs by 8-fold and is required for virulence of NTHi in a chinchilla model of otitis media (39). SapA binds chinchilla BD-1 as well as human CAMPs (e.g., LL-37, HBD-2 and -3, and HNP-1) (40). The binding of CAMPs to SapA upregulates expression of the *sap* operon (*sapABCDFZ*) (39), promoting transfer of the CAMPs into the cytosol where they are degraded (41). Further research by this group

proposed a mechanism where CAMPs are taken up by the periplasmic binding protein SapA then transferred to the cytoplasm through the SapBCDF transporter and SapZ accessory protein (42). Interestingly, this mechanism might increase the intracellular levels of nutrients, since the amino acids from the degraded CAMPs could be recycled. SapA of *H. ducreyi* also increases bacterial resistance to LL-37, but not to α- and β-defensins. However, the most important effect of SapA in *H. ducreyi* was observed in the human forearm model of chancroid in that SapA production was found to increase virulence, probably by promoting resistance to the higher concentrations of LL-37 that are secreted at infection sites in the dermis (22).

2.3 Hindering CAMP localization to the bacterial surface

When bacteria find themselves in environments rich in CAMPs, they have strategies other than proteolysis to neutralize or repulse CAMPs, thereby reducing their susceptibility to these antimicrobials. Non-proteolytic mechanisms of CAMP resistance include 1) the presence of CAMP-binding agents, 2) expression of hydrophilic bacterial biopolymers to retard the passage of amphipathic CAMPs in the electronegative bacterial surface, and 3) architectural constraints imposed by biofilms.

Binding or repulsion of CAMPs by extracellular compounds reduces their capacity to interact with negatively-charged target sites on the bacterial surface. Several CAMP-binding compounds have been described. For example, staphylokinase (Sak) and the streptococcal inhibitor of complement (SIC) bind to and neutralize CAMPs such as HNP 1-3 and LL-37 in the extracellular milieu, effectively decreasing the local CAMP activity as much as 80% (43, 44). The production of extracellular polymers shields bacteria with an extra layer of protection against CAMPs. Polysaccharide intercellular

adhesin (PIA) and poly-γ-glutamic acid (PGA) polymers, produced by staphylococci, inhibit HBD-3 and LL-37 activity (45, 46). It is thought that the charges present on these polymers are a triple threat as they are able to repulse similarly charged antimicrobials, neutralize and sequester oppositely charged antimicrobials, and behave as a mechanical barrier to their entry. Alginic acid produced by *P. aeruginosa* inhibits CAMPs in a similar fashion (47).

The production of capsule, or glycocalyx (literally, "sugar coat"), is a common defense mechanism also utilized by other human bacterial pathogens, including *N. meningitidis*, *Klebsiella pneumoniae*, *Legionella pneumophila*, *Streptococcus pneumoniae*, *S. aureus*, and *Bacillus anthracis*. It is notable that many of these pathogens are the cause of mucosal and respiratory tract infections that may progress to the bloodstream, an environment where encapsulated organisms are at a distinct advantage over other bacteria (48). However, not all prokaryotic glycocalyces are produced by the pathogen itself, and can sometimes be stolen from the host (see pathogenic *Neisseria*, below). In the environment, several species of both *Eubacteria* and *Archaea* produce S-layers, which is a glycoprotein shroud that completely surrounds the prokaryotic cell. Interestingly, some eubacterial S-layers can be glycosylated with up to 150 carbohydrate moieties per protein unit (49). S-layers may be another protective mechanism against CAMPs in the highly competitive soil and water microbiome.

As with proteolysis, biopolymer sequestration of CAMPs can still function even when physically separated from the cell. Certain Gram-negative bacteria can release membrane vesicles rich in CAMP-binding sites called "blebs", and the act of blebbing could provide an extracellular sink for CAMPs. Some bacteria may also release

negatively-charged capsular polysaccharides (CPS) that titrate CAMPs by electrostatic interactions. Llobet *et al.* have described this mechanism of resistance in several clinically relevant pathogens, such as *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *S. pneumoniae* (50). They show that CPS from different bacteria at concentrations as low as 1 μg/mL (*E. coli*, *K. pneumoniae*, *P. aeruginosa*) can increase the MIC of HNP-1 between 5 and 30 fold, regardless of the CPS source. However, this mechanism can be rendered inadequate by the presence of polycations that preferentially bind to CPS, freeing CAMPs to react with the bacterial cell wall and kill the targeted cell (50). Additionally, Campos *et al.* showed that a *K. pneumoniae* mutant lacking CPS is more sensitive to CAMPs such as HNP-1, HBD-1, protamine sulfate and polymyxin B, compared to the wild-type strain expressing CPS (51). They also showed that the CPS mutant binds more polymyxin B than the wild type strain, suggesting that CPS protects the bacteria, either by mechanically shielding the bacteria or by titrating the CAMP. Moranta *et al.* injected wild-type or CPS mutant *K. pneumoniae* into mice and showed decreased levels of β-defensins produced in response to the wild-type strain, as compared to higher levels produced for the CPS mutant isogenic strain (52). This suggested that CPS not only prevented the action of CAMPs at the surface of the bacteria, but also prevented signaling to the host immune system that would normally increase levels of β-defensins. In *N. meningitidis*, Jones *et al.* observed that expression of LOS and capsule are directly linked to increased resistance to LL-37 compared to the LOS-deficient and capsule-deficient mutants (53). Furthermore, they show that incubation of the wild-type bacteria with sublethal concentrations of LL-37 induced the expression of the capsule-

associated genes *siaC* and *siaD*, which results in upregulation of capsule biosynthesis (53).

CAMPs are typically amphipathic molecules whose hydrophobic domain allows membrane insertion, an event required for killing the target microbe. Accordingly, the presence of bulky hydrophilic structures on the bacterial surface may hinder the migration of CAMPs from the extracellular milieu to the negatively charged bacterial surface structures that form the gateway to hydrophobic lipid bilayers. This may partly explain why the presence of the hydrophilic O-antigen in the LPS of Gram-negative enterics endows them with greater CAMP resistance than rough mutants lacking this hydrophilic glycopolymer. Indeed, early work with *S. typhimurium* (54) showed that when the O-antigen is lost and the inner core sugar chain is progressively truncated, bacterial susceptibility to granule extracts from human neutrophils increases proportionally. These granules are rich in defensins and cationic antimicrobial proteins (55). Although these "deep rough" mutants have increased exposure of their negatively charged lipid A phosphate groups, which are important for CAMP-binding, their surface is generally more hydrophobic. This characteristic is conducive for CAMP/membrane interactions and enhances the likelihood of membrane insertion.

The above mechanisms have been defined with planktonic bacteria. However, it is now recognized that many bacterial species form a specialized, highly organized community termed a biofilm. Due to their ultrastructural organization, bacteria within biofilms can exhibit increased resistance to antimicrobials, including CAMPs, compared to their planktonic counterparts (56). Biofilms were correlated with persistent bacterial infections, such as chronic lung infection in cystic fibrosis (57). Antimicrobial resistance

displayed by bacteria within biofilms appears to be due to mechanisms different from those typically observed in free-floating cells. Since biofilms are multicellular communities of bacteria encased in a hydrated matrix of polysaccharide, proteins and/or nucleic acids, the capacity of antimicrobials to interact with all members of the community is reduced. Biofilms can also trap and inactivate CAMPs in the complex matrix imposed by its structure. Leid *et al.* demonstrated that biofilms from *S. aureus* can be penetrated by leukocytes that are active and secrete antimicrobial compounds (including CAMPs). However, though these leukocytes were able to phagocytose planktonic *S. aureus*, they could not engulf sessile cells (58). The authors suggested that the structure of the biofilm is more of a porous hydrogel than a fixed impenetrable structure. Three main hypotheses have been advanced to explain the increased antimicrobial resistance displayed by bacteria in biofilms (59). One hypothesis invokes a less permeable and less diffusible environment created by the biofilm with negatively charged compounds (i.e. nucleic acid, polysaccharide) in the matrix that could retard CAMP diffusion. The second hypothesis emphasizes the ultrastructural architecture of the biofilm, where microenvironments might present unfavorable conditions of pH, salt, and anaerobiosis that render CAMPs inactive or inefficient. The third hypothesis speculates that bacteria go through a cell-differentiation process and reach a spore-like metabolic state while in the biofilm, which allows some of them to be resilient to higher concentrations of antibiotics. These hypotheses are not mutually exclusive and other defense mechanisms described throughout this chapter could contribute to biofilm-mediated CAMP resistance. Another possible mechanism of resistance that needs to be

considered is that biofilm formation may trigger an alternate gene expression profile that modulates resistance phenotypes.

Biofilm formation is a dynamic process and can be influenced by environmental conditions, including the presence of certain CAMPs, other host compounds, and bacterial gene products. In the first instance, LL-37 and lactoferrin can prevent biofilm formation by *P. aeruginosa* by promoting bacterial motility (60, 61). Due to its structural organization, the availability of molecular oxygen may differ at sites within the biofilm complex, and this could influence the antimicrobial action of CAMPs. Accordingly, Schroeder *et al.* (6) found that hBD-1 was highly efficient against various human flora and select pathogens in its reduced form but lacked efficient antimicrobial activity in its oxidized form. This suggests that an oxidative environment (perhaps present at different degrees within biofilms) could preclude some CAMPs from their killing activity. Finally, a number of bacterial products directly enhance survival of CAMP attack in sessile cells. For example, inducible resistance can be controlled by two-component regulatory (TCR) systems (62, 63) and stand alone regulators (164-165, 154); the hair-like surface appendage termed Curli promotes formation of biofilm structures in UPEC strains of *E. coli* (25); and periplasmic glucans may bind to CAMPs on their way to the cytoplasmic membrane and sequester them (64).

2.4 Envelope modifications that decrease CAMP binding and permeability

Nearly fifty years ago, Spitznagel and co-workers found that the antimicrobial, arginine-rich, cationic peptides present in neutrophil granules (now known as defensins) rapidly coat the surface of ingested bacteria (65-67). This electrostatic interaction between CAMPs and bacterial surface structures, and how bacteria modify these

structures to inhibit said interaction, is perhaps the most studied mechanism of CAMP resistance [the reader is also directed to several excellent reviews especially those by Andreas Peschel (published in 2002) and Kim Brogden (published in 2005)]. In general, eukaryotic membranes are zwitterionic and have low affinity for CAMPs, which may provide them with some immunity to the lytic activity of these peptides. Prokaryotic cell surfaces, on the other hand, are typically negatively charged, and so have a higher affinity for CAMPs. In order to prevent the deadly consequences of this attraction, many bacteria have evolved ways to decrease the net negative charge of their exteriors and modify the permeability of their membrane(s). Importantly, these mechanisms are not always specific to CAMPs, and may provide protection against a broad spectrum of host and pharmacological cationic antimicrobials, including myeloperoxidase, phospholipase A2, lysozyme, vancomycin, moenomycin, and daptomycin (68).

Though both groups are negatively charged on their exteriors, Gram-negative bacteria are generally more resistant to CAMPs than Gram-positive bacteria, due to the presence of an outer membrane that can retard the passage of CAMPs to the inner membrane and cytoplasm. This is perhaps facilitated by LPS molecules that are held tightly together by (i) Van der Waals interactions that exist between acyl chains, and (ii) salt bridges formed by divalent cations between neighboring carbohydrate chains and between lipid A phosphates. Early studies with deep-rough LPS mutants of *S. typhimurium* (54), and the *pmrA* mutants tested by Vaara and co-workers (69, 70) and Shafer *et al.* (71) as well as Farley *et al.* (72, 73), support the concept that the availability of exposed, unsubstituted lipid A phosphate groups are critical to the ionic (and hydrophobic) interactions between CAMPs and the bacterial surface. More recent studies

also support this model. For instance, a knockout insertion of a putative LPS synthesis gene *galU* in *Campylobacter jejuni*, a leading food-borne pathogen, decreased the length of LPS and reduced bacterial resistance to polymyxin B (74). Similarly, *Bordetella bronchiseptica*, an upper respiratory tract pathogen and close relative of *B. pertussis* (the etiologic agent of whooping cough), appears to require the addition of a negatively charged trisaccharide to LPS by the *wlbA* and *wlbL* genes for full resistance to several phylogenetically diverse CAMPs. It is thought that the uronic acid sugar moieties present in this trisaccharide shield the membrane from antimicrobial attack, perhaps by sequestering the peptides or providing a bulky barrier to entry (75). Phosphorylcholine is produced by *H. influenzae* (76) and can increase the membrane fraction of zwitterionic phospholipids in the bacterial inner membrane. This would decrease the net negative charge normally present at the exoplasmic leaflet of the cytoplasmic membrane and slow the rate of CAMP self-promoted uptake (see below). Importantly, the investigators also observed modification of *H. influenzae* lipooligosaccharide (LOS) with phosphorylcholine. Such a modification is hypothesized to mimic host membranes (which contain phosphatidylcholine) and further reduce LL-37 binding. The viscosity of the periplasm may also contribute, since this space is densely packed with hydrophilic proteins that may non-specifically hinder CAMPs on their way to the inner membrane and cytoplasm, similar to the nonspecific binding of drugs by plasma proteins in the human body (77).

Gram-negative bacteria can also decrease the net negative charge of their exterior by decorating LPS/LOS lipid A with positively charged small molecules. In *N. meningitidis*, *N. gonorrhoeae*, *Helicobacter pylori*, *E. coli*, and *S. enterica* serovar

Typhimurium, the addition of phosphoethanolamine (PEA) not only removes the negative charge once provided by free lipid A phosphate, but also adds a positive charge, thereby decreasing the net negative charge of the outer membrane and perhaps membrane permeability as well (78-80). Alternatively, 4-amino-4-deoxy-L-arabinose (L-Ara4N) may be added to the same phosphates in some Gram-negative bacteria and provides resistance to CAMPs in a similar manner to phosphoethanolamine (81). Bacteria may also use the LpxE lipid A phosphatase to simply remove phosphate from lipid A and reduce negative charge, a phenomenon seen in the plant symbiont *Rhizobium leguminosarum*, as well as the human pathogen *H. pylori*. LpxE orthologues are present in *Francisella tularensis*, *Brucella melitensis*, and *Legionella pneumophila* (81, 82). These modifications are not only important for bacterial survival, but also impact the immune response to LOS/LPS or "endotoxin", one of the most potent inducers of septic shock (68). The regulation of these modifications has been very thoroughly studied in *S. enterica*, and is controlled by combined efforts of the PhoP/PhoQ and PmrA/PmrB TCR systems (79, 83); these regulatory systems are discussed in more detail below.

In Gram-positive organisms, polyalditol, polyglycerol or polyribitol phosphate polymers [teichoic acids (TA)] create a "continuum of negative charge" (84) with deprotonated phosphate residues present along each chain. The cationic antimicrobial lysosomal protein cathepsin G appears to use TA as a binding site on *S. aureus* (85). Seminal work by Andreas Peschel and co-workers first characterized the CAMP repulsive effect caused by the D-alanylation of TA in *S. aureus*, an ability that endows this pathogen (and others) with decreased susceptibility to diverse CAMPs from different sources (86). The enzymatic pathway encoded by the *dltABCD* operon processes the

esterification of D-alanine to TA alditol residues and transforms TA into partly zwitterionic polymers, reducing the net negative charge at Gram-positive surfaces. The expression of the *dlt* operon in *S. aureus* is under the control of the Aps/GraRSX regulatory system (87, 88). This appears to be a widespread defense mechanism present in other Firmicutes such as *Bacillus, Enterococcus*, and *Streptococcus*. It is also important to mention that *S. aureus* mutants lacking a functional *dlt* operon are more susceptible to the glycopeptide antibiotic vancomycin (89), and in the future it may be possible to counter vancomycin resistant *S. aureus* (VRSA) with drugs that block D-alanylation of TA.

Changes in membrane rigidity can also influence levels of CAMP resistance. PhoP/PhoQ control of *pagP*, which encodes a palmitoyltransferase that hepta-acylates *S. enterica* lipid A in response to stresses typically found in a phagosomal environment, can influence membrane rigidity and the capacity of CAMPs to productively insert into bacterial membranes. These stresses include low pH, varying Ca^{2+} and Mg^{2+} ionic strength, and high concentrations of CAMPs (90, 91). The MsbB protein in *Vibrio cholerae* plays an analogous role by adding an acyl chain to the same position as PagP. This modification was found to greatly enhance resistance to polymyxin B, LL-37 (and its mouse homologue CRAMP), and magainin 2 (92). Interestingly, *msbB* deletion mutants were unable to induce a TLR4 response in human embryonic kidney cells, which suggests that efficient recognition and binding of bacterial endotoxin is largely due to lipid A structure. This finding also supports the notion that CAMP resistance mechanisms may protect not only directly, but indirectly through modulating the host immune response. The actions of both PagP and MsbB ultimately increase the stability and

hydrophobicity of the outer membrane and decrease its permeability, thus enhancing the fortitude of an already formidable barrier to CAMP entry (68). In *S. aureus*, pigment production through the *crtOPQMN* operon performs a similar function in CAMP resistance by increasing membrane rigidity (93). The cold shock system of *S. aureus* may also be important in resistance as mutants lacking CspA (94) and CspB (95) have reduced pigment levels and altered susceptibilities to certain antimicrobials, including cathepsin G-derived CAMPs.

Conversely, decreased membrane rigidity may also provide CAMP resistance. *S. aureus* tPMP (thrombin-induced platelet microbicidal protein) resistant strains were consistently found to have greater membrane fluidity than their tPMP susceptible counterparts, caused by a preponderance of longer chain, unsaturated fatty acids (96). It has also long been known that *S. aureus* membranes contain unsaturated sexa-, hepta- and octa-isoprenoid menaquinones (97). Apart from their function as redox molecules in the electron transport chain, they also increase membrane fluidity. Thus, it has been hypothesized that large fluctuations in membrane fluidity to either extreme may distance membrane order from the "sweet spot" required for optimum CAMP bactericidal activity (48, 93).

One of the most nonspecific and ubiquitous mechanisms of CAMP defense in bacteria is mediated by the MprF (multiple peptide resistance factor) protein (98). Originally described by Peschel and colleagues in 2001 (98), it soon became clear that MprF provides significantly enhanced bacterial resistance to neutrophils and several evolutionarily distinct CAMPs. MprF is present in a wide variety of Gram-positive, Gram-negative, and acid-fast bacteria, and is also present in the *Archaea*. This CAMP

resistance mechanism is remarkable in that it only requires substrates that are abundant in the bacterial cell—charged tRNAs and membrane phospholipids—and is somewhat indiscriminant when recognizing tRNA donor and phospholipid acceptor molecules. This is thought to be why the MprF mechanism is so widespread (99). MprF is an integral cytoplasmic membrane protein and may add the positively charged L-lysine, L-alanine and perhaps, in *Mycobacterium tuberculosis*, L-ornithine (100, 101) amino acids to phosphatidylglycerol and cardiolipin (diphosphatidylglycerol). *S. aureus* MprF consists of (i) a transesterase domain which adds the amino acid residue to phosphatidylglycerol (PG) on the cytoplasmic leaflet of the membrane and (ii) a "flippase" domain that flips the nascent lysyl-PG to the exoplasmic leaflet of the membrane where it can serve to repulse CAMPs by reducing the net negative charge of the membrane's outer surface. It is noteworthy that MprF is the first flippase to be discovered in prokaryotes (100). In *M. tuberculosis*, the *lysX* gene is actually a fusion of *mprF* and *lysU*, a lysyl-tRNA synthase. Here, lysyl-tRNA can be made at the cytoplasmic membrane level by the LysU domain, then shuttled into the MprF reactions described above (102). *Clostridium perfringens* produces two MprF proteins, 1 and 2, which produce alanyl-PG and lysyl-PG, respectively (99). It is probable that, like other CAMP resistance mechanisms, the activities of MprF are under the control of two- or three-component regulatory systems; indeed, MprF appears to be under the control of the VirR protein of the VirRS TCR in *Listeria monocytogenes* (103), and the Aps/GraRSX TCR in *S. aureus* (87, 88, 104).

Intriguingly, it seems that CAMPs themselves are not the only stimuli that can induce CAMP resistance mechanisms in bacteria. A brief but elegant study by Dorrer

and Teuber in the 1970's (105) demonstrated that phosphate starvation induced polymyxin B resistance in *Pseudomonas fluorescens* by increasing the membrane fraction of ornithylated lipids, which decreases the net negative charge of the bacterial envelope. Notably, it was later discovered that survival inside of macrophages induced the expression of phosphate importers 9.4 fold in *Salmonella typhimurium* (106). This might indicate that (i) low phosphate levels preclude the use of phosphate on membrane lipids and require that other groups (e.g., ornithine) provide the hydrophilic portion of the membrane lipid to maintain a stable bilayer structure, and (ii) the host environment may unwittingly hinder its own efforts to kill with CAMPs by inducing the production of these cationic lipid species.

Though the mechanisms described above allow the microorganism to change the envelope structure without dire consequences for growth, there are other CAMP resistance strategies that come at great fitness cost to the bacterium. In *S. aureus*, small colony variants (SCVs) are typically deficient in electron transport and have a diminished membrane potential ($\Delta\psi$) (48). They also arise much more readily (10,000 fold) in the host than in laboratory culture (107). This suggests that slowing cell growth may represent a "niche-specific" defense mechanism, triggered by growth in a host, that allows bacteria to depolarize their membrane and decrease the rate of "self-promoted uptake" by host CAMPs (68) [see (108) for a description of this phenomenon]. Interestingly, the *S. aureus cspB* mutant studied by Duval *et al.*, described above, exhibited many of the characteristics of SCVs (95). In Gram-negative bacteria, reduced growth rates may also help stave off death by CAMP by reducing the occurrence of nascent septa that are a critical component during bacterial binary fission. Sochacki and

colleagues used real-time fluorescence microvideography to show that rhodamine-labeled LL-37 consistently binds *E. coli* cells at their nascent septa first, then proceeds outward in a continuous “circumferential band” towards the distal poles of each developing daughter cell (109), though LL-37 was still able to bind to non-septated cells.

2.5 Export of CAMPs

Even if CAMPs successfully traverse the formidable barriers described above, bacteria can still circumvent their action by the use of drug efflux pumps that capture and export structurally diverse antimicrobials after they breach the cell envelope. Drug efflux pumps are grouped into superfamilies based on their component stoichiometry, number of transmembrane regions in the transporter, energy source, and type of substrates recognized. Five superfamilies of efflux pumps are known: the resistance-nodulation-division (RND) superfamily, the major facilitator (MFS) superfamily, the multidrug and toxin extrusion (MATE) superfamily, the ATP-binding cassette (ABC) transporter superfamily, and the small multidrug resistance (SMR) superfamily [see (110) for an excellent review of bacterial efflux pumps]. The MtrCDE efflux pump of *N. gonorrhoeae* is a member of the RND superfamily and was the first efflux pump shown to export CAMPs to the extracellular milieu (111). This pump has been studied in detail and will be described later (see below); the analogous pump in *N. meningitidis* also can export CAMPs (112). Other Gram-negative pathogens have been found to use efflux pumps to resist CAMPs. *Yersinia enterocolitica* can protect itself from CAMP activity by expressing the RosAB efflux pump, which is induced upon growth at 37°C. The RosA pump activity is powered by the potassium antiporter RosB and is thought to provide resistance through (i) efflux of CAMPs and (ii) acidification of the cytoplasm

(113). *K. pneumoniae* expresses the AcrAB-TolC efflux pump that mediates resistance to human antimicrobial peptides, as an AcrB mutant was more sensitive to HBD-1 and HBD-2 (114). The homologous efflux pump in *E. coli*, AcrAB-TolC, is arguably the most structurally characterized efflux pump to date (115-117), and will likely become an invaluable tool for the design and testing of an emerging class of antibiotics, the efflux pump inhibitors (EPIs) (118).

Efflux pumps also function in Gram-positive bacteria for CAMP resistance. For instance, the EpiFEG efflux pump of *S. epidermidis* exports and increases staphylococcal resistance to various CAMPs. EpiFEG is an ABC transporter that is known to export bacterial derived CAMPs, such as gallidermin, nisin, and epidermin (119). The MefE/Mel efflux pump possessed by certain strains of *S. pneumoniae* is a mechanism used by this pathogen to develop resistance to macrolides. Expression of this pump was found (194) to be inducible by 14- and 15-membered macrolides as well as LL-37/CRAMP and that such induction enhanced pneumococcal resistance to macrolides and LL-37. Maximal constitutive and inducible cathelicidin resistance expressed by pneumococci required a functional MefE/Mel pump system, although it is yet to be determined if these CAMPs are actual pump substrates. It also appears that some efflux pumps might actually enhance CAMP resistance independently of their efflux capacity. In *S. aureus*, QacA (a plasmid-encoded multidrug MFS efflux pump) mediates resistance to tPMP, but does not affect levels of resistance to HNP-1 or protegrin-1 (120). Curiously, later studies found that QacA-mediated resistance to tPMP was not due to efflux activity, and that membrane fluidity seemed to diminish slightly in strains bearing

the *qacA* gene (121), but the exact mechanism of how QacA expression protects against tPMP remains to be determined.

Of note is a very intriguing pattern that has emerged among the Gram-positive bacteria that teams ABC-transporter efflux pumps and TCR systems into very close functional associations called "resistance modules". Each component of the module is dependent on the other for resistance against antimicrobial peptides. Extensive phylogenetic analysis suggests a co-evolution of efflux pumps and TCR systems in the phylum *Firmicutes* (over 250 resistance modules are estimated). In this partnership, sensor domain-deficient inner membrane histidine kinases (IMHKs) still relay signals through their cognate response regulators, but recognition of the environmental stimulus is carried out by a neighboring permease/transporter protein in the membrane (122). Well-characterized examples of such TCR/efflux pump couplings include the BceRS TCR and BceAB pump in *Bacillus subtilis* and *Streptococcus mutans* (123, 124), and the BraRS TCR and BraED pump in *S. aureus* (125). The VraED pump also appears to play a role in *S. aureus* resistance, but is required only for efflux and not for sensing.

2.6 Modification of internal targets

Since the antimicrobial action of most CAMPs is independent of stereochemistry, it has generally been thought that they do not recognize targets with a chiral center (126, 127). Furthermore, their killing activity has been linked to processes such as loss of membrane integrity and depolarization. Due to these broad spectrum killing mechanisms, CAMPs have been likened to "dirty bombs" in contrast to the "smart bomb"-like action of many antibiotic drugs (128). This analogy may not be completely correct as it is now clear that CAMPs may also kill bacteria by interfering with internal

cellular functions like DNA/RNA/protein synthesis, protein folding, peptidoglycan polymerization, and septum formation (129, 130). Just as the bacterial envelope can accumulate changes to hinder CAMP attack, antimicrobial stress selects for mutants containing modified and thus less CAMP-accessible cytoplasmic targets. One example of this is a mutation in the *gyrB* gene in *E. coli*. *gyrB* encodes DNA gyrase, a type II topoisomerase that maintains a level of DNA supercoiling necessary for replication, transcription, and recombination. GyrB is a target of the well-known class of antibiotics called quinolones, and also the bacteriocin microcin B17, which is produced by *E. coli*. del Castillo and colleagues (131) found that mutation of residue W751 to hydrophilic amino acids like lysine or arginine imparts a great deal of resistance to microcin B17. The authors hypothesize that this residue may be located on the entry gate through which the intact DNA can be transported (T-segment), and that microcin B17 normally binds and inhibits GyrB activity, leading to cell death (131).

2.7 Inducible mechanisms of CAMP-resistance

Inducible mechanisms of CAMP resistance allow bacteria to promptly respond to stressful changes in their environments. TCR systems sense potentially harmful changes, orchestrate a response to the imposed stress and adapt gene expression to the new context. TCR systems consist of a sensor histidine kinase on the inner membrane and a cytoplasmic regulatory protein. Typically, the sensor kinase detects a signal in the environment, becomes autophosphorylated and in turn phosphorylates the cognate intracellular regulator, activating it. The activated regulator binds to DNA and alters the expression of different genes. Numerous and functionally distinct TCR systems are found in bacteria. The PhoP/PhoQ TCR system was initially studied in *S. typhimurium* (132-

134), and homologs were subsequently found and studied in *P. aeruginosa* (135), various *Enterobacteriaceae*, and in *Neisseria* where it is named MisR/MisS (136).

The PhoP/PhoQ and the PmrA/PmrB are well-studied examples of TCRs that have important roles in CAMP resistance. Under favorable conditions, PhoQ is bound by divalent cations such as Mg^{2+} and Ca^{2+} in the environment, and is not active. At low concentrations of divalent cations, PhoQ phosphorylates PhoP, which in turn regulates many genes involved in AMP resistance, such as *pagP*, *pgtE*, *slyA* and *pmrD* (137). PmrD is required for activation of the PmrA/PmrB TCR system (28, 104, 135, 138, 139). When CAMPs are present in the media, it is thought that they displace divalent cations bound to an acidic patch of PhoQ and induce activation of the TCR system (91). In *S. enterica*, PhoP/PhoQ is activated by CAMPs and upregulates genes related to CAMP resistance such as *pagP*, *pagL*, and *lpxO* (140, 141). Using a mutant strain of *S. enterica* showing increased resistance to polymyxin B, azurocidin and CAP57 (71) due to a *pmrA* mutation, Roland *et al.* identified the TCR system PmrA/PmrB (142). This TCR regulates expression of genes involved in CAMP resistance such as *pmrHFIJKLM* (or *pbg* operon), *cld* and *cptA*, which are responsible for LPS modifications (143). In *P. aeruginosa*, PmrA/PmrB is induced by low concentrations of Mg^{2+} and by LL-37, which promotes expression of genes, such as *pbgP*, *pbgE* and *ugd,* involved in LPS modification and resistance to polymyxin B (144-146). It was shown that mutating *pmrAB* in *P. aeruginosa* rendered the bacteria hypersusceptible to killing by LL-37 or by other CAMPs such as polymyxin B (147).

Li *et al.* uncovered a novel regulatory system in *S. epidermidis* that they named *aps* for antimicrobial peptide sensor (87, 88), also observed in *S. aureus* and named *gra*

for glycopeptide resistance associated genes (148, 149). The system consists of a TCR system with a sensor kinase (*apsS*) and a regulator (*apsR*), and a third protein with unknown yet essential function (*apsX*) (87, 88). Their research showed that deletion of any or all of these components led to downregulation of the *dlt* and *mprF* genes, which modify cell surface structures and enhance resistance to CAMPs (87). Furthermore, Lai *et al.* have described *agr* and *sarA* in *S. aureus* and *S. epidermidis* as major regulators that are induced in the presence of the anionic AMP dermcidin. These gene regulators increase expression and proteolytic activity of the SepA metalloprotease in presence of dermcidin (150).

3 CAMP resistance in clinically relevant pathogens

In the above sections, we reviewed major mechanisms that bacteria have evolved to resist CAMPs. In order to highlight how such CAMP resistance systems can influence the efficacy of host resistance to infection and bacterial pathogenesis, we discuss them in the context of three major clinically relevant pathogens of public health concern. In this respect, we focus on the obligate human Gram-negative pathogens *N. gonorrhoeae* and *N. meningitidis*, and on the Gram-positive bacteria *S. aureus*, particularly the methicillin resistant strains (MRSA).

3.1 *Neisseria gonorrhoeae* and *N. meningitidis*

N. gonorrhoeae and *N. meningitidis* are Gram-negative diplococci and strict human pathogens (154). Gonococci (GC) cause the sexually transmitted infection gonorrhea. In contrast, meningococci (MC) are present as commensals in 8-25% of the human population, but can cause bacterial meningitis and fulminant septicemia (151). Gonorrhea is the second most reported infection in the United States, though many cases

are asymptomatic, and can enhance HIV transmission (152, 153). Furthermore, although there are vaccines available for many MC serogroups that cause disease, a protective vaccine for serogroup B MC is still under development; no vaccine has been developed that blocks GC infection. Both of these pathogens are also becoming increasingly resistant to antibiotics (154). Worryingly, a recent report described a strain of gonorrhea that is resistant to the last remaining first-line antibiotic used in empirical treatment, ceftriaxone (155). Thus, the pathogenic *Neisseria* represent a significant threat to global health.

As strictly human pathogens, GC and MC have evolved remarkable and redundant mechanisms to defend themselves against host CAMPs. These include capsule production by MC (53, 156), host-molecule "cloaking" using the highly anionic polymers heparin/heparan sulfate and short, cationic polyamines (3, 53, 157), MC sequestration of LL-37 in the bacterial cytosol (158), downregulation of host LL-37 production (27), export of CAMPs by the MtrCDE efflux pump (111), decoration of lipid A with PEA (78, 112, 159), and hexa-acylation of lipid A (112). Some of these mechanisms have been shown to be under the control of the MisR/MisS TCR system, named for its regulation of meningococcal LOS inner core structure, which itself is necessary for resistance to CAMPs (136, 160, 161). Other mechanisms may be induced by different inputs, e.g. upregulation of *mtrCDE* expression in GC upon exposure to hydrophobic pump substrates typically present at infection sites (162).

Is there evidence that any of these resistance mechanisms influence bacterial survival during infection? Briefly, yes: in support of this idea, elegant experiments performed in the laboratory of A. Jerse have shown that loss of the MtrC-MtrD-MtrE

efflux pump due to its genetic inactivation decreased the ability of GC to survive in an experimental model of lower genital tract infection in female mice (163). Further work by her group (164, 165) showed that over-expression of MtrC-MtrD-MtrE increases fitness of GC during infection by nearly three orders of magnitude. In contrast, loss of the ability to activate transcription of *mtrCDE* decreased fitness *in vivo* by 500-fold. Finally, *lptA* mutants of GC that are unable to decorate their lipid A with PEA are more susceptible to CAMPs and less fit *in vivo* than the parental wild type strain (Jerse, personal communication, 2011).

3.2 *Staphylococcus aureus*

S. aureus is a Gram-positive bacterium that has evolved to survive in a commensal capacity on the human host. It can be found on the skin and in the nares in 20% of the population, but when staphylococci breach host defenses they can cause many different illnesses, including skin infections, abscesses, and life-threatening diseases such as endocarditis, pneumonia, meningitis, toxic shock syndrome, and sepsis. Importantly, *S. aureus* is one of the most frequent causes of hospital- and community-acquired infections. The incidence of multiple antibiotic-resistant strains of *S. aureus* continues to increase, restricting the options for treatment. *S. aureus* has become one of the most difficult bacterial infections to treat as multi-drug resistant strains have emerged; a typical example is methicillin resistant *S. aureus*, or MRSA. MRSA colonizes 2 % of the population, many of whom are immuno-compromised due to age (e.g., the elderly and young children) or medical condition (e.g. pregnant women, HIV-positive and cancer patients) (166). MRSA can cause life-threatening infections such as pneumonia, septicemia and infections following surgery. MRSA resist most β-lactam antibiotics

(penicillins and cephalosporins) including penicillin, methicillin, and amoxicillin. Furthermore, it is quite common to see resistance develop when MRSA infection is treated with macrolides and/or fluoroquinolones. Importantly, as of 2007, MRSA infections caused more deaths (>17,000) in the United States than HIV/AIDS (167).

S. aureus has found strategies to impair all of the events associated with CAMP killing activity, and sometimes in more than one way. For instance, *S. aureus* secretes proteolytic enzymes, V8 and aureolysin, which are able to degrade and inactivate CAMPs such as LL-37 (30). It was suggested that loss of these enzymes by molecular modification could render *S. aureus* more susceptible to CAMPs *in vitro*, *ex vivo* and *in vivo* (30). Staphylokinase (Sak) is a secreted protein that sequesters LL-37 and increases virulence *in vivo* (168). Burlak *et al.* demonstrated that *S. aureus* express Sak *in vivo*, since injection of *S. aureus* in mouse elicited the production of specific antibodies against Sak (169). *S. aureus* produces positively charged polysaccharide intercellular adhesin (PIA) and negatively charged poly-γ-glutamic acid (PGA) at its surface, which increases the net positive charge of the cell surface and, as it was described for *S. epidermidis*, could impair binding of positively charged CAMPs by electrostatic repulsion; however, other mechanisms might be involved as well, since PIA also protected *S. aureus* from the negatively charged AMP dermcidin (45, 46). Moreover, PIA, which is produced by the intercellular adhesion *ica* locus has been involved in biofilm formation in *S. aureus* (170). *S. aureus* can also express D-Ala and L-Lys at its surface, modifying the net charge, through the *dlt* operon and *mprF* gene, respectively (86, 98, 171-173). These mechanisms are also efficient against other CAMPs, such as those derived from lactoferrin and phospholipase A2 (174).

S. aureus regulates these genes and many others involved in CAMP resistance with Aps/GraRSX, an inducible system that is activated in presence of CAMPs (88, 175). Li *et al.* showed that a mutant with a deletion of *aspS* was less virulent in an intraperitoneal mouse infection model than the wild-type strain (88). Other inducible mechanisms described in *S. aureus* and implicated in CAMP resistance involve Agr and SarA (176). Modifications of the membrane involve carotenoid production by the *crtOPQMN* operon, which can suppress nonoxidative host defenses mediated by CAMPs (93). *S. aureus* is also able to prevent CAMP activity by expressing efflux pumps such as the plasmid-encoded QacA and the ABC transporter EpiFEG (though QacA-mediated resistance is independent of efflux activity). Finally, *S. aureus* is able to form biofilms, which are ultrastructures that promote bacterial resistance to AMPs and other killing agents. Internal targets are probable in *S. aureus*, since CAMPs are able to kill *S. aureus* without significant depolarization or disruption of the membrane (177).

4 Conclusions and Perspectives

Bacteria have constantly evolved novel mechanisms to overcome attacks by CAMPs. It seems that for every way CAMPs kill, bacteria have developed a resistance mechanism(s) in response. As mentioned above, the mechanisms of CAMP resistance are for all purposes similar to those developed by bacteria to resist classical antibiotics. At first glance, mechanisms of bacterial resistance to CAMPs would seem to favor the microbe and not the host. However, this view may be overly simplistic; most of the bacteria we interact with on a daily basis are not normally pathogenic and many are associated with good health. If such commensally carried, helpful bacteria were to be reduced or eliminated in the presence of CAMPs, how would this impact our health?

Perhaps CAMP resistance mechanisms evolved not as a way for pathogens to avoid elimination, but rather as a way for the helpful commensals to survive.

CAMPs have been promoted as a new class of therapeutic antimicrobials for treating multi-antibiotic-resistant pathogens, some of which cause infections that are becoming untreatable. Studies exploring various characteristics of CAMPs (charge, amphipaticity, hydrophobicity, etc.) will help gain insight in the design of ever more efficient synthetic CAMPs. Alternatively, further research focusing on specific bacterial metabolic states could prevent formation of structures such as biofilms that are extremely hard to destroy and that increase the risk of chronic infections and antibiotic resistance development. In this respect, the laboratory of Robert Hancock is working on CAMPs that prevent formation of biofilms, which could one day be bound to internal medical devices. The continued advancement of these peptides as therapeutics will require additional studies to further analyze their potential short and long-term toxic effects, their specificity, their pharmacokinetics, the appearance of resistance patterns, and immunomodulatory/immunostimulatory secondary effects.

Continued studies on mechanisms of CAMP resistance are also warranted. As therapeutic antimicrobial peptides pass through clinical trials, we can use the knowledge gained from such experiments to predict how bacteria will respond to their presence during treatment (which will likely be at higher levels than what occurs naturally), and if resistance (especially broad spectrum) will develop. We must, however, be cognizant of the possibility that resistance to administered CAMPs may negatively impact innate host defenses mediated by the natural CAMPs that function at different sites in the human

body. How this might influence decisions to move forward with the therapeutic application of CAMPs is a matter of future consideration.

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Table 1: Examples of CAMP resistance mechanisms expressed by bacteria.

Product name/gene	**Organism**	**Reference**
CAMP binding/inactivation		
Staphylokinase	*S. aureus*	(43, 168)
SIC	*S. pyogenes*	(178)
M1 surface protein	*S. pyogenes*	(179)
CAMP proteolytic cleavage		
V8 protease	*S. aureus*	(30)
Aureolysin	*S. aureus*	(30)
ZapA	*P. mirabilis*	(34, 180)
LasA	*P. aeruginosa*	(34, 35)
Elastase LasB	*P. aeruginosa*	(34)
Gelatinase GelE	*E. faecalis*	(34)
Surface protease PgtE	*S. enterica*	(28)
Metalloproteinase SepA	*S. epidermidis*	(150)
Metalloprotease DegP	*E. coli*	(181)
Cysteine protease SpeB	*S. pyogenes*	(34, 182, 183)
Production of capsular polysaccharides		
PIA, PGA	*S. epidermidis*	(45, 46)
Capsule-synthesis gene cluster		
cps cluster	*N. meningitidis*	(184)
	K. pneumoniae	(51)
Mycolic acid synthesis		
kasB	*M. marinum*	(185, 186)
D-Ala modification of teichoic acids		
dltABCD	*S. pyogenes*	(187)
	L. monocytogenes	(188)
	S. aureus	(86)

Product name/gene	Organism	Reference
Modification of membrane phospholipids with amino acids		
mprF, lysS	*S. aureus*	(98, 132, 189)
lysX	*M. tuberculosis*	(102)
mprF1 and *mprF2*	*C. perfringens*	(99)
Lipid A modifications		
Aminoarabinose addition: *pmrAB, pmrE, pmrFHIJKL, pmrC*	*S. enterica* ser. Typhimurium	(79, 83, 143)
Acetylation: O-acetyltransferase	*P. mirabilis*	(190)
PEA addition: LptA	*N. gonorrhoeae*	(78)
	N. meningitidis	(112)
Phosphorylcholine substitution: GlpQ	*H. influenzae*	(76, 191)
Decreased membrane fluidity		
htrB	*H. influenzae*	(192)
Production of carotenoids		
crtOPQMN	*S. aureus*	(93)
Plasmid encoded efflux pump		
QacA	*S. aureus*	(120)
CAMP expulsion		
MtrCDE	*N. gonorrhoeae*	(111)
	N. meningitidis	(193)
MtrCD-GlmU	*H. ducreyi*	(21)
AcrAB-TolC	*K. pneumoniae*	(114)
EpiFEG	*S. aureus*	(119)
MefE/Mel	*S. pneumoniae*	(194)
Controlled import leading to degradation		
Sap	*S. enterica*	(38)
Sap	*H. influenzae*	(41)
SapA	*H. ducreyi*	(22)

Product name/gene	Organism	Reference
Induction mechanisms		
phoP/phoQ	*S. enterica*	(37, 134)
phoP/phoQ	*P. aeruginosa*	(135)
pmrA/pmrB	*S. enterica*	(142)
pmrA/pmrB	*P. aeruoginosa*	(145)
misR/misS	*N. meningitidis*	(136, 160)
covR/covS	*S. pyogenes*	(10)
agr, sarA, aps/graRSX	*S. aureus*, *S. epidermidis*	(150, 175)
Biofilm formation		
icaB	*S. aureus*	(46)
Bacterial regulation of host AMP expression		
Plasmid DNA-mediated decrease in LL-37 and HBD-1 expression	*S. dysenteriae, S. flexneri*	(26)
Decreased LL-37 expression	*N. gonorrhoeae*	(27)
Diminished membrane potential		
Small colony variants	*S. aureus*	(48, 107)

Figure 1

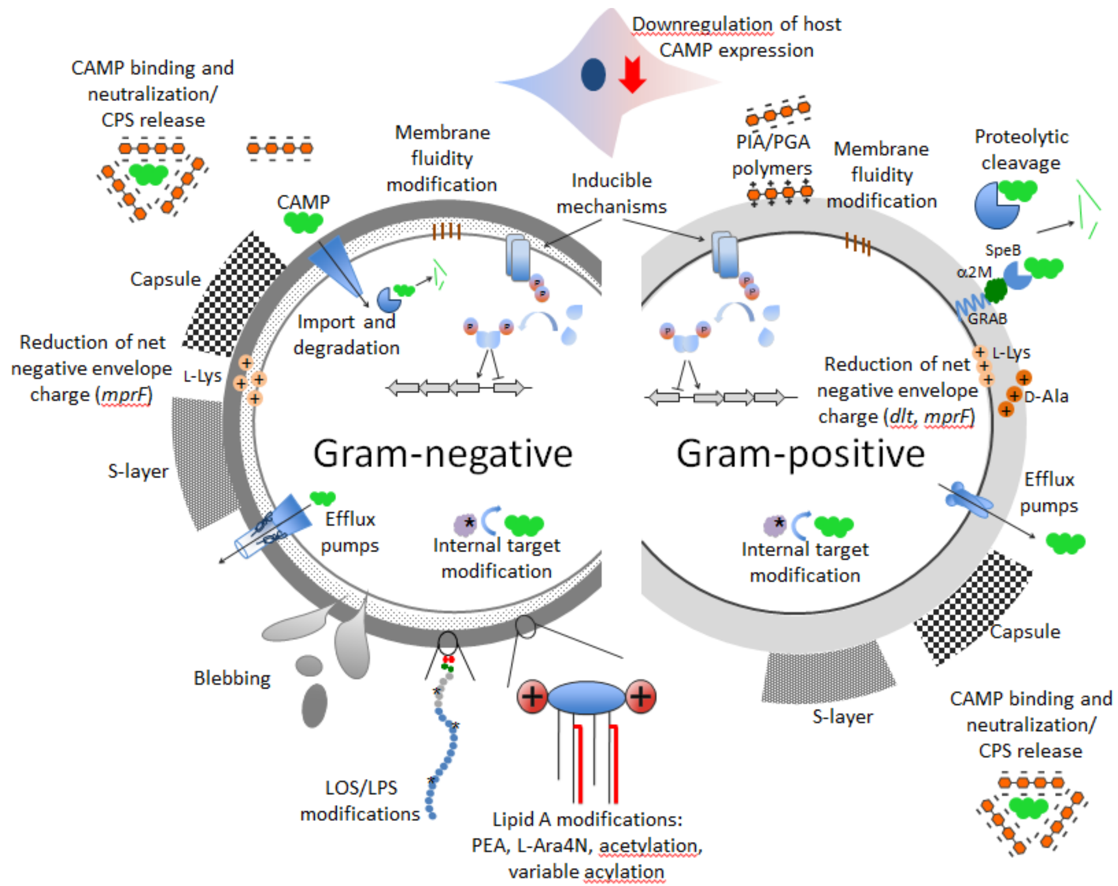

Figure 1: Schematic representation of the mechanisms of resistance to cationic antimicrobial peptides (CAMP) used by Gram-negative and Gram-positive bacteria. Key: CAMP: cationic antimicrobial peptide; CPS: capsule polysaccharide; D-Ala: D-alanine; L-Ara4N: 4-amino-4-deoxy-L-arabinose; L-Lys: L-lysine; LOS: lipooligosaccharide; LPS: lipopolysaccharide; PEA: phosphoethanolamine. These examples are of specific mechanisms of CAMP-resistance expressed by Gram-positive and –negative bacteria and should not to be considered exhaustive.

Chapter 7: Unpublished Results

As mentioned in Chapter 1, we became interested in studying the MisR/MisS two component regulatory system (TCS) in *Neisseria gonorrhoeae* because of its importance for cationic antimicrobial peptide (CAMP) resistance and pathogenesis in the closely related species, *N. meningitidis*. Gonococci are subjected to CAMP stress in the host, though a small percentage can actually survive the heavy infiltration of phagocytes characteristic of symptomatic gonorrhea. Thus, we hypothesized that MisR/MisS would likely regulate factors that respond to CAMP-related stress to help maintain a viable population during disease. Prior to this work, there were no reports describing the role of MisR/MisS in gonococci, though we suspected that it would be similar in both species of pathogenic *Neisseria*. Below are the results of several experiments exploring the regulatory targets of MisR.

a. Generation of mutants, growth phenotype, and CAMP susceptibility

We began by generating *misR*- or *misS*-deficient mutants of the wild type (WT) strain FA19 using PCR products amplified from a kind gift of meningococcal genomic DNA of mutants constructed previously (1, 2), and these strains were subsequently complemented as described previously (3). Each mutant carries a nonpolar kanamycin resistance cassette (4) that interrupts *misR* or *misS*. Preliminary studies on the growth of these mutants showed that the *misR::kan* mutation does not impact growth on GC agar plates or morphology by Gram stain, but does cause a markedly reduced growth rate and endpoint culture density in GC broth growth curves. Since growth of *phoP*$^-$ mutants of *Salmonella typhimurium* (5) and *misR*$^-$ mutants of *N. meningitidis* (6) was Mg^{2+}-dependent, we wondered if supplementation of our GC broth with $MgCl_2$ or

$MgSO_4$ would alleviate the *misR::kan* growth defect. Indeed, addition of 10 mM Mg^{2+} (regardless of which salt) resulted in WT-level endpoint culture densities. However, the *misR::kan* mutant still appeared to replicate more slowly than the isogenic WT parent, which became apparent when viable cell counts and cell density were compared for both strains (Appendix Figures 1A, 1B, and 1C). We later discovered that the long lag phase and decreased growth rate associated with the *misR::kan* mutation could be mostly alleviated by starting broth cultures using cells from plates no more than 12-14 hours after passage (i.e. minimizing the number of stationary/death phase gonococci in the broth culture inoculum). In contrast, the *misS::kan* mutation had no apparent impact on growth.

Next, we tested the susceptibility of *misR*- and *misS*-deficient mutants to polymyxin B (PMB), a model CAMP. As had been observed previously in *misR*$^-$ mutants of meningococci (7, 8), the FA19 *misR::kan* mutant was more susceptible to PMB than the FA19 WT parent in 2-fold agar dilution minimum inhibitory concentration (MIC) assays, and this phenotype was reversed by complementation (Appendix Table 3A). In contrast, the *misS::kan* mutation did not seem to impact susceptibility to PMB under our experimental conditions [the PMB MIC was equal to that of FA19 WT for both the FA19 *misS::kan* mutant and the FA19 *misS::kan*, *misS*$^+$ (pGCC4) complemented strain]. Preliminary results of minimum bactericidal concentration (MBC) assays also suggested that FA19 *misR::kan* gonococci are more susceptible to the human cathelicidin, LL-37 (MBC_{90} = 0.98 ug/mL) than the isogenic WT parent strain (MBC_{90} = 3.92 ug/mL), and that overexpression of *misR* in the complemented strain restores WT levels of LL-37 resistance (MBC_{90} = 7.84 ug/mL). However, the necessity for Mg^{2+} supplementation in

misR::kan mutant culture made it difficult to conduct further experiments with this highly salt-sensitive peptide (9)—thus, we decided to continue our work using PMB.

Our initial hypothesis was that MisR/MisS might contribute to gonococcal CAMP resistance by directly sensing antimicrobial peptides with MisS [in a manner similar to that of the *Salmonella* sensor kinase, PhoQ; see (10)], so we next tested if MisR/MisS was necessary for gonococci to adapt to sublethal levels (0.1x the MIC) of PMB using MBC assays. Indeed, we found that WT gonococci pre-treated with PMB were 4-fold more resistant to subsequent challenge with PMB than buffer-treated gonococci (Appendix Figure 2). Loss of *misR* not only prevented this adaptation effect, but also resulted in a 4-fold decrease in constitutive resistance to PMB (buffer pre-treatment alone); complementation of *misR* returned the phenotype to that of WT gonococci. On the other hand, loss of *misS* had no impact on the adaptation effect (data not shown).

b. MisR/MisS does not respond to low Mg^{2+} concentrations

As mentioned above, we observed that the *misR::kan* mutation caused a growth defect in broth that resulted in a much lower endpoint OD_{600} value. Supplementation with 10 mM $MgCl_2$ seemed to reverse this effect, so we considered the possibility that MisR/MisS might be responding to low Mg^{2+} concentrations in WT gonococci to allow growth at higher cell densities, during which divalent cations would become scarce and unshielded negative charges present on LOS would cause outer membrane instability. To test whether the MisR/MisS could respond to different concentrations of $MgCl_2$, we generated a *misR-lacZ* translational fusion at a second site in the chromosome (the *proAB* locus) using the pLES94 system (11). MisR has been shown to autoregulate its own promoter in *N. meningitidis* by binding to a 15-bp consensus sequence [reported in (12)]

~43 bp upstream of the *misRS* -35 promoter element (2). Sequence alignment of the *N. meningitidis* MC58 MisR~P binding site (2) and the *N. gonorrhoeae* FA19 (NCBI accession number NZ_ABZJ01000036) *misRS* upstream region shows that this binding site is also present upstream of the FA19 *misRS* operon (with 100% identity), and was included in the *misR-lacZ* translational fusion. We hypothesized that if gonococcal MisS was sensing levels of $MgCl_2$ in the growth medium like *Salmonella* PhoQ [see (13)], then expression of *misR-lacZ* would increase during $MgCl_2$ starvation due to phosphorylation of MisR and activation of the *misR-lacZ* promoter by MisR~P. However, decreased levels of $MgCl_2$ and even treatment with the divalent cation chelator, EDTA, had no effect on *misR-lacZ* expression (Appendix Figure 3). These results suggested that MisR/MisS was not sensing levels of Mg^{2+} in the medium, in agreement with similar data from meningococcal studies (2).

c. RNA-Seq—a global view of MisR regulation

From the work described above it seemed to us that gonococcal MisR/MisS, like meningococcal MisR/MisS, was probably not a functional homologue of *Salmonella* PhoP/PhoQ, since it is unresponsive to Mg^{2+} concentration and MisS was not necessary for adaptation to PMB pretreatment. In order to determine the regulatory targets of MisR/MisS that contribute to the CAMP-susceptible phenotype of *misR*-deficient mutants, we performed RNA-Seq to measure transcription of gonococcal genes on a global scale in the presence or absence of MisR. The methods used for this experiment, and a link to the raw data, have been reported [see Methods section of (14)].

MisR is a global regulator. Using a regulation impact cutoff of 2-fold and a Bonferroni statistical significance cutoff of <0.05, the RNA-Seq data revealed that MisR

is a global regulator that regulates 95 genes (40 activated, 55 repressed) spanning several functional categories (replication, metabolism, protein folding, hypothetical, nutrient transport, transcriptional regulation, phage-related, and IS elements). The RNA-Seq data also show that MisR regulates numerous virulence factors known to be important for gonococcal survival and/or pathogenesis [including *tbpB* and *tbpA* (transferrin binding; (15)), *macA* and *macB* (resistance to macrolides; (16)), *bfrA* and *bfrB* (iron storage and protection from oxidative stress; (17)), and many others]. Genes significantly impacted by MisR can be seen in Appendix Table 2A.

***trans*-regulation of *tbpBA* by the MisR response regulator.** Despite its necessity for *in vivo* survival (15), little is known about factors that impact gonococcal TbpBA expression (see Chapter 1 section VIa). As just mentioned, the results of the MisR RNA-Seq experiment (Appendix Table 2A) were our first indication that MisR could be a novel regulator of this vital iron acquisition system, and revealed that transcript levels for both *tbpB* and *tbpA* were increased in the absence of MisR (5.76- and 3.05-fold, respectively). We next tested if this regulation was direct by performing DNA-binding assays on the *tbpBA* upstream region using purified, His-tagged, phosphorylated MisR protein (unphosphorylated MisR failed to shift any *tbpBA* upstream region tested) and radioactively labeled DNA. Using a PCR product spanning the *tbpBA* -221 to +75 upstream region, we found that MisR~P bound specifically to the *tbpBA* promoter as determined by electrophoretic mobility shift assays (EMSA), and that this binding occurred most strongly at the *tbpBA* -10 element on the antisense strand as determined by DNaseI footprinting (Appendix Figure 4). These results are consistent with our

hypothesis that MisR represses *tbpBA*, and suggest that MisR competes with RNA polymerase holoenzyme for binding to the *tbpBA* promoter to repress transcription.

MisR does not impact decoration of lipid A with the positively charged small molecule, phosphoethanolamine (PEA). Unexpectedly absent from the MisR RNA-Seq regulon was *lptA*, whose product catalyzes the addition of PEA to the 4' (and rarely, the 1) position on the lipid A moiety of gonococcal lipooligosaccharide (3, 18). PEA-modified lipid A is less electronegative than unmodified lipid A, and is thus less attractive to the cationic charges that initiate CAMP binding to Gram-negative outer membranes. This modification greatly decreases gonococcal susceptibility to PMB; in contrast, gonococcal mutants lacking *lptA* [FA19 *lptA::spc*; see (3)] do not have PEA-modified lipid A and are quite sensitive to PMB killing. Since MisR also decreased susceptibility to PMB (Appendix Table 3A), we performed several additional experiments to more thoroughly explore whether or not MisR could impact PEA decoration lipid A through regulation of *lptA*.

We first determined the transcriptional start point (TSP) of *lptA* and mapped a potential promoter sequence for *lptA* by primer extension [Figure 2B in (19)]. Next, we confirmed that this promoter could drive expression of the *lptA* gene by generating a translational fusion using the pLES94 vector (11). This fusion links a 323 bp upstream region of *lptA* (including the mapped promoter, the ribosome binding site, and the 1st three codons) to a promoterless *lacZ* gene; thus, expression of the *lptA* gene was measured through beta-galactosidase assays. We found that this upstream region could indeed drive expression of *lptA* in our WT strain FA19, and that maximum expression of *lptA* occurred during early- to mid-log phase in broth culture (Appendix Figure 5).

Expression of *lptA-lacZ* decreased 2-3 fold in the presence of the *misR::kan* mutation (Appendix Figure 6), suggesting that MisR could play a role in regulation of the proximal *lptA* promoter.

While unlikely, due to the nonpolar nature of the kanamycin cassette (4), we wished to eliminate the confounding possibility that polar effects of the *misR::kan* mutation could have impacted *lptA-lacZ* expression, and attempted to transform the *misR*$^+$ complemented strain with the *lptA-lacZ* fusion [FA19 *misR::kan*, *misR*$^+$ (pGCC4), *lptA-lacZ*]. However, after multiple attempts at transformation (we frequently generated merodiploid transformants that were kanamycin resistant and had both *misR::kan* and a WT copy of *misR*, and our efforts were further hindered by the nontransformable phenotype of *misR*-deficient cells), we were unable to generate this strain. Therefore, we used qRT-PCR to test more directly for differences in *lptA* transcription.

Importantly, we suspected [and demonstrated; Figure 2A in (19)], that *lptA* might be part of an operon and that as a consequence, transcription of *lptA* could be driven both from the proximal promoter 61 bp upstream of the *lptA* start codon and from a distal promoter upstream of *serC* [see Figure 1 in (19)]. RNA-Seq visualization maps the beginning of *serC* transcript to be 15 bp upstream of the *serC* ATG start codon and ~ 19 bp downstream of a near consensus -10 element TAAAAT (Appendix Figure 7), but our attempts to confirm this promoter location by primer extension were inconclusive. To account for the presence of two transcripts that could encode *lptA*, we tested transcription of *lptA* in the presence or absence of MisR using two sets of primers (Appendix Table 1A): the product of the 1st set (lptA_qRT_F/R) corresponds to a site completely within the *lptA* open reading frame, and as a result measures total *lptA* transcription; the 2nd set

(lptA_qRT_F2/R2) corresponds to a site preceding the TSP of the *lptA* proximal promoter and extending to the 5'-most region of the *lptA* open reading frame. The second primer set would be unable to generate an amplicon representing transcript initiated at the *lptA* proximal promoter, and would thus only measure transcription of *lptA* from any upstream promoter(s).

Using strains FA19 WT, FA19 *misR::kan*, and FA19 *misR::kan*, $misR^+$ (pGCC4), transcription of *lptA* from the proximal and distal promoters was measured from early, mid, and late log broth cultures and calculated using the $2^{(-\Delta\Delta C_T)}$ method (20). In keeping with our beta-galactosidase assay results, loss of *misR* resulted in a 2-4 fold reduction both in total *lptA* transcription (lptA_qRT_F/R) and in transcription originating from a distal site upstream (lptA_qRT_F2/R2). However, a similar decrease in *lptA* transcription also occurred in the complemented strain for both primer sets, suggesting that the *misR::kan* mutation might be having some indirect effect impacting *lptA* transcript levels. Importantly, a positive control gene *dsbD*, which is strongly upregulated by MisR in the meningococcus (12, 21) and in our own RNA-Seq data (Appendix Table 2A) was also included and confirmed that our complemented strain was functional.

Our results indicate that while the *misR::kan* mutation does have an impact on expression of *lptA*, it is likely to be small (~2 fold) and is probably not indicative of a direct MisR activation of the *lptA* gene, as complementation of *misR* did not restore *lptA* expression to WT levels. In agreement with this, 1) the triplicate RNA-Seq global transcriptome profiling of the FA19 WT and FA19 *misR::kan* strains [see Appendix Table 2A and Methods in (14)] demonstrated that MisR has essentially no impact on *lptA* transcription (average fold change of -1.13 in FA19 *misR::kan*), and 2) DNaseI protection

assays (Appendix Figure 8) show that MisR~P protein occludes the *lptA* proximal promoter and 5' UTR (which would not be expected for a transcriptional activator).

Most importantly, MALDI-TOF mass spectrometry analysis of purified gonococcal lipid A demonstrated that lipid A was still decorated with PEA in the FA19 *misR::kan* mutant (Appendix Figure 9), but (as expected) the lipid A of the FA19 *lptA::spc* control strain was not. This experiment in particular—regardless of any expression differences in *lptA* caused by loss of MisR—shows that the impact of MisR on PEA decoration of lipid A is not likely to be biologically significant. Finally, overexpression of *lptA* from a second site does not return the PMB MIC of a *misR::kan* mutant to WT levels (Appendix Figure 10), which confirms that reduced *lptA* expression is not the cause of CAMP-hypersusceptibility in *misR*-deficient gonococci.

A bioinformatic tool for location of potential MisR binding sites. RNA-Seq is a tremendously powerful tool for finding the transcriptional regulatory targets of DNA-binding proteins. However, it is often necessary to elucidate the precise mechanism of regulation with DNA-binding studies. Such studies rely on knowledge of the regulator binding site at the gene of interest, which can initially be a guessing game. Using the PRODORIC Virtual Footprint website http://prodoric.tu-bs.de/ (22), which has recently been utilized in a similar bacterial transcriptome study (23), the 15-bp consensus MisR binding site [IUPAC code: KWWWTGTAARGNNWH with a mismatch tolerance of 1; (12)] was used to probe the completely closed genome of gonococcal strain FA 1090 (NCBI reference sequence NC_002946) for potential target sites of MisR regulation. Of the 95 MisR-regulated genes that we discovered by RNA-Seq analysis, 13 were found to

have sequences matching the MisR consensus binding site within 350 bp of the start codon for the gene (Appendix Table 2B).

While the number of PRODORIC-mapped MisR target sequences was much smaller than the number of MisR-regulated genes, it is important to note that some of the MisR regulated genes may be within operons, and thus MisR-binding sites may be further than 350 bp away from a target gene. Additionally, MisR significantly regulates 4 genes that encode transcriptional regulatory proteins, and thus is likely to regulate many genes indirectly. Furthermore, MisR may regulate non-coding transcripts that could have a post-transcriptional impact on targets in the RNA-Seq results. Despite its limitations, the PRODORIC Virtual Footprint tool is a helpful starting point for any future MisR DNA-binding studies, wherein sites may be experimentally confirmed by EMSA and DNaseI footprinting.

Rescue of *dsbD* expression does not return PMB susceptibility to WT levels. As mentioned above, MisR did not transcriptionally regulate *lptA* in the RNA-Seq experiment. Surprisingly, MisR also had no transcriptional impact on any of the known genes important for gonococcal efflux of CAMPs (fold-changes for the *mtrCDE* efflux pump genes, the *mtrF* efflux accessory gene, the *mtrR* and *mtrA* regulatory genes were all <1.5; data not shown). We considered the possibility that MisR might affect CAMP resistance through an indirect, post-transcriptional mechanism (e.g. effects on protein function). Therefore, we looked at the RNA-Seq for MisR-regulated genes whose products are important for protein folding, transport, or activity. One of the major regulatory targets of MisR is *dsbD*, which is strongly activated by MisR in meningococci (12, 21) and in our own gonococcal RNA-Seq data (Appendix Table 2A). Since the LptA

catalytic domain contains multiple disulfide bonds (24) and the Dsb (disulfide bond) system was recently shown to impact LptA function in meningococci (25), we hypothesized that MisR's regulatory impact on *dsbD* might indirectly affect LptA activity and thus PEA modification of lipid A and PMB susceptibility.

In the meningococcus, DsbD is an essential inner membrane thiol-disulfide interchange protein that provides reducing power to DsbC, a periplasmic isomerase that shuffles disulfide bonds to achieve the correct conformation for membrane and secreted proteins in the oxidizing environment of the periplasm; misfolded proteins are generally nonfunctional and susceptible to proteolytic degradation (21, 26, 27). In order to determine if overexpression of *dsbD* could bring back WT levels of PMB resistance in a *misR*-deficient background, we generated a plasmid construct using the pGCC4 system as described previously [see (3)] in which expression of a WT copy of *dsbD* could be controlled by IPTG induction. We then used transformation to generate strains FA19 *dsbD*$^{+}$ (pGCC4) and FA19 *misR::kan, dsbD*$^{+}$ (pGCC4), and tested them for PMB susceptibility with or without IPTG induction.

We found that two independent clones of FA19 *misR::kan, dsbD*$^{+}$ (pGCC4) retained the PMB susceptibility (3.13-6.25 ug/mL MIC) characteristic of *misR*-deficient gonococci [as compared to a WT background control (50 ug/mL MIC)] in both the absence and presence of 1 mM IPTG (Appendix Table 3B). We also confirmed that our construct could indeed overexpress *dsbD* transcript in the *misR::kan* background by qRT-PCR (Appendix Figure 11). Taken together, these results show that rescue of *dsbD* transcription could not restore PMB resistance to WT levels, and suggest that lack of *dsbD* expression in *misR*-deficient gonococci is probably not the root cause of the PMB-

susceptible phenotype. This finding is in agreement with the mass spectrometry data demonstrating that PEA modification of lipid A still occurs in *misR::kan* gonococci (see Appendix Figure 9).

MisR impacts protein quality control genes regulated by heat shock and RpoH. Thus far, all of our data had indicated that MisR does not impact gonococcal CAMP resistance through any effects on PEA decoration of lipid A. Furthermore, transcriptional regulation of the *mtr* efflux system components by MisR did not occur under our RNA-Seq conditions. We therefore looked to regulatory clues in the RNA-Seq data for a possible lead. Aside from autoregulation of the *misR* gene (nearly significant at 1.77-fold activation by MisR), MisR regulated 4 other loci with predicted regulatory function as determined by RNA-Seq (an XRE-family regulator encoded by NGO0867, *marR2* encoded by NGO1244, *mpeR* encoded by NGO0025, and *rpoH* encoded by NGO0288; see Appendix Table 2A). We decided to focus on *marR2* for a number of reasons; its ORF (NGO1244) was the most strongly regulated of all the above regulators (5.90-fold MisR repressed), MarR family proteins are named for their association with multiple antibiotic resistance (28), and our laboratory had previously generated a *marR2* (NGO1244) knockout [(29); there are two MarR-family regulators in gonococci, MarR1 (renamed FarR due to its impact on the FarAB efflux system) and MarR2, whose gene was interrupted by a nonpolar kanamycin cassette].

We first tested the FA19 *marR2::kan* mutant for PMB resistance, but found that it had a similar phenotype to WT gonococci (100 ug/mL MIC). Thus, like *dsbD*, *marR2* cannot be solely responsible for the CAMP-susceptible phenotype of *misR*-deficient gonococci. However, we noted that previous studies had shown *marR2* to be regulated by

heat shock (30) and that intriguingly, several MisR-regulated genes in the RNA-Seq data were also affected by heat shock and RpoH overexpression (30, 31) (see Appendix Table 2A). Since many of the stress-responsive genes in the gonococcus encode chaperones or proteases, it seemed reasonable to speculate that protein folding, function, and degradation would be dysregulated in *misR*-deficient gonococci. Furthermore, proper protein structure is important for normal cell envelope permeability (see Chapter 1 section Vb), and MisR's regulation of stress response genes provided an important clue in the search for an explanation of its role in CAMP susceptibility.

In order to confirm the regulation of gonococcal stress response genes by MisR we had seen in the RNA-Seq (and weigh the impact of the regulator MarR2) broth cultures of FA19 WT, FA19 *misR::kan*, and FA19 *marR2::kan* were grown at 37°C. Cells were harvested at mid-log, RNA purified, and qRT-PCR performed to measure transcription of stress response genes. Expression of these genes was clearly different between each strain (Appendix Figure 12). For our experiment, we chose to measure transcription of *marR2*, *clpB*, *htpX*, *lon*, *rpoH*, NGO1245, *dnaK*, *grpE*, *yjoH*, *secB*, and *grxC*. Most of these genes were regulated by both heat shock (30, 31), and MisR (in the RNA-Seq) except for *secB*, which was upregulated by overexpression of RpoH/σ^{32}, the stress response sigma factor (30). *misR* was measured as a control to show that no transcription of the *misR* gene occurs in the FA19 *misR::kan* mutant. Expression of *misR* and *marR2* was not detectable in their respective mutants due to interruption of the primer amplicons by the kanamycin cassette.

As expected, *marR2* transcription was increased in the absence of its repressor, MisR. *clpB*, *dnaK*, and *grpE* transcription was also upregulated in the absence of MisR

(Appendix Figure 12). These qRT-PCR results agreed with our RNA-Seq results, and suggest that MisR is an important repressor of these stress-related genes. In further confirmation of our RNA-Seq data, MisR strongly activated *htpX*. In *E. coli*, HtpX is a zinc-metalloprotease that cleaves misfolded proteins in the cell envelope (32) and is transcriptionally controlled by the CpxR/CpxA TCS. Finally, transcription of the hypothetical gene, NGO1245 (which is immediately downstream of and likely shares an operon with *marR2*) was increased in the absence of its repressor, MisR. NGO1245 encodes a putative ABC transporter integral membrane protein, but its function is as yet unclear.

Interestingly, NGO1245 expression was decreased in the absence of MarR2, which suggests that MarR2 may autoactivate the *marR2*-NGO1245 locus. Lastly, as we had seen in the absence of MisR, *grpE* transcription was increased in the absence of MarR2. In contrast, *lon*, *rpoH*, *yjoH*, *secB*, and *grxC* were not significantly affected by loss of MisR or MarR2 under these experimental conditions.

d. MisR is required for resistance to antimicrobials that enter cells by self-promoted uptake and to substrates of the MtrCDE efflux pump

The impact of MisR on the gonococcal stress response genes described above demonstrated that several protein quality control mechanisms are dysregulated in a *misR::kan* mutant. Since proteins must be folded correctly in order to be tightly embedded into membranes (33), we hypothesized that *misR*-deficient gonococci would be generally more susceptible to antimicrobial agents that enter the cell through self-promoted uptake because their cell wall would contain a higher concentration of misfolded proteins than WT gonococci. We tested our hypothesis by examining the

susceptibility of *misR*-deficient gonococci to various antimicrobials using MICs. We included PMB and a number of aminoglycosides, which are known to enter Gram-negative bacteria through self-promoted uptake (see Chapter 1, Section Vb).

We also wished to test our previous hypothesis that MisR might affect post-transcriptional mechanisms that impact CAMP resistance, and to do so generated *misR*-deficient mutants of the *mtrCDE*-overexpressing strains JF1 [which carries a deletion of gene encoding the *mtrCDE* repressor, *mtrR*; see (34, 35)] and KH15 [which carries a single base-pair deletion in the *mtrR*-*mtrCDE* intergenic region that greatly increases expression of *mtrCDE* and greatly decreases expression of *mtrR*; see (35, 36)]. These *mtrCDE*-overexpressor parent strains generate much more *mtrCDE* transcript than WT FA19 gonococci and are much more resistant to substrates of the MtrCDE efflux pump. This pump is large and complex, and would be expected to require at least some of the protein folding mechanisms regulated by MisR for optimum functionality. While there did not appear to be an impact of MisR on the *mtr* system genes in the WT background of the RNA-Seq experiment, we reasoned that overexpression of *mtrCDE* would burden the cellular protein folding and translocation machinery, and make any *misR*-deficient phenotypes more pronounced.

As expected, *misR*-deficient gonococci were more 4-8 fold more susceptible to PMB than their parent strain in all genetic backgrounds (Appendix Table 3C). Importantly, this same fold increase in susceptibility was also true in the context of the aminoglycosides gentamicin, streptomycin, and tobramycin, which suggests a common mechanism and supports our hypothesis that self-promoted uptake is likely to be enhanced in the absence of MisR [resistance to spectinomycin, a bacteriostatic

aminocyclitol similar to but distinct from aminoglycosides (see Chapter 1, section Vb) also appeared to be *misR*-dependent but to a lesser extent]. Ciprofloxacin, which enters via porins and not through the membranes (37, 38), had the same MIC value for all strains. Kanamycin was also tested as a control drug due to the presence of the *aphA3* cassette in *misR::kan* gonococci, and indeed *misR::kan* gonococci were many times more resistant to kanamycin than the parent strains. When substrates of the MtrCDE efflux pump were tested, the *misR*-deficiency often resulted in a 2-fold increase in susceptibility in both the WT and JF1 (Δ*mtrR*) backgrounds (Appendix Table 3D).

However, in the KH15 background, where *mtrC* transcription is dually increased due to the absence of MtrR and the enhanced affinity of RNA polymerase holoenzyme for the *mtrC* promoter, loss of *misR* resulted in a more diverse phenotype (Appendix Table 3D). The impact was smallest for azithromycin, ceftriaxone, rifampicin, and sodium deoxycholate (2-fold increase in susceptibility); a larger effect was seen for crystal violet, erythromycin, and penicillin G (4-fold increase in susceptibility), but the most impacted substrate was the nonionic detergent triton X-100 (32-fold increase in susceptibility). Curiously, MisR appeared to be more important for ceftriaxone resistance in a WT background than in an *mtrCDE*-overexpressor background (4-fold vs. 2-fold increase in susceptibility, respectively).

We hypothesized that MisR could be affecting MtrCDE efflux in two ways; 1) loss of MisR impacts the amount of efflux pump present, or 2) loss of MisR prevents MtrCDE from functioning properly. In order to test the 1st possibility, mid-log phase broth cultures of FA19 WT, JF1, KH15 (and their isogenic *misR::kan* mutants) were harvested and whole cell lysates were run on an SDS-PAGE gel for Coomassie staining

and Western blot against MtrE and MisR. As expected, MisR protein was absent from all strains bearing the *misR::kan* mutation (Appendix Figure 13). Importantly, the loss of MisR in the *mtrCDE*-overexpressor strains did not impact levels of MtrE protein, which suggests that the increased susceptibility of *misR*-deficient mutants to substrates of MtrCDE is probably not due to lower amounts of MtrCDE efflux pump.

e. Suppressor mutants of FA19 *misR::kan* with a near-WT resistance to PMB

Thus far, all of our results seemed to point to an increase in membrane permeability as the cause of increased antimicrobial susceptibility in *misR*-deficient gonococci. This change in permeability did not seem to come from differences in phosphoethanolamine modification, and was associated with a decreased potency of mutations that normally enhance MtrCDE efflux and antibiotic resistance. In order to locate the precise genetic factors regulated by MisR to control susceptibility to CAMPs, we selected for suppressor mutants of FA19 *misR::kan* that could survive on 1x-4x the normal MIC of PMB (the FA19 *misR::kan* PMB MIC is 12.5-25 ug/mL). We reasoned that these suppressor mutants, if stably PMB-resistant, might harbor genetic changes that could be detected by whole genome sequencing. At least some of these changes could be expected to influence the expression of MisR-regulated genes, thus reversing the phenotype imposed by the loss of MisR and allowing for increased PMB resistance.

In order to generate these PMB-resistant FA19 *misR::kan* suppressor mutants, lawns of FA19 *misR::kan* cells (originating from a single colony) were resuspended to a concentration of 2.4×10^9 cfu/mL in unsupplemented GC broth. A 1:10 dilution of this resuspension was made, and 100 uL (number of gonococcal cfu/plate was 2.4×10^8 for undiluted and 2.4×10^7 for the 1:10 dilution) of gonococci were spread plated onto 10 GC

agar plates containing PMB at 25 or 50 ug/mL (1-2x and 2-4x the MIC of FA19 *misR::kan*, respectively). As a control, FA19 WT cells were struck for isolation on these same plates to ensure that lack of FA19 *misR::kan* growth was not due to defective media, but was a result of killing by PMB. The plates were allowed to incubate for approximately 72 hours at 37°C in a 5.0% CO_2 incubator prior to examination.

All plates that had been spread with the undiluted resuspension grew 1000's of colonies or complete lawns, which suggested that an excess of cell envelope material was probably neutralizing the PMB and reducing the actual concentration available for killing sensitive gonococci. Of the 2.4×10^8 total viable gonococci plated from the 1:10 dilution onto 10 GC agar plates supplemented with PMB at 25 ug/mL, only 2 PMB-resistant colonies could be found. Thus, PMB-resistant suppressor mutants of FA19 *misR::kan* arise at a frequency of approximately 1×10^{-8}. A single, well-isolated colony was also picked from the undiluted plate group for further testing. No growth could be observed on the plates supplemented with PMB at 50 ug/mL.

Using the 3 suppressor mutants isolated above, a series of passages on GC agar containing PMB at 25 ug/mL was performed to ensure that no susceptible gonococci remained. Next, an efficiency of plating (EOP) assay was performed to confirm that the FA19 *misR::kan* parent strain was phenotypically more susceptible to PMB than the suppressor mutants. Furthermore, all 3 suppressor mutants grew on plain GC agar and GC agar supplemented with kanamycin (50 ug/mL) or PMB (25 ug/mL). In contrast, the FA19 *misR::kan* parent strain could not grow on PMB-containing agar (Appendix Figure 14A). Additionally, the efficiency of plating was similar between WT and all 3 suppressor mutants (Appendix Figure 14B). We next tested the MIC values for the

suppressor mutants using PMB, aminoglycosides, and MtrCDE pump substrates (to determine if the PMB resistance phenotype was due to increased MtrCDE activity)(Appendix Table 3E). The increased PMB resistance of the suppressor mutants appeared to be stable, as passage onto plain GC agar prior to the assay had no impact on the PMB MIC. Further, the suppressor mutants had PMB MICs approaching that of WT gonococci and were 4-fold more resistant to PMB than the FA19 *misR::kan* parent strain. As would be expected, MICs against the aminoglycosides streptomycin, gentamicin, and tobramycin resembled those of the FA19 *misR::kan* parent strain. Additionally, MICs against erythromycin and TX-100 did not appear to be elevated in the suppressor mutants, which ruled out the possibility that overexpression or increased activity of the MtrCDE efflux pump system was responsible for the rescue of PMB resistance. Importantly, distinct MIC profiles between suppressor 1 and suppressor 3 suggest that these two mutants are not clonal.

By using whole genome sequencing on purified genomic DNA from suppressor mutants 1 and 3, and the parent strain FA19 *misR::kan*, future work will determine the genetic factors that directly link MisR and PMB resistance.

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Chapter 8: **Discussion**

Neisseria gonorrhoeae is a strictly human pathogen. Though gonococci can be grown in the laboratory under certain conditions, and a female mouse model of lower genital tract infection is frequently used in gonococcal pathogenesis studies, in nature *N. gonorrhoeae* is exclusively found in *Homo sapiens*. In men, lower genital tract gonococcal infections are typically symptomatic. However, often in women (and occasionally in men), the presence of gonococci in the lower genital tract produces no symptoms, and pharyngeal gonorrhea is usually asymptomatic in both sexes. In these instances, the gonococcus has essentially entered into a commensal-like state; that is, gonococci receive the benefits of living in an animal host without inducing a bactericidal immune response, and the human host remains unharmed. However, as is the case for *N. meningitidis* in the nasopharynx and *Staphylococcus aureus* on the skin, movement of the gonococcus from a silent commensal state to a damaging pathogenic state can occur. Thus, the gonococcus transitions fluidly between these two lifestyles, and therefore must possess mechanisms that prevent or delay detection by the immune system *and* defenses that prepare it for the possibility of a proinflammatory innate immune response.

In this work, I describe novel regulatory mechanisms that are likely to impact both of these gonococcal lifestyles. These mechanisms are focused around three important virulence factors; LptA (enzymatic modification of lipid A with PEA), TbpBA (acquisition of host iron), and MisR (CAMP resistance).

I. LptA

As stated in Chapter 1, acquired CAMP resistance through PEA modification of lipid A in *Salmonella* is carried out by the enzyme EptA, whose encoding gene *eptA* is regulated by the PmrAB two component regulatory system (TCS)(1, 2). PmrAB activity is in turn a target of phosphorylated PhoP (PhoP~P); thus, modification of lipid A can be indirectly induced in *Salmonella* by the presence of PhoQ-detected stimuli such as antimicrobial peptides, low divalent ion concentration, and acid stress. Since the gonococcus can also modify its own lipid A with PEA through the action of a similar enzyme, LptA, and this modification is important for resistance to CAMPs and complement (3), we became interested in the regulation of this important virulence gene. To our surprise, *lptA* does not appear to be under the control of the TCS response regulator, MisR, which we had originally thought might play an analogous role to PhoP in this regard. However, we did discover novel *cis*-regulatory elements that influence *lptA* transcription (two promoters) and translation (a phase variable tract in the open reading frame of *lptA*).

During our exploration of the *lptA* gene, we found an unexpected mechanism of *lptA* regulation, which we have published (4). The sequence of the *lptA* gene has been determined previously for strain FA 1090 (http://www.genome.ou.edu/gono.html). Interestingly, *lptA* is not designated as an ORF in the online FA 1090 genome, and we found that this is due to a frame shift mutation in a poly-T tract in the 5’ end of the *lptA* gene. Were it not for this serendipitous omission, we would probably not have noticed the poly-T tract, nor suspected that *lptA* is phase variable. In addition to the presence of two

promoters that can drive transcription of *lptA*, this phase variation mechanism provides a third way for gonococci to modulate the decoration of lipid A with PEA.

Certainly, the presence of two promoters hints that as yet unknown transcriptional regulatory proteins are likely to play a role in *lptA* expression, even if MisR is not among them. This raises the possibility that *lptA* transcription can respond to inputs from multiple transcriptional regulators, and potentially become more (or less) transcribed than the flanking genes *serC* and *nfnB*. Data from the RNA-Seq analysis show that *serC*, *lptA*, and *nfnB* have roughly similar transcriptional levels under our *in vitro* conditions (all genes were transcribed at a level of ~300-500 reads-per-kilobase-per-million-reads a.k.a. "RPKM"), which is consistent with their linkage on the same transcript. However, it is not yet clear to what degree each of the two promoters contributes to the amount of *lptA* mRNA. Perhaps under some inducing condition requiring additional PEA modification of lipid A, the *lptA* proximal promoter might become activated by a regulatory protein. In this way, the gonococcus might modulate *lptA* transcript levels independently of *serC* message.

In order to isolate the impact of the upstream distal promoter on *lptA* expression, we tried numerous times but failed to generate a fully *serC*-deficient gonococcal mutant in either of the FA19 or FA 1090 wild type (WT) backgrounds (though we did generate several merodiploid clones) using both nonpolar (*serC::kan*) and polar (*serC::*Ω) cassettes. *serC* encodes a putative phosphoserine aminotransferase, which is necessary for the synthesis of the amino acid serine and the enzyme cofactor pyridoxal-5'-phospate (P5P) in *E. coli* (5). *serC* appears to be essential in at least four other bacteria [*Francisella novicida*, (6) supplementary table 9; *Helicobacter pylori*, (7) supplementary

table 1; *Mycobacterium tuberculosis*, (8) supplementary table S1 ORF HI1167; *Acinetobacter baylyi,* (9)], *E. coli serC*-deficient mutants only grow under special conditions (5, 10), and *serC* was recently shown to be essential in *N. gonorrhoeae* strain MS11 [see NCBI accession number YP_208349.1 in Supplementary Table S4 in (11)]. This new evidence hints that *serC* is probably essential in most if not all strains of *N. gonorrhoeae*, which suggests that in general, gonococcal *lptA* is constitutively transcribed as a result of the linkage between *serC* and *lptA*. This linkage, when considered in light of the phase variable nature of the *lptA* ORF, raises an interesting question: did the phase variable tract evolve to shut off PEA modification of lipid A without altering the transcript levels of *lptA* or its flanking genes *serC* and *nfnB*?

In the pathogenic *Neisseria*, *lptA* is encoded between two metabolically important genes: *serC* (whose product was described above) and *nfnB* [which encodes a putative nitroreductase likely to be important for degradation of nitroaromatic compounds toxic to the cell; see (12)—*nfnB* does not appear to be essential according to the (11) study]. In contrast, most commensal *Neisseria* do not seem to encode *lptA* at all, and genetically link *serC* and *nfnB* directly (13). Furthermore, it has also reported (13) that there are a few strains of commensals (in species *N. subflava* and *N. flavescens*) that have a >7 kb insertion between *serC* and *nfnB*. When taken together with the placement of *lptA* in pathogenic species, this evidence suggests that the *serC*-*lptA*-*nfnB* operon is a site of considerable genetic variability among the *Neisseria*.

Thus, it seems that *lptA* is a relatively recent addition between these two more-established flanking genes, and as such has probably had less time to evolve more sensitive, protein-attracting genetic elements like regulator binding sites. The evolution of

a phase variable poly-T tract in *lptA* is, by comparison, a much simpler way to modulate production of an active LptA enzyme. As stated above, *lptA* is only present in the pathogenic *Neisseria* (and 1 strain each of *N. lactamica* and *N. polysaccharea*)(13). Importantly, a BLAST search using a 78 bp region surrounding the FA19 *lptA* poly-T_8 tract resulted in all phase-ON *lptA* hits for *N. meningitidis*. Therefore, it seems possible that meningococcal *lptA* may phase vary as well, though to our knowledge this has not been directly tested.

LptA-mediated decoration of lipid A with PEA greatly increases the inflammatory potential of gonococci and meningococci (13). In contrast, lower genital tract infection of female BALB/c mice with gonococci that lack *lptA* induces approximately the same quantity of proinflammatory cytokines and chemokines as mock infection with phosphate buffered saline (14), and these *lptA*-deficient gonococci are less fit in competitive infections of both mice and men (15). These observations suggest that phase variation of *lptA* could play an important role in the precipitation of symptoms during gonococcal (and perhaps meningococcal) colonization, due to its impact on the structure of the TLR4 ligand, lipooligosaccharide (LOS).

Inflammation by the innate immune system has profound effects on the generation of immune memory (16). As master pathogens of humans, gonococci have evolved ways to dysregulate the connections between the innate and adaptive arms of the host immune system (17), thereby limiting immune memory and ensuring their transmission among the global population. Interestingly, TLR4-deficient female BALB/c mice produce much less T_H17-type cytokines upon infection with PEA-decorated gonococci than do WT BALB/c mice (18). Since the upregulation of T_H17-type responses

is an important step in gonococcal subversion of host adaptive immunity (19) this result suggests that host-sensing of gonococcal LOS structure could be a key tool used by gonococci to control the host immune response. Furthermore, because *lptA*-deficient gonococci do not produce a significant inflammatory response in mice (14), it is tempting to speculate the following: 1) phase-OFF *lptA* may allow gonococci to live quietly without inducing inflammation, and 2) phase-ON *lptA* may be a necessary player in both CAMP resistance and in the suppression of immune memory during the potent antigonococcal proinflammatory response by ensuring that lipid A is decorated with PEA and T_H17-type cytokines are kept high.

Since phase-ON *lptA* appears to be common among symptomatic clinical isolates of gonococci (4), one obviously lacking piece of the puzzle is the phase-state of *lptA* in gonococcal isolates from individuals who are colonized but have no symptoms. Unfortunately, this information is hard to acquire since people without symptoms would have no reason to seek medical treatment or provide specimens. Furthermore, clinical isolates from patients being tested for gonorrhea are routinely grown on Thayer-Martin or Martin-Lewis agar (20), which contain sufficient quantities of colistin to kill *lptA*-deficient gonococci (21). Thus, our finding (4) that clinical isolates of gonococci are typically *lptA* phase-ON may be skewed by the loss of phase-OFF specimens during diagnostic culture.

It is clear from competitive infection experiments that PEA-modification of lipid A is essential for gonococcal survival of the innate immune response in the murine female lower genital tract and in the human male urethra (15). Therefore, the only situation in which *lptA* phase-OFF gonococci would be likely to survive would be in the

absence of CAMP and phagocyte influx [*lptA* phase-OFF gonococci would also be at a disadvantage in the blood due to the importance of PEA-decorated lipid A for complement resistance; (3)]. Importantly, *lptA*-deficient gonococci are approximately as fit as WT gonococci in noncompetitive infections of female mice (14), which confirms that gonococci can survive *in vivo* without PEA modification of lipid A, provided there is no triggering of the potent proinflammatory response by WT gonococci. One intriguing possibility is that phase-OFF *lptA* might play an important role maintaining a silent commensal state during pharyngeal, cervical, and rectal colonization, which are often asymptomatic.

II. TbpBA

Gonococci do not produce siderophores, and thus must steal iron from xenosiderophores produced by other bacteria (22) or directly from human iron-binding proteins (23). Among the major human iron-binding proteins is transferrin, which safely sequesters free iron in blood and extracellular fluids (24). Unlike the other two component iron-uptake systems that remove and take up iron from lactoferrin and hemoglobin, the gonococcal transferrin-binding proteins are universally expressed in all tested gonococci (23). Additionally, we found the amount of *tbpBA* transcript (and that of other iron-responsive genes) to be greatly increased after phagocytosis by human macrophage-like monocytes (25), which demonstrates at the cellular level that iron acquisition is an important survival strategy employed by gonococci to resist host innate immune factors.

Previous work from the Shafer laboratory linked iron-homeostasis and gonococcal resistance to host antimicrobials (26) via *mpeR*, a Fur+Fe^{2+}-repressed gene

whose product (MpeR) modulates MtrCDE expression and function when iron is limiting by regulating the *mtrR* and *mtrF* genes (27, 28). Unexpectedly, we discovered another iron-antimicrobial resistance linkage when we found that *tbpB* and *tbpA* were among the most MisR-repressed genes as determined by RNA-Seq. Our DNA binding studies supported this regulatory scheme by showing that MisR~P can bind to the *tbpBA* promoter at a site that overlaps the Fur box and the -10 element.

It is not clear why *tbpBA* would require further repression by any other regulator, when Fur+Fe^{2+} so strongly blocks transcription [see Figure 1, (29)]. However, it should be noted that MisR~P protection was strongest on the antisense strand at the -10 element, and could perhaps enhance the affinity of Fur+Fe^{2+} for the Fur box. Alternatively, MisR might also control *tbpBA* in the absence of Fur (i.e. under iron-limiting conditions). Since our RNA-Seq experiment was performed under iron-replete conditions (Fur+Fe^{2+} is bound at the *tbpBA* promoter and strongly represses transcription), and our DNA-binding studies did not utilize purified Fur protein, the true relationship between MisR and Fur at the *tbpBA* promoter requires further exploration.

One of the advantages of the next generation sequencing method RNA-Seq is that it maps transcriptional data to a completed genome. Thus, using sequence-mapping software, investigators may actually visualize the precise nucleotides of the genome where transcription occurs. In addition to quantifying the transcription of known open reading frames (as with microarray technology), RNA-Seq can also reveal new areas of transcription in non-coding regions of a genome. Therefore, we utilized the MisR RNA-Seq data to determine whether or not transcription occurs in a large, mostly non-

coding region upstream of *tbpBA*, whose expression and function has remained unexplored.

Using SeqMan Pro software (DNASTAR) to visually inspect transcription of the *tbpBA* upstream region, we determined that NGO1497 was not transcribed and that NGO1498 was not likely to be transcribed (due to orientation) under our RNA-Seq *in vitro* conditions [Figure 8, (29)]. However, we also discovered a very large region of transcription upstream of *tbpBA* (spanning approximately 1.8 kb) that had not previously been described, and through various transcriptional and translational assays our collaborators demonstrated that interruption of this long transcript with the polar Ω cassette decreased *tbpBA* mRNA levels but increased TbpA and TbpB protein levels [Table 2 and Figure 7, (29)]. Therefore, we concluded that [in addition to the action of Fur+Fe^{2+} and the hairpin loop between *tbpB* and *tbpA* (Chapter 1)], this upstream RNA plays an important role in the expression of transferrin-binding proteins. In this regulatory scheme, expression of the upstream RNA might help stabilize a large pool of available *tbpBA* transcript, while minimizing needless translation of the Tbps when iron is available. In this way, the gonococcus could in theory be ready at a moment's notice for iron-limiting conditions. Furthermore, since disruption of the upstream RNA results in enhanced production of TbpB and TbpA protein, future studies might use this phenotype to increase the availability of TbpBA antigens at the surface of gonococcal vaccine outer membrane vesicles or killed whole cells.

The interruption of the upstream RNA causes a unique phenotype [we were unable to find any precedents for this kind of RNA-mediated regulation—see Discussion in (29)] and raises several questions: 1) what is the mechanism by which this upstream

RNA influences *tbpBA* transcript and protein levels? 2) is the upstream RNA one contiguous transcript, or is it comprised of multiple transcripts spanning the region? (alternatively, could the RNA-Seq have inappropriately mapped transcript reads from homologous repeats at other locations in the genome to the *tbpBA* upstream region?) and 3) is the upstream RNA itself regulated, and if so by what mechanism(s)? More studies are needed to elucidate the complex relationship between the TbpBA system and the upstream RNA.

III. MisR

We had originally become interested in the MisR/MisS TCS due to its importance for resistance to CAMPs, its ability to globally regulate gene expression, and its role in pathogenesis in the closely related species, *N. meningitidis* (see Chapter 1). The MisR response regulator is highly conserved among the *Neisseria*, and the amino acid sequences of MisR from gonococcal strain FA19 (NGEG_00293) and *N. meningitidis* strain MC58 (NMB0595) are 100% identical. Thus, MisR is likely to a play similar role in both pathogens. Therefore, we initially hypothesized that MisR/MisS might be a CAMP-sensing TCS in the gonococcus, because like PhoP/PhoQ, meningococcal MisR/MisS is necessary for pathogenesis and CAMP resistance and impacts *lptA* expression. As of the start of this work in 2008, no studies had yet been published regarding the role of MisR/MisS in gonococci. Since gonococci are subjected to a plethora of killing mechanisms during symptomatic infection (not the least of which is direct killing by CAMPs), and can resist these immune responses, it seemed reasonable that initially low levels of CAMPs present at the genital mucosal surface might be sensed

by MisR/MisS, leading to an adaptive transcriptional response that would enhance the chances of gonococcal survival of a subsequent potent proinflammatory response.

Indeed, MisR was required for constitutive and inducible resistance to PMB, which supported the idea that CAMPs might be sensed by MisR/MisS. However, we were unable to reconcile that hypothesis with our observations that 1) FA19 *misS::kan* gonococci had the same MIC against PMB as FA19 WT gonococci, and 2) the adaptation effect after pretreatment with a sublethal level of PMB still occurred in the absence of MisS. Thus, it seems unlikely that MisS senses CAMPs (at least PMB) directly. In support of this, we found that in gonococci [as in meningococci; see (30)] Mg^{2+} levels had no impact on *misRS* expression, which is a hallmark of the divalent cation-sensing PhoP/PhoQ system (31). Furthermore, loss of MisS had no apparent effect on growth, which was surprising given the importance of MisR for growth in broth. Therefore, in gonococci MisS probably does not function as a PhoQ homologue [this statement is supported by several meningococcal studies of MisR/MisS led by Yih-Ling Tzeng (30, 32, 33)]. Since a phenotype for MisS sensor kinase-deficient gonococci was not forthcoming, we turned our attention to the DNA-binding response regulator of the MisR/MisS system, MisR.

To determine the transcriptional regulatory targets of MisR that might contribute to CAMP resistance, we performed an RNA-Seq global transcriptome analysis comparing gene expression in FA19 WT to FA19 *misR::kan*. Surprisingly, the two most prominent CAMP resistance mechanisms (efflux by MtrCDE and decoration of lipid A with PEA by LptA) did not appear to fall under the transcriptional control of MisR, as neither *lptA* nor any of the *mtr* system genes (*mtrC*, *mtrD*, *mtrE*, *mtrF*, *mtrR*, *mtrA*) met

our significant fold-change cutoff of 2-fold. In keeping with this trend, 1) beta-galactosidase assays and qRT-PCR experiments demonstrated that loss of MisR had little effect upon expression of *lptA*, 2) MIC assays showed that overexpression of *lptA* could not restore *misR::kan* gonococci to WT levels of PMB resistance, and 3) mass spectrometry analysis confirmed that PEA decoration of lipid A is unaffected by MisR.

We therefore sought out other reasons why gonococci lacking *misR* would have an increased susceptibility to CAMPs, using the RNA-Seq results as a guide. Notably, MisR regulated several stress response genes that are members of the heat shock and RpoH regulons. When we tested the expression of these genes by qRT-PCR, our results recapitulated what we had seen in the RNA-Seq data (MisR is a repressor of *marR2*, *clpB*, NGO1245, *dnaK*, and *grpE*, but a strong activator of *htpX*). In *E.coli*, DnaK is a chaperone, and utilizes ATP hydrolysis to refold damaged proteins and prevent protein aggregation, working with the nucleotide exchange factor GrpE to more efficiently cycle ADP and ATP binding. It has also been hypothesized that the DnaK machinery can work in conjunction with the “unfolder” chaperone ClpB to disaggregate clumped proteins (34). Currently, there are no published reports of *marR2* or NGO1245 function in gonococci, however they were both upregulated by heat shock (35) and may be cotranscribed.

While the DnaK/DnaJ/GrpE system and ClpB are all cytosolic, HtpX is an integral membrane protein that endoproteolytically cleaves misfolded membrane proteins, making them more accessible to FtsH degradation (36)(gonococcal *ftsH*, NGO0382, was not significantly regulated by MisR). Furthermore, it is well-established that MisR is an activator of the *dsbD* gene, which encodes a thiol:disulfide interchange

protein that is important for generating the correct disulfide bonding schemes in periplasmic proteins and has been shown to be essential in both *N. meningitidis* (37) and *N. gonorrhoeae* [see NCBI accession number YP_208076.1 in Supplementary Table S4 in (11)].

Since MisR appeared to be playing an important role in protein quality control (further demonstrated by its RNA-Seq impact on genes encoding peptidyl-prolyl *cis-trans* isomerase B, oligopeptidase A, the Lon serine protease, and ClpA ATP-binding component of the Clp protease), we considered the possibility that the increased CAMP susceptibility of *misR*-deficient gonococci was due to altered membrane permeability not because of surface charge differences, but in the sense that membranes would be more leaky in a *misR::kan* mutant. While the cytosolic chaperones DnaK/GrpE, and ClpB were all repressed by MisR and thus would be more plentiful in a *misR::kan* cell, strong MisR activation of *htpX* and *dsbD* would be crucial to maintaining correctly formed, functional proteins in the membranes and periplasm. Thus, at first glance MisR appears to control the degradation of misfolded membrane proteins and the disulfide bonding conformation of periplasmic proteins. If this is true, it then follows that *misR*-deficient gonococci would be more susceptible to antimicrobial agents that enter Gram-negative bacteria through the membranes, since misfolded membrane proteins would be expected to disrupt the phospholipid bilayers of the envelope.

Indeed, when we tested the susceptibility of *misR::kan* gonococci to aminoglycosides, which, like CAMPs, enter Gram-negative bacteria via self-promoted uptake through the membranes (see Chapter 1 Section Vb), we found a 4-8 fold increase in susceptibility to gentamicin, tobramycin, and streptomycin in the absence of MisR as

compared to WT cells. It is worth mentioning that this 4-8 fold increase in susceptibility was the very same that we had seen with PMB, which supports our hypothesis that the increased susceptibility of *misR::kan* cells to these two classes of antimicrobials is through a common mechanism. Since leaky membranes would be expected to also impact susceptibility to hydrophobic antimicrobials (HA) such as substrates of the MtrCDE efflux pump, we generated *misR*-deficient isogenic mutants of two *mtrCDE*-overexpressor strains, JF1 and KH15.

Again, loss of MisR resulted in increased susceptibility, and across a number of different antibiotic classes [azithromycin and erythromycin (macrolides), ceftriaxone (a cephalosporin), crystal violet (a dye), penicillin G (a beta-lactam), rifampicin (a rifamycin), sodium deoxycholate (a bile salt), and TritonX-100 (a nonionic detergent)]. In contrast, susceptibility to the fluoroquinolone ciprofloxacin was identical between all strains, and demonstrates that porin-mediated influx of drugs is not likely to be affected by MisR. Interestingly, the *misR*-deficiency caused increased susceptibilities to HA of varying degrees (between 2 and 32-fold) across the range of low- to high-*mtrCDE*-expressing strains, which suggests that the level of efflux pump is probably not the root cause of the HA-hypersusceptible *misR*$^{-}$ phenotype (if that were the cause, fold-changes in susceptibility would be expected to be approximately equal for all substrates in a given parent/*misR*-deficient strain pair). Indeed, levels of MtrE protein were the same regardless of MisR expression in each of the three parent/*misR*-deficient strain pairs.

Perhaps MtrCDE-substrates are more potent against *misR*-deficient gonococci because efflux pump activity is inhibited in some way, or because the membranes are leaky enough to expedite antimicrobial entry into the cytoplasm before they can be bound

by MtrD. However, although MtrCDE protein levels appear to be the same regardless of MisR expression, whole-cell lysate Western immunoblotting does not confirm that MtrCDE has been translocated to the envelope, nor that it has been assembled correctly. As mentioned above, MisR regulation impacts protein folding and quality control machinery, which we confirmed by qRT-PCR. Furthermore, *tatC*, which encodes the integral membrane protein TatC necessary for recognition of twin-arginine motifs in signal peptides, was the most strongly MisR-activated gene in the RNA-Seq experiment (*tatB* was also activated by MisR). The Tat system is responsible for the translocation of fully-folded proteins and protein complexes across the cytoplasmic membrane (38), and it is likely that *misR::kan* gonococci have impaired Tat function which could impact the installation of larger protein complexes in the envelope.

Aside from protein quality control mechanisms, MisR also regulated several other interesting genes, some of which may be categorized into groups based on function. Specifically, these groups involve:

- metabolite transporters and associated components
    - MisR-repressed: *nadC*, *pitB*, NGO2014, *putP*, *glnQ*, *cytX*, *potD*, *tnaB*
    - MisR-activated: *arsB/nhaD*
- iron acquisition and storage proteins
    - MisR-repressed: *tbpB*, *tbpA*
    - MisR-activated: *bfrA*, *bfrB*
- drug efflux factors
    - MisR-repressed: NGO1245, *mpeR*
    - MisR-activated: *macA*, *macB*

- redox enzymes
    - MisR-repressed: *grx*, *thiO*, *sdhD*, *sdhA*, *yojH*, *trxI*
    - MisR-activated: *nrdA*, *nrdB*
- methyltransferases and signaling
    - MisR-repressed: *ppk2*, NGO1546, NGO0651, NGO1287
    - MisR-activated: *mraW*
- metabolism
    - MisR-repressed: *opdA*, *GAPDH*, *rpe*, *glnA*, *acnB*, *thiC*, *tmp*/*tenI*, *gltA*, *shmt*
- DNA repair and recombination
    - MisR-repressed: *recN*, *rusA*
    - MisR-activated: *exoIII*
- outer membrane proteins
    - MisR-repressed: *opcA*, *mafA3*, *mafA2*
    - MisR-activated: *ompA*
- cell division proteins
    - MisR-repressed: *scpA*
    - MisR-activated: *nlpC*, *ftsL*, *dca*, *pbp2*, *mraZ*, *maf*
- ribosomal proteins
    - MisR-activated: *rpmB*, *rpmE*, *rplL*

Notably, the TbpBA system and OpmA are currently gonococcal vaccine candidates (39), and future studies might capitalize on MisR's impact on these genes. Another interesting observation is that MisR activates NGO1852 (*rplL*). *rplL* encodes the ribosomal L7/L12

protein, which has been shown to “moonlight” as an invasin of human endometrial cells by mimicking the host lutropin receptor (40). Thus, perhaps MisR-mediated activation of *rplL* could result in enhanced invasion of endometrial cells and promote expansion of gonococcal populations to the female upper genital tract.

Intriguingly, MisR seems to strongly activate an as yet undescribed immunity/antitoxin protein, *imm22* (NGO1590). This gene is part of a toxin/antitoxin cluster encoded by NGO1589-NGO1592. NGO1589 and NGO1592, the putative toxin genes, encode RNase toxin 46 (pfam15526) and HNH endonuclease (cd00085) domains, respectively, which in theory would target nucleic acids. These two genes flank NGO1590 and NGO1591, which each encode an immunity protein 22 (pfam15592) domain. It is not yet clear if these toxin/antitoxin genes are functional in gonococci, or whether the toxins target the nucleic acids of gonococci, competing microbes, or host cells. Nevertheless, the involvement of MisR could suggest that whatever signal induces MisR/MisS would in turn upregulate immunity protein 22 expression, and perhaps bring gonococci out of a quiescent state induced by self-toxicity. Future studies are needed to elucidate the true nature of this toxin/antitoxin locus, as these systems are seldomly studied in gonococcal biology (41) and are prevalent in many other bacteria (42).

These are just a few of the interesting MisR-regulatory targets shown by the RNA-Seq experiment. Importantly, most of these regulatory phenotypes still need to be confirmed by a secondary method (e.g. qRT-PCR or beta-galactosidase assays), and this RNA-Seq table does not describe the non-coding regions of the FA19 chromosome that may be affected by the loss of MisR. However, the regulatory targets of MisR that contribute to CAMP resistance may become clearer by using whole genome sequencing

to map any mutations that may have occurred in the FA19 *misR::kan* PMB-resistant suppressor mutants due their selection from the FA19 *misR::kan* parent strain on agar containing PMB (see Chapter 7 Unpublished Results). Potential mutations that increase PMB resistance might be found in loci such as *lptA*, *mtrCDE*, genes that are important for protein quality control, and genes that are important for proton motive force. One of the suppressor mutants (#3; see Table 3E in Chapter 7 Unpublished Results) had concomitant increases in both PMB and aminoglycoside resistance, which could suggest a return to more WT-levels of membrane permeability. Sequencing and analysis of the FA19 *misR::kan* parent and FA19 *misR::kan*, PMB-resistant suppressor mutants are currently underway.

Though MisR is unlikely to be a direct homologue of any other bacterial response regulator, this section cannot be concluded without some mention of the similarity between phenotypes associated with MisR and those associated with another response regulator, CpxR. First and foremost, both MisR (this work) and CpxR (43-45) are required for resistance to aminoglycosides. Second, both regulators activate *htpX* [this work and (46, 47)], which encodes an important membrane protease. Third, improper disulfide bonding appears to be an inducer of MisR and CpxR activities [meningococci: (37); (48)], though the precise signal remains elusive for the MisR/MisS system in both meningococci and in gonococci. Thus, we propose that MisR/MisS is likely to be in large part responsible for the gonococcal response to membrane-damaging stimuli, a hitherto unexplored mechanism in this organism.

IV. Final Summary

At the beginning of this work, we set out to determine the role of the two component regulatory system response regulator, MisR, in gonococcal resistance to CAMPs, which are a crucial component of the human innate immune response to the sexually transmitted infection gonorrhea. While our initial hypothesis that MisR controlled CAMP susceptibility by regulating *lptA* appears to be incorrect, we discovered that *lptA* can be transcribed from two distinct promoters, and is post-transcriptionally regulated by a phase variable poly-T_8 tract present within the *lptA* open reading frame.

Transcriptome analysis of the MisR regulon by RNA-Seq revealed that MisR is a global regulator that significantly impacts the expression of nearly 100 genes (including the transferrin-binding protein genes *tbpB* and *tbpA*). Further, visualization of transcription from the *tbpBA* upstream region at nucleotide-resolution demonstrated the expression of a previously unknown RNA species that impacts *tbpBA* transcript and TbpBA protein levels by a yet to be defined mechanism. Phagocytosis of gonococci by human macrophage-like monocytic cells greatly upregulated transcription of *tbpBA* (and other iron-responsive genes), and did not kill 100% of the internalized bacteria. These data suggest that the iron-limiting environment of the macrophage interior can be sensed by gonococci, and that this process may enhance the chances of gonococcal survival during the iron-limiting innate immune response. Furthermore, we have expanded the pool of *tbpBA* regulators to include MisR and a novel RNA.

Many of the genes regulated by MisR in the RNA-Seq profile are involved in protein quality control, and antimicrobial susceptibility testing of the *misR::kan* mutation in various genetic backgrounds shows that loss of MisR increases susceptibility to

CAMPs and aminoglycosides by approximately the same factor (4-8 fold). Furthermore, function of the MtrCDE efflux pump was impaired by loss of MisR in both WT and in *mtrCDE*-overexpressor isogenic mutant backgrounds, which are common among gonococcal clinical isolates. The characterization herein of a novel CAMP/aminoglycoside/HA resistance mechanism in gonococci is of special interest in light of the dwindling number of curative antibiotics for gonorrhea, and the approaching implementation of an aminoglycoside (gentamicin) as a first-line therapy in the United States.

In closing, these studies have determined the genetic landscape of the gene encoding the lipid A phosphoethanolamine transferase, *lptA*, discovered a novel regulatory RNA upstream of the transferrin binding protein operon encoding *tbpB* and *tbpA*, and characterized the regulatory targets and antibiotic susceptibility profile of the MisR response regulator in *N. gonorrhoeae*.

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Chapter 9: Appendix

Appendix Table 1A
Primers used for studies in Chapter 7: Unpublished Results

Primer name	Primer sequence (5’→3’)	Purpose
16Smai-RTF[a]	CCATCGGTATTCCTCCACATCTCT	Test levels of 16S rRNA transcription as a housekeeping reference gene
16Smai-RTR[a]	CGTAGGGTGCGAGCGTTAATC	Test levels of 16S rRNA transcription as a housekeeping reference gene
5’lptA-Z[b]	TTGGATCCTCCTTTTCAATCCGGG CGTGATGC	Construction of pLES94-*lptA* (forward)
3’lptA-Z[b,c]	TTGGATCC**TT/TAT/CAT**TTGGAA TGTGTCGG	Construction of pLES94-*lptA* (reverse)
5’misRS-lacZ[b]	TTGGATCCCACCGCTGCTGCCCG AACTGCTC	Construction of pLES94-*misR* (forward)
3’misRS-lacZ[b,c]	TTGGATCC**CG/GCT/CAT**GGTGTT TCCTTTTC	Construction of pLES94-*misR* (reverse)
clpB_qRT_F[a]	TCCAACAAGCCCTTGCAGAA	test levels of *clpB* expression
clpB_qRT_R[a]	GCTGCTGCAAACGCTGTTTA	test levels of *clpB* expression
dnaK_qRT_F[a]	ATGGCTCTGCAACGTCTGAA	test levels of *dnaK* expression
dnaK_qRT_R[a]	CGAATTTGGCGCGGGTAATT	test levels of *dnaK* expression
dsbD_FPacI[d]	CGTTAATTAAATGCGGACAAATT ATTTGTCTGA	Amplification of the *dsbD* gene for complementation in pGCC4
dsbD_RPmeI[d]	CGGTTTAAACTCAGCGGTTTTGT TCATACCACT	Amplification of the *dsbD* gene for complementation in pGCC4
dsbD_qRT_F[a]	CGCAGGTTGCCTTTCCTTATG	test levels of *dsbD* expression
dsbD_qRT_R[a]	TCGGTTTGCGGATGGTAAGT	test levels of *dsbD* expression

grpE_qRT_F[a]	AAATGTGGAGGCGGTGGAAA	test levels of *grpE* expression
grpE_qRT_R[a]	GCGCAGTTGTTCGTCTTTCA	test levels of *grpE* expression
grxC_qRT_F[a]	GGTGTCGGACATATCGACGAA	test levels of *grxC* expression
grxC_qRT_R[a]	AAATCCTCCGACGTGCGTTT	test levels of *grxC* expression
htpX_qRT_F[a]	TCGGCTTCACTGGTTCGATT	test levels of *htpX* expression
htpX_qRT_R[a]	TTGGGCTTCGACAGTGTTCA	test levels of *htpX* expression
lon_qRT_F[a]	AGGCGTAAGAAACCCGTTGT	test levels of *lon* expression
lon_qRT_R[a]	CATCACGTCGCTCAAATCGT	test levels of *lon* expression
lptA_qRT_F[a]	GGCATCGCGATGTTGCAATA	Test total levels of *lptA* transcription
lptA_qRT_R[a]	CACGACCGCCATATCCAATTG	Test total levels of *lptA* transcription
lptA_qRT_F2[a]	GCTTTGCTCCGTTGCCTTAT	Test levels of *lptA* transcription initiated from the distal promoter upstream of *serC*
lptA_qRT_R2[a]	CCAGCGAAGAATACAGGGAAAG	Test levels of *lptA* transcription initiated from the distal promoter upstream of *serC*
LptA11	CCGGTTCGAATTTTGCTTACG	DNA binding studies at the *lptA* promoter
LptA7_R	AAGAATACAGGGAAAGAAAGGC	DNA binding studies at the *lptA* promoter
marR2_qRT_F[a]	TCGGGCAGCAGGATTTGAAT	test levels of *marR2* expression
marR2_qRT_R[a]	TCGCCTTCCTGCCATTCAAT	test levels of *marR2* expression
misR_qRT_F[a]	TGAGCGGGCAATACGATGTA	test levels of *misR* expression
misR_qRT_R[a]	ACCCATTTCCAAGCCGATGA	test levels of *misR* expression

NGO1245_qRT_F[a]	TGGGCGATTAATGCCGTGAT	test levels of NGO1245 expression
NGO1245_qRT_R[a]	CTGCCGTTGTTCCAACACAA	test levels of NGO1245 expression
RnpB1F	CGGGACGGGCAGACAGTCGC	Amplification of *rnpB* nonspecific competitor probe for EMSAs
RnpB1R	GGACAGGCGGTAAGCCGGGTTC	Amplification of *rnpB* nonspecific competitor probe for EMSAs
rpoH_qRT_F[a]	AACGGCAGCCTCGAACAATA	test levels of *rpoH* expression
rpoH_qRT_R[a]	GGTGGGACAGGATGAGTTGTT	test levels of *rpoH* expression
secB_qRT_F[a]	AGTGGATATGCGCGTTTCCA	test levels of *secB* expression
secB_qRT_R[a]	ACCGCTTTGGGTTACTTCGT	test levels of *secB* expression
tbpBA_1090F	CTTGTGTTTTAGAAGACTCAGGG	DNA binding studies at the *tbpBA* promoter
tbpBA_1090R	CACAGGCAACACCATAGCAGC	DNA binding studies at the *tbpBA* promoter
yjoH_qRT_F[a]	AGATGTGGCGTTGGAATCGT	test levels of *yjoH* expression
yjoH_qRT_R[a]	AACTGGCGGCTGACATGAAA	test levels of *yjoH* expression

[a]All primers used for qPCR were designed using Primer3Plus software (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi) with the parameters outlined in Appendix Table 1B. All primer sets were verified for accuracy and a PCR efficiency of 90-105% by qPCR with 10-fold serial dilutions of FA19 WT genomic DNA as template, melt-curve analysis, and electrophoresis of products on a 1% agarose gel

[b]The engineered BamHI cleavage sites are underlined

[c]The first two codons and partial third codon of the ORF are shown in boldface

[d]The engineered PacI (or PmeI) cleavage sites are underlined

Appendix Table 1B
Primer3Plus settings used for qPCR primer set design
http://primer3plus.com/cgi-bin/dev/primer3plus.cgi

Primer3 File - http://primer3.sourceforge.net
P3_FILE_TYPE=settings
P3_FILE_ID=User settings
P3P_PRIMER_NAME_ACRONYM_INTERNAL=IN
P3P_PRIMER_NAME_ACRONYM_LEFT=F
P3P_PRIMER_NAME_ACRONYM_RIGHT=R
P3P_PRIMER_NAME_ACRONYM_SPACER=_
PRIMER_DNA_CONC=50.0
PRIMER_DNTP_CONC=0.6
PRIMER_EXPLAIN_FLAG=1
PRIMER_FIRST_BASE_INDEX=1
PRIMER_GC_CLAMP=0
PRIMER_INSIDE_PENALTY=-1.0
PRIMER_INTERNAL_DNA_CONC=50.0
PRIMER_INTERNAL_DNTP_CONC=0.0
PRIMER_INTERNAL_MAX_GC=80.0
PRIMER_INTERNAL_MAX_HAIRPIN_TH=47.00
PRIMER_INTERNAL_MAX_LIBRARY_MISHYB=12.00
PRIMER_INTERNAL_MAX_NS_ACCEPTED=0
PRIMER_INTERNAL_MAX_POLY_X=5
PRIMER_INTERNAL_MAX_SELF_ANY=12.00
PRIMER_INTERNAL_MAX_SELF_ANY_TH=47.00
PRIMER_INTERNAL_MAX_SELF_END=12.00
PRIMER_INTERNAL_MAX_SELF_END_TH=47.00
PRIMER_INTERNAL_MAX_SIZE=27
PRIMER_INTERNAL_MAX_TM=63.0
PRIMER_INTERNAL_MIN_GC=20.0
PRIMER_INTERNAL_MIN_QUALITY=0
PRIMER_INTERNAL_MIN_SIZE=18
PRIMER_INTERNAL_MIN_TM=57.0
PRIMER_INTERNAL_MISHYB_LIBRARY=NONE
PRIMER_INTERNAL_OPT_GC_PERCENT=50.0
PRIMER_INTERNAL_OPT_SIZE=20
PRIMER_INTERNAL_OPT_TM=60.0
PRIMER_INTERNAL_SALT_DIVALENT=0.0
PRIMER_INTERNAL_SALT_MONOVALENT=50.0
PRIMER_INTERNAL_WT_END_QUAL=0.0
PRIMER_INTERNAL_WT_GC_PERCENT_GT=0.0
PRIMER_INTERNAL_WT_GC_PERCENT_LT=0.0
PRIMER_INTERNAL_WT_HAIRPIN_TH=0.0

PRIMER_INTERNAL_WT_LIBRARY_MISHYB=0.0
PRIMER_INTERNAL_WT_NUM_NS=0.0
PRIMER_INTERNAL_WT_SELF_ANY=0.0
PRIMER_INTERNAL_WT_SELF_ANY_TH=0.0
PRIMER_INTERNAL_WT_SELF_END=0.0
PRIMER_INTERNAL_WT_SELF_END_TH=0.0
PRIMER_INTERNAL_WT_SEQ_QUAL=0.0
PRIMER_INTERNAL_WT_SIZE_GT=1.0
PRIMER_INTERNAL_WT_SIZE_LT=1.0
PRIMER_INTERNAL_WT_TM_GT=1.0
PRIMER_INTERNAL_WT_TM_LT=1.0
PRIMER_LIBERAL_BASE=1
PRIMER_LIB_AMBIGUITY_CODES_CONSENSUS=0
PRIMER_LOWERCASE_MASKING=0
PRIMER_MAX_END_GC=2
PRIMER_MAX_END_STABILITY=9.0
PRIMER_MAX_GC=60
PRIMER_MAX_HAIRPIN_TH=47.00
PRIMER_MAX_LIBRARY_MISPRIMING=12.00
PRIMER_MAX_NS_ACCEPTED=0
PRIMER_MAX_POLY_X=3
PRIMER_MAX_SELF_ANY=8.00
PRIMER_MAX_SELF_ANY_TH=47.00
PRIMER_MAX_SELF_END=3.00
PRIMER_MAX_SELF_END_TH=47.00
PRIMER_MAX_SIZE=25
PRIMER_MAX_TEMPLATE_MISPRIMING=12.00
PRIMER_MAX_TEMPLATE_MISPRIMING_TH=47.00
PRIMER_MAX_TM=64
PRIMER_MIN_3_PRIME_OVERLAP_OF_JUNCTION=4
PRIMER_MIN_5_PRIME_OVERLAP_OF_JUNCTION=7
PRIMER_MIN_END_QUALITY=0
PRIMER_MIN_GC=40
PRIMER_MIN_LEFT_THREE_PRIME_DISTANCE=3
PRIMER_MIN_QUALITY=0
PRIMER_MIN_RIGHT_THREE_PRIME_DISTANCE=3
PRIMER_MIN_SIZE=18
PRIMER_MIN_TM=59
PRIMER_MISPRIMING_LIBRARY=NONE
PRIMER_NUM_RETURN=10
PRIMER_OPT_GC_PERCENT=50.0
PRIMER_OPT_SIZE=20
PRIMER_OPT_TM=60.0
PRIMER_OUTSIDE_PENALTY=0.0
PRIMER_PAIR_MAX_COMPL_ANY=8.00

PRIMER_PAIR_MAX_COMPL_ANY_TH=47.00
PRIMER_PAIR_MAX_COMPL_END=3.00
PRIMER_PAIR_MAX_COMPL_END_TH=47.00
PRIMER_PAIR_MAX_DIFF_TM=1
PRIMER_PAIR_MAX_LIBRARY_MISPRIMING=24.00
PRIMER_PAIR_MAX_TEMPLATE_MISPRIMING=24.00
PRIMER_PAIR_MAX_TEMPLATE_MISPRIMING_TH=47.00
PRIMER_PAIR_WT_COMPL_ANY=0.0
PRIMER_PAIR_WT_COMPL_ANY_TH=0.0
PRIMER_PAIR_WT_COMPL_END=0.0
PRIMER_PAIR_WT_COMPL_END_TH=0.0
PRIMER_PAIR_WT_DIFF_TM=0.0
PRIMER_PAIR_WT_IO_PENALTY=0.0
PRIMER_PAIR_WT_LIBRARY_MISPRIMING=0.0
PRIMER_PAIR_WT_PRODUCT_SIZE_GT=0.0
PRIMER_PAIR_WT_PRODUCT_SIZE_LT=0.0
PRIMER_PAIR_WT_PRODUCT_TM_GT=0.0
PRIMER_PAIR_WT_PRODUCT_TM_LT=0.0
PRIMER_PAIR_WT_PR_PENALTY=1.0
PRIMER_PAIR_WT_TEMPLATE_MISPRIMING=0.0
PRIMER_PAIR_WT_TEMPLATE_MISPRIMING_TH=0.0
PRIMER_PICK_ANYWAY=1
PRIMER_PICK_INTERNAL_OLIGO=0
PRIMER_PICK_LEFT_PRIMER=1
PRIMER_PICK_RIGHT_PRIMER=1
PRIMER_PRODUCT_MAX_TM=
PRIMER_PRODUCT_MIN_TM=
PRIMER_PRODUCT_OPT_SIZE=
PRIMER_PRODUCT_OPT_TM=
PRIMER_PRODUCT_SIZE_RANGE=125-175
PRIMER_QUALITY_RANGE_MAX=100
PRIMER_QUALITY_RANGE_MIN=0
PRIMER_SALT_CORRECTIONS=1
PRIMER_SALT_DIVALENT=1.5
PRIMER_SALT_MONOVALENT=50.0
PRIMER_SEQUENCING_ACCURACY=20
PRIMER_SEQUENCING_INTERVAL=250
PRIMER_SEQUENCING_LEAD=50
PRIMER_SEQUENCING_SPACING=500
PRIMER_TASK=generic
PRIMER_THERMODYNAMIC_OLIGO_ALIGNMENT=1
PRIMER_THERMODYNAMIC_TEMPLATE_ALIGNMENT=0
PRIMER_TM_FORMULA=1
PRIMER_WT_END_QUAL=0.0
PRIMER_WT_END_STABILITY=0.0

PRIMER_WT_GC_PERCENT_GT=0.5
PRIMER_WT_GC_PERCENT_LT=0.5
PRIMER_WT_HAIRPIN_TH=0.0
PRIMER_WT_LIBRARY_MISPRIMING=0.0
PRIMER_WT_NUM_NS=0.0
PRIMER_WT_POS_PENALTY=0.0
PRIMER_WT_SELF_ANY=0.0
PRIMER_WT_SELF_ANY_TH=0.0
PRIMER_WT_SELF_END=0.0
PRIMER_WT_SELF_END_TH=0.0
PRIMER_WT_SEQ_QUAL=0.0
PRIMER_WT_SIZE_GT=1.0
PRIMER_WT_SIZE_LT=1.0
PRIMER_WT_TEMPLATE_MISPRIMING=0.0
PRIMER_WT_TEMPLATE_MISPRIMING_TH=0.0
PRIMER_WT_TM_GT=1.0
PRIMER_WT_TM_LT=1.0

Appendix Table 2A
RNA-Seq analysis of the MisR regulon in *N. gonorrhoeae* FA19

<u>MisR-repressed genes</u>				
Gene	**ORF** (FA 1090 designation)	[a]**Fold Change** (*misR*-/WT)	[b]**Bonferroni value**	[c]**Function**
nadC	NGO0377†	10.42	0.00E+00	putative NadC family sodium/dicarboxylate symporter
$lctP_{trunc}$	NGO1361†	9.54	1.60E-11	putative L-lactate permease (truncated)
marR2	NGO1244†	5.90	2.04E-05	MarR family protein (regulator of multiple antibiotic resistance)
tbpB	NGO1496†	5.76	0.00E+00	transferrin-binding protein, TbpB
[d]tdf_{trunc}	NGO1362†	5.65	1.90E-04	putative TonB-dependent iron siderophore receptor (truncated)
ppk2	NGO2113	5.05	0.00E+00	putative polyphosphate kinase 2 (catalyzes the transfer of inorganic phosphate from poly P to GDP to create GTP, an important signalling molecule)
clpB	NGO1046	5.01	0.00E+00	ClpB molecular chaperone
	NGO1546	4.15	3.33E-11	putative S-adenosyl-L-methionine-dependent methyltransferase
opdA	NGO1770	4.03	4.45E-13	oligopeptidase A
	NGO1245†	4.02	4.07E-06	putative integral membrane protein (multidomain homology to an ABC-type multidrug transport system—ATPase and permease components)

	NGO0651	3.32	0.00E+00	S-adenosylmethionine-dependent methyltransferase
	NGO1287	3.15	0.00E+00	Ras-like GTPase
tbpA	NGO1495†	3.05	0.00E+00	transferrin-binding protein TbpA
GAPDH	NGO1776	3.04	0.00E+00	glyceraldehyde-3-phosphate dehydrogenase
opcA	NGO0868	2.84	1.33E-05	outer membrane protein (adhesion and invasion)
lon	NGO0775	2.79	0.00E+00	Lon serine protease—hydrolyses ATP to degrade protein substrates
	NGO1588	2.78	0.00E+00	uncharacterized conserved *Neisseria*l protein
grx	NGO0114	2.69	2.87E-09	glutaredoxin 3 [GRX is a glutathione (GSH) dependent reductase, catalyzing the disulfide reduction of target proteins]
pitB	NGO1581	2.66	0.00E+00	predicted phosphate transport permease
	NGO2014†	2.64	0.00E+00	predicted periplasmic binding protein; ABC transporter probably involved in glutamine transport
ppiB	NGO0376†	2.64	0.00E+00	peptidyl-prolyl cis-trans isomerase B
grpE	NGO1422	2.62	0.00E+00	GrpE co-chaperone; GrpE is the adenine nucleotide exchange factor of DnaK (Hsp70)-type ATPases
mpeR	NGO0025	2.55	2.17E-02	Mtr protein efflux regulator
rpe	NGO0758	2.55	0.00E+00	ribulose-5-phosphate 3-epimerase

scpA	NGO0961	2.49	5.29E-02	segregation and condensation protein A; involved in chromosomal partitioning during cell division
recN	NGO0318	2.41	0.00E+00	ATP-binding DNA repair protein
glnA	NGO1600	2.40	7.02E-03	glutamine synthetase
putP	NGO1552	2.37	0.00E+00	highly conserved bacterial sodium/proline symporter
glnQ	NGO2013†	2.35	2.81E-10	GlnQ ATP-binding component of the bacterial periplasmic glutamine permease
	NGO0834	2.32	4.18E-09	CsgG-family lipoprotein (CsgG is involved in curli formation in *E. coli*)
thiO	NGO2008†	2.27	2.75E-06	putative glycine oxidase with minimal identity to ThiO
cytX	NGO2009†	2.24	0.00E+00	putative hydroxymethyl-pyrimidine transporter CytX involved in thiamine production
rusA	NGO0489	2.23	3.24E-02	putative endodeoxyribonuclease/ Holliday junction resolvase RusA
sdhD	NGO0922†	2.23	1.49E-02	succinate:quinone oxidoreductase
kdtA	NGO1915	2.23	2.18E-05	putative KdtA (3-deoxy-D-manno-octulosonic-acid transferase a.k.a. kdotransferase)
acnB	NGO1231	2.23	0.00E+00	aconitate hydratase B (catalyses the formation of cis-aconitate from citrate as part of the TCA cycle)

dnaK	NGO1429	2.20	0.00E+00	DnaK (a.k.a. Hsp70) chaperone
potD	NGO1494	2.19	1.60E-11	PotD (spermidine/putrescine-binding periplasmic protein)
	NGO0165	2.18	2.86E-02	conserved hypothetical protein of unknown function
	NGO0115	2.18	3.33E-10	hypothetical protein of unknown function
	NGO1582†	2.18	2.97E-06	conserved hypothetical protein of unknown function
mafA3	NGO1584†	2.17	1.69E-07	putative adhesin MafA3
thiC	NGO2041	2.14	1.11E-03	ThiC; participates in the formation of 4-amino-5-hydroxymethyl-2-methylpyrimidine from AIR, an intermediate in de novo pyrimidine biosynthesis
tmp/tenI	NGO2007†	2.10	1.38E-06	TMP thiamine monophosphate synthase; catalyzes an important step in the thiamine biosynthesis pathway
mafA2	NGO1393	2.08	2.22E-12	putative adhesin MafA2
gltA	NGO0918	2.07	0.00E+00	GltA-like citrate synthase (CS)
shmt	NGO0866†	2.06	4.89E-12	serine/glycine hydroxymethyl-transferase
	NGO0865†	2.02	1.07E-11	hypothetical protein of unknown function
rpoH	NGO0288	2.01	0.00E+00	alternative sigma factor RpoH; sigma-32, sigma factor H, heat shock sigma factor
yojH	NGO1980	2.00	0.00E+00	YojH malate:quinone-oxidoreductase

tnaB	NGO2073	2.00	4.45E-13	TnaB tryptophan permease
sdhA	NGO0921†	1.99	1.78E-12	SdhA succinate dehydrogenase/ fumarate reductase
trxI	NGO0652	1.99	1.59E-06	TRX(I) thioredoxin I
	NGO1864	1.97	8.78E-04	conserved bacterial protein of unknown function
hsp33	NGO1189	1.86	0.00E+00	Hsp33
MisR-activated genes				
nlpC	NGO1190†	-2.02	6.54E-05	NlpC/P60 family cell envelope protein
	NGO0420	-2.03	3.14E-07	COG3471, predicted periplasmic/secreted protein
ftsL	NGO1543†	-2.04	2.62E-07	FtsL cell division protein
dca	NGO1540†	-2.08	1.69E-23	Dca putative membrane associated sulfatase
clpA	NGO0408	-2.10	1.21E-14	ClpA ATP-binding subunit of the Clp protease
rpmB	NGO1680	-2.23	1.19E-09	RpmB 50S ribosomal protein L28
	NGO1282	-2.23	2.72E-02	hypothetical protein of unknown function (3’ of *serC* sequence and 5' of *lptA* sequence)
exoIII	NGO1561	-2.24	1.28E-12	highly conserved DNA exonuclease III (exodeoxyribonuclease III)
pbp2	NGO1542†	-2.31	6.84E-59	PBP2 penicillin-binding protein 2 peptidoglycan transpeptidase
nrdA	NGO0614†	-2.32	1.29E-30	NrdA ribonucleotide-diphosphate reductase subunit alpha
	NGO0867	-2.34	3.94E-22	predicted transcriptional regulator (Helix-turn-helix XRE-family like protein)

tatB	NGO0182†	-2.40	7.49E-77	twin arginine-targeting protein translocase TatB (Sec-independent protein translocase)
	NGO1556	-2.45	1.95E-02	conserved hypothetical protein (100% homologous duplication of NGO1462)
arsB/nhaD	NGO1411	-2.46	1.97E-36	predicted anion permease ArsB/NhaD; these permeases have been shown to translocate sodium, arsenate, antimonite, sulfate and organic anions across biological membranes
mraW	NGO1544†	-2.46	4.98E-19	MraW S-adenosyl-methyltransferase
	NGO0722	-2.46	1.10E-05	putative phage associated protein of unknown function
	NGO0618	-2.57	1.66E-24	conserved hypothetical protein of unknown function
	NGO1191†	-2.58	2.88E-14	conserved hypothetical protein of unknown function
macA	NGO1440†	-2.69	1.68E-49	MacA (membrane fusion component of the MacAB macrolide efflux pump)
nrdB	NGO0615†	-2.69	2.73E-53	NrdB ribonucleoside-diphosphate reductase
	NGO1462	-2.77	9.06E-03	hypothetical protein of unknown function
mraZ	NGO1545†	-2.83	6.04E-04	MraZ cell division protein
rpmE	NGO2126	-3.07	6.17E-29	RpmE 50S ribosomal subunit protein L31
bfrA	NGO0794†	-3.26	2.98E-05	BfrA bacterioferritin
macB	NGO1439†	-3.31	5.07E-38	MacB (ATP-binding permease protein component of the MacAB macrolide efflux pump)

	NGO1412	-3.46	3.00E-02	IS1016 transposase
	NGO1861	-3.51	1.76E-15	conserved gonococcal hypothetical protein
rplL	NGO1852	-3.59	3.44E-18	L7/L12 ribosomal protein
laz	NGO0994	-3.76	4.81E-03	Laz azurin (a copper-binding protein in the plastocyanin/azurin family)
bfrB	NGO0795†	-3.79	8.18E-80	BfrB bacterioferritin
htpX	NGO0399	-3.84	1.04E-05	HtpX heat shock protein that endoproteolytically cleaves misfolded membrane proteins
	NGO1215	-5.65	6.64E-03	COG2847; putative copper (I) binding envelope protein
	NGO1981	-6.00	3.71E-13	hypothetical protein of unknown function
	NGO0179†	-6.90	6.42E-03	hypothetical protein of unknown function
hlyIII	NGO1289	-7.76	1.69E-08	conserved protein homologous to the HlyIII channel-forming cytolysin
dsbD	NGO0978	-9.21	1.89E-42	DsbD (thiol:disulfide interchange protein)
maf	NGO0180†	-11.90	7.11E-10	predicted Maf family nucleotide binding protein involved in septation
ompA	NGO1559	-17.48	4.56E-03	OmpA family protein
imm22	NGO1590	-18.50	7.72E-11	putative immunity/antitoxin protein (encoded next to a putative toxin gene encoded by NGO1589)
tatC	NGO0181†	-24.34	2.71E-57	TatC Sec-independent protein translocase component

†genes that are adjacent to and encoded in the same orientation as other MisR regulatory targets (i.e., possibly coregulated by MisR as part of an operon).

Repressed groups: NGO0376/NGO0377 (*ppiB*/*nadC*); NGO0865/NGO0866 (hypo/*shmt*); NGO0921/NGO0922 (*sdhA*/*sdhD*); NGO1244/NGO1245 (*marR2*/hypo ATPase and permease); NGO1361/NGO1362 ($lctP_{trunc}$/tdf_{trunc});

NGO1495/NGO1496 (*tbpA*/*tbpB*);
NGO1582/NGO1584 (hypo/*mafA3*); NGO2007/NGO2008/NGO2009 (*tmp*/*thiO*/*cytX*); NGO2013/NGO2014 (*glnQ*/amino acid transporter).
Activated groups: NGO0179/NGO0180/NGO0181/NGO0182 (hypo/*maf*/*tatC*/*tatB*); NGO0614/NGO0615 (*nrdA*/*nrdB*); NGO0794/NGO0795 (*bfrA*/*bfrB*);
NGO1190/NGO1191 (*nlpC*/hypo); NGO1439/NGO1440 (*macB*/*macA*); NGO1540/NGO1542/NGO1543/NGO1544/NGO1545 (*dca*/*pbp2*/*ftsL*/*mraW*/*mraZ*)

[a] Fold change is calculated as the ratio of reads-per-kilobase-per-million-reads (RPKM) values for each gene in the FA19 *misR::kan* cells/FA19 WT cells. These data are representative of three independent comparisons.

[b] The Bonferroni Correction (1), which is a multiple-comparison correction used when several dependent or independent statistical tests are being performed simultaneously, was performed as a more stringent method of determining whether or not fold change values show statistical significance. Fold changes with Bonferroni values >0.05 were considered non-significant.

[c] Protein functions were determined using a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) on the NGO designation of the gene, followed by a characterization of any conserved domains present using the Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

[d] "*tdf*" refers to the "TonB-dependent function" naming scheme. See (2).

Appendix Table 2B
Genes regulated by MisR in the RNA-Seq experiment (Appendix Table 2A) that have PRODORIC[a] Virtual Footprinting sites

ORF (FA 1090 designation)	**Gene name**	**MisR binding site sequence**[b] (KWWWTGTAARGNNWH)	**Start position** (FA 1090)	**End position** (FA 1090)
NGO0377	*nadC*	**GATATGTAAGGGGAA**	372470	372484
NGO0614	*nrdA*	**GAATTGGAAGGGCTT**	601746	601760
NGO0722	NGO0722	**GTTTTGGAAAGGATT**	722214	722228
NGO0794	*bfrA*	**GATTTGGAAGGCATC**	784521	784535
NGO0865	NGO0865	**GATTTCTAAAGACTA**	847689	847703
NGO1282	NGO1282	**TAATTATAAGGCAAC**	1234710	1234724
NGO1362	tdf_{trunc}	**GTTTCGTAAAGTAAT**	1320961	1320975
NGO1552	*putP*	**GATTTGTACGGCAAA**	1528996	1529010
NGO1582	NGO1582	**TAATTGTAATGGGTA**	1557645	1557659
NGO1588	NGO1588	**TTATTGTAAAGGAGA**	1562803	1562817
NGO2008	*thiO*	**TTTTTTTAAAGAAAA**	1979937	1979951
NGO2073	*tnaB*	**GGTTTGTAAGGTATT**	2053095	2053109
NGO2126	*rpmE*	**TTTTTGAAAGGAAAT**	2108940	2108954

[a]http://prodoric.tu-bs.de/

Input parameters:

Strain ATCC 700825= *N. gonorrhoeae* FA 1090
(NCBI reference sequence: NC_002946)

Single pattern
IUPAC code: KWWWTGTAARGNNWH
Mismatch tolerance=1
Maximum distance to gene=350 bp
Ignored match orientation and removed palindromic matches

[b]MisR binding site nucleotides that match the consensus sequence reported in (3) are bolded; mismatches are underlined. K=G/T, W=A/T, R=A/G, N=any nucleotide, H=A/T/C

Appendix Table 3
Minimum inhibitory concentrations (MICs)

A

Strain	PMB MIC (ug/mL)
FA19 WT	100.00
FA19 *misR::kan*	12.50
FA19 *misR::kan*, *misR*+ (pGCC4)	200.00
FA19 *lptA::spc*[a]	0.39

[a]*lptA* is required for resistance to polymyxin B (4, 5)

B

	PMB MIC (ug/mL) (1 mM IPTG broth pregrowth /1 mM IPTG MIC plate)			
Strain	-/-	-/+	+/-	+/+
FA19 *dsbD*+ (pGCC4)	50.00	50.00	50.00	50.00
FA19 *misR::kan*	12.50	12.50	12.50	12.50
FA19 *misR::kan*, *misR*+ (pGCC4)	100.00	100.00	100.00	100.00
FA19 *misR::kan*, *dsbD*+ (pGCC4) clone #1	6.25	3.13	6.25	3.13
FA19 *misR::kan*, *dsbD*+ (pGCC4) clone #2	6.25	3.13	6.25	3.13

C

	MIC (ug/mL)						
Strain	**PMB**	**Gent**	**Strep**	**Tob**	**Spec**	**Kan**	**Cipro**
FA19 WT	100.00	10.00	12.50	10.00	25.00	30	0.0025
FA19 *misR::kan*	12.50	1.25	3.13	1.25	12.50	480	0.0025
JF1	200.00	10.00	12.50	10.00	25.00	15	0.0025
JF1 *misR::kan*	25.00	1.25	3.13	1.25	12.50	960	0.0025
KH15	200.00	10.00	25.00	10.00	25.00	30	0.0025
KH15 *misR::kan*	50.00	1.25	3.13	1.25	6.25	480	0.0025
FA19 *mtrD::kan* (KH14)	25.00	5.00	12.50	5.00	12.50	>100	0.0025
FA19 *lptA::spc*[a]	0.39	5.00	>50.00	5.00	100.00	25	0.0025
FA19 *misR::kan*, *misR*+ (pGCC4)	200.00	10.00	25.00	10.00	25.00	>480	0.0025

[a]FA19 *lptA::spc* is resistant to both spectinomycin and streptomycin due to the *aadA* gene encoded in the Ω cassette that interrupts *lptA* (6)

Appendix Table 3 (continued)

D

MIC (ug/mL)

Strain	Azi	CRO	CV	Ery	Pen G	Rif	SDC	TX-100
FA19 WT	0.128	0.00060	0.625	0.250	0.016	0.064	125	125.0
FA19 *misR::kan*	0.064	0.00015	0.313	0.125	0.008	0.032	62.5	125.0
JF1	0.256	0.00060	1.250	0.500 - 1.000	0.016	0.128	125	250.0
JF1 *misR::kan*	0.256	0.00030	0.625	0.500	0.008	0.128	125	125.0
KH15	0.512	0.00060	2.500	1.000 - 2.000	0.032	0.256	250	8000.0
KH15 *misR::kan*	0.256	0.00030	0.625	0.500	0.008	0.128	125	250.0
FA19 *mtrD::kan* (KH14)	0.032	0.00120	0.078	0.031	0.032	0.008	15.6	15.6
FA19 *lptA::spc*	0.128	0.00060	0.625	0.250	0.032	N/A	125	125.0
FA19 *misR::kan*, *misR*$^+$ (pGCC4)[a]	>0.512	0.00060	0.625	4.000	0.016	N/A	125	125.0

[a]FA19 *misR::kan*, *misR*$^+$ (pGCC4) is resistant to erythromycin (and likely azithromycin) due to the *ermC* gene encoded within the pGCC4 construct that integrates into the gonococcal chromosome between *aspC* and *lctP* (7)

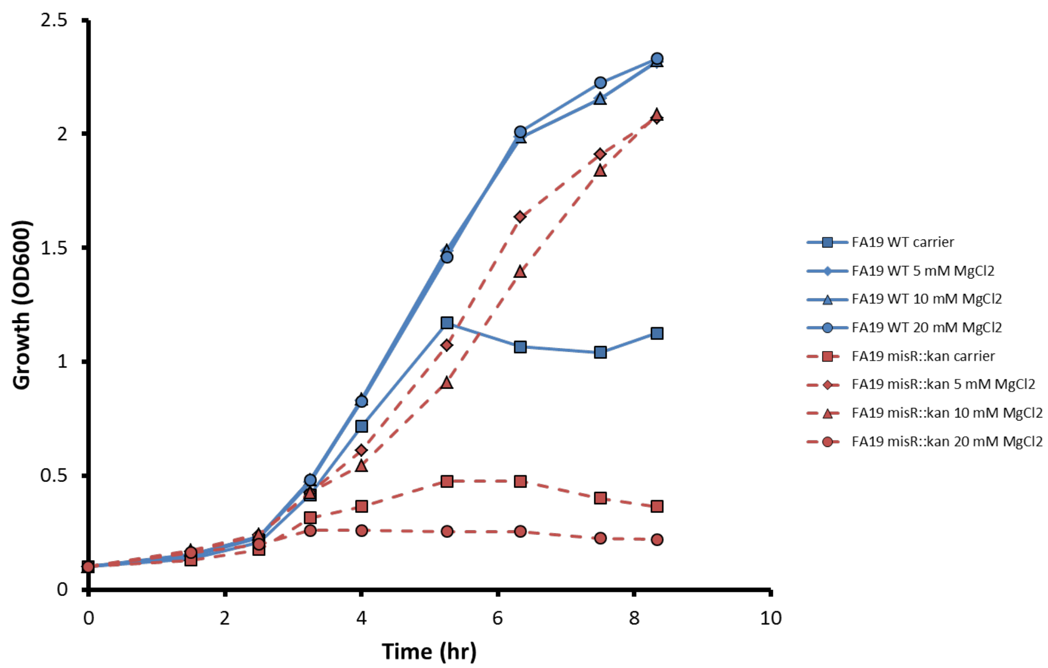
E

MIC (ug/mL)

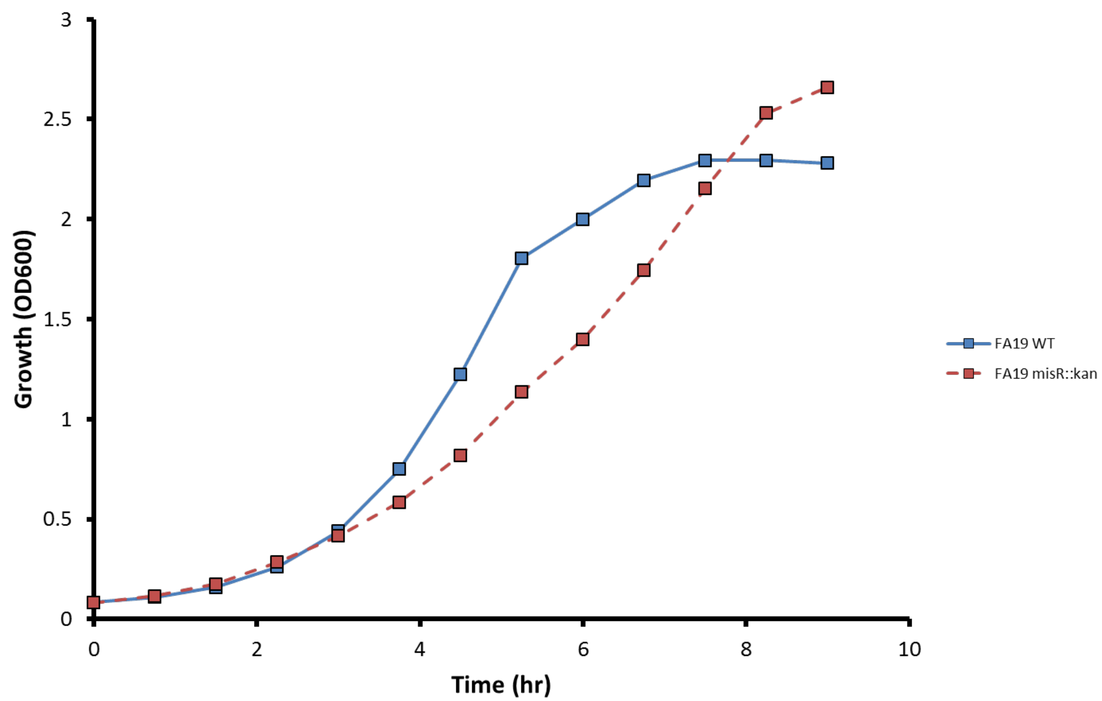
Strain	PMB	Gent	Strep	Tob	Ery	TX-100	Cipro
FA19 WT	100.00	10.00	12.50	10.00	0.250	125.0	0.0025
FA19 *misR::kan*	12.50	1.25	3.13	1.25	0.125	125.0	0.0025
FA19 *misR::kan*, *misR*$^+$ (pGCC4)	100.00	10.00	25.00	20.00	>4.000	125.0	0.0025
FA19 *misR::kan*, supp. 1	50.00	1.25	3.13	1.25	0.250	125.0	0.0025
FA19 *misR::kan*, supp. 2	50.00	N/A	3.13	N/A	0.063	62.5	0.0025
FA19 *misR::kan*, supp. 3	50.00	2.50	6.25	2.50	0.125	62.5	0.0025

Appendix Figure 1

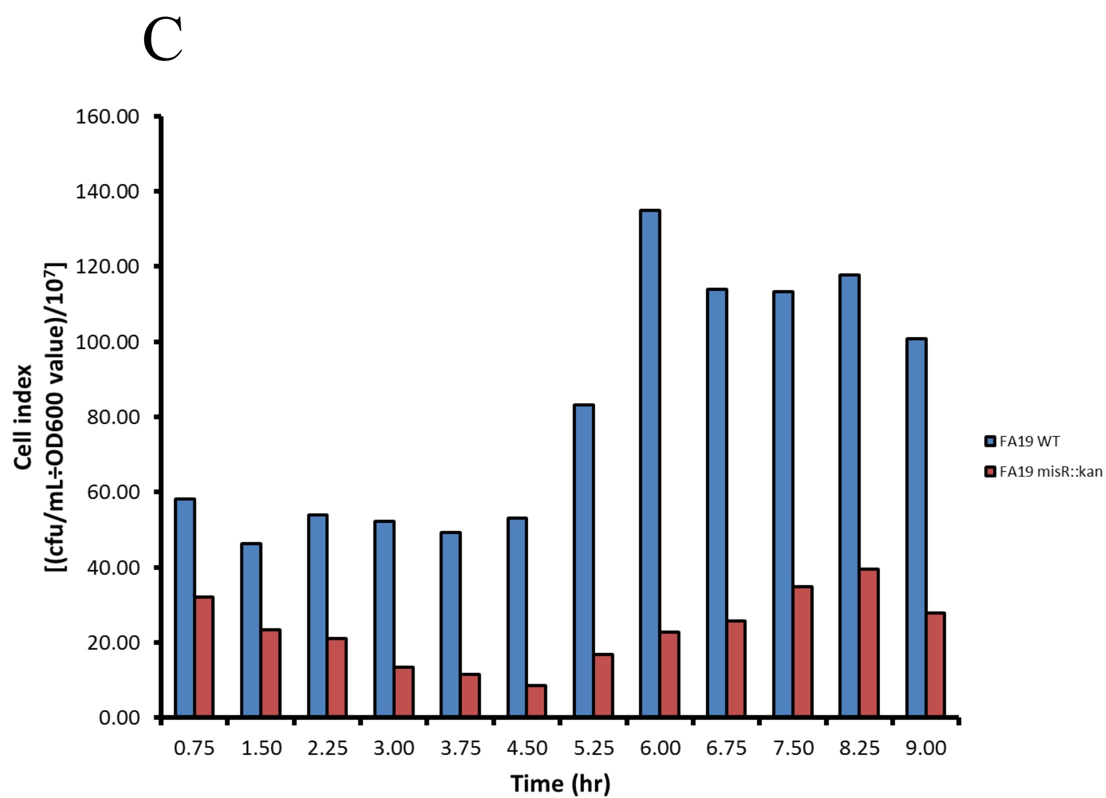
A



B

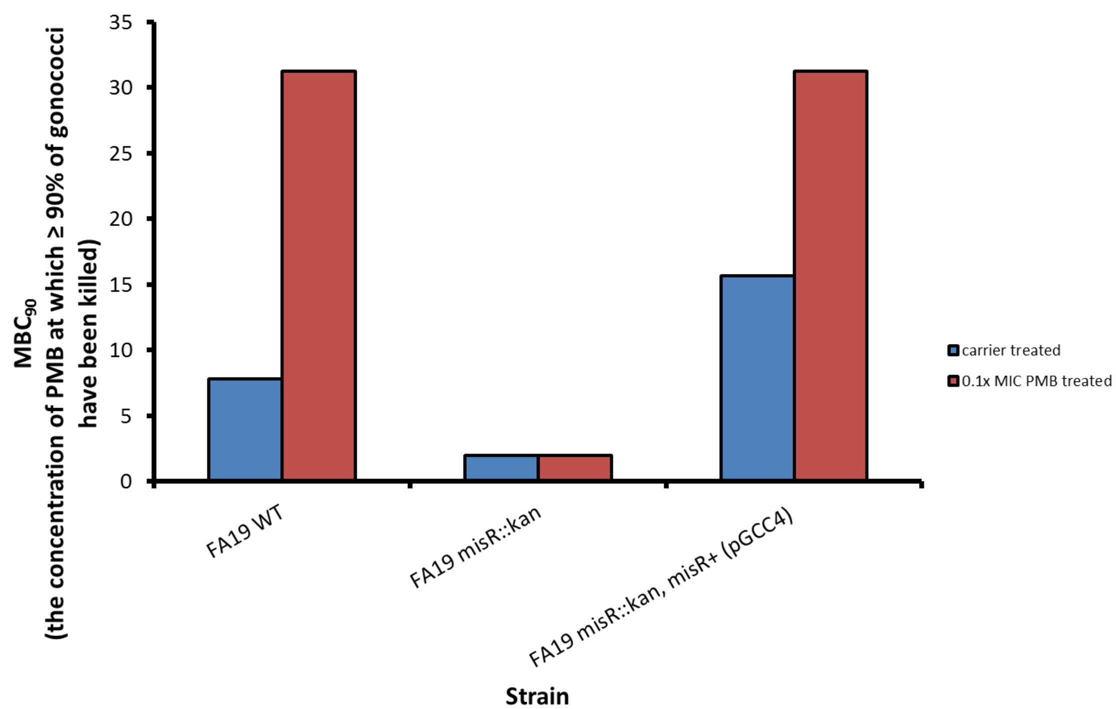


Appendix Figure 1 (continued)



Appendix Figure 1: Loss of MisR results in a severe growth defect in broth which may be partially alleviated by supplementation with $MgCl_2$. Panel A) Gonococci were grown in supplemented GC broth containing Kellogg's supplement I and iron, 0.043% (w/v) sodium bicarbonate, and various amounts of $MgCl_2$ in a 37°C water bath shaking at 200 rpm. Growth was monitored by OD_{600} measurement of cell density. **Panel B)** Gonococci grown as in panel A (only with 10 mM $MgCl_2$) were monitored by OD_{600} measurement of cell density. Serial dilutions were taken to monitor viable cfu/mL at several time points, and the ratio of viable cfu/mL:OD_{600} was calculated as described in (8)**(panel C)**. **Panels A and B** are representative of many experiments that show the same trend. Cell indices in **panel C** were calculated from a single experiment.

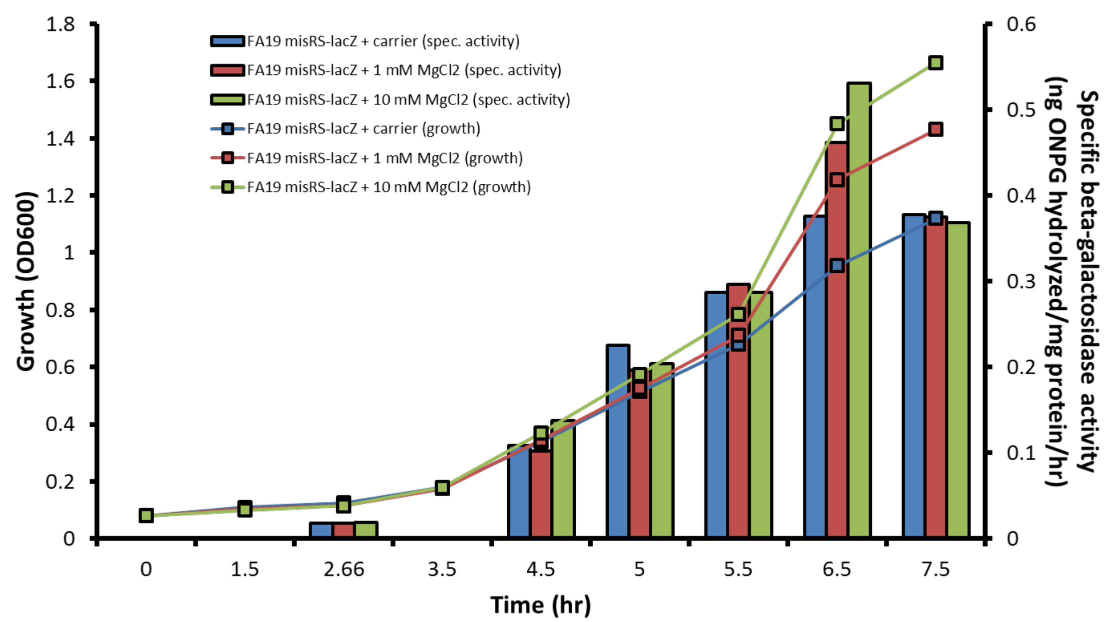
Appendix Figure 2



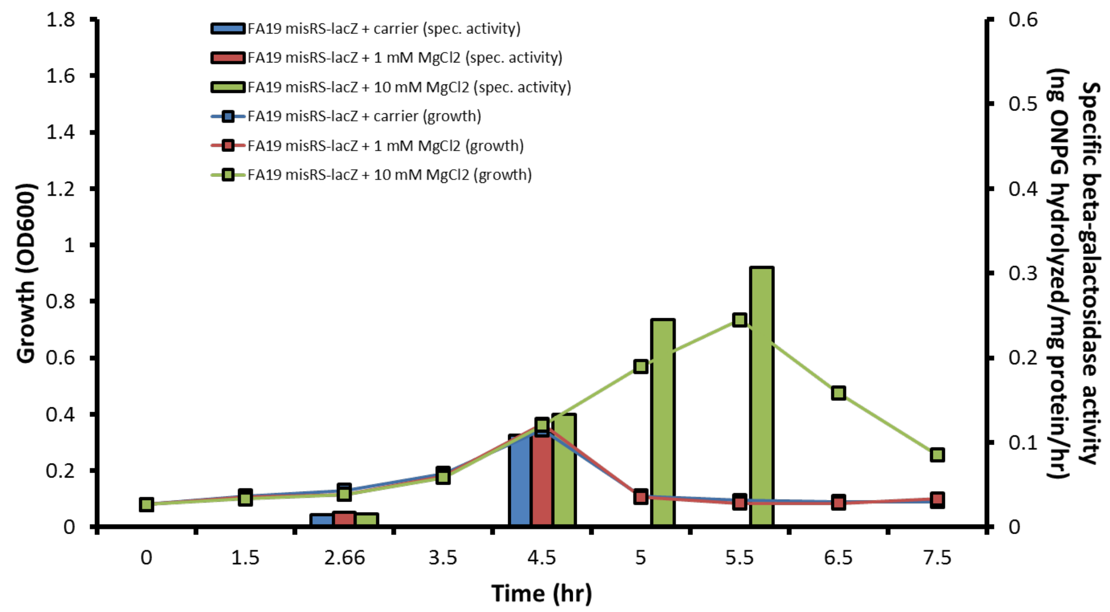
Appendix Figure 2: MisR is required for constitutive and inducible resistance to PMB. Shown is the modal MBC_{90} value of three independent experiments performed in triplicate, which was determined by the 2-fold broth dilution method (9). Broth cultures were split at early log and treated with either carrier (sterile ddH_2O) or a sublethal level (0.1x the strain's plate MIC) of PMB. Treated cultures were induced for 3 hours, normalized to an OD_{600} of 0.4 in unsupplemented GC broth, further diluted 1:100 in 0.2x unsupplemented GC broth (diluted using sterile ddH_2O), and tested for resistance to PMB challenge in sterile polypropylene microtiter plates for 45 minutes in a 4.3% CO_2, 37°C incubator. All cultures were grown in the presence of 10 mM $MgCl_2$ and 1 mM IPTG.

Appendix Figure 3

A

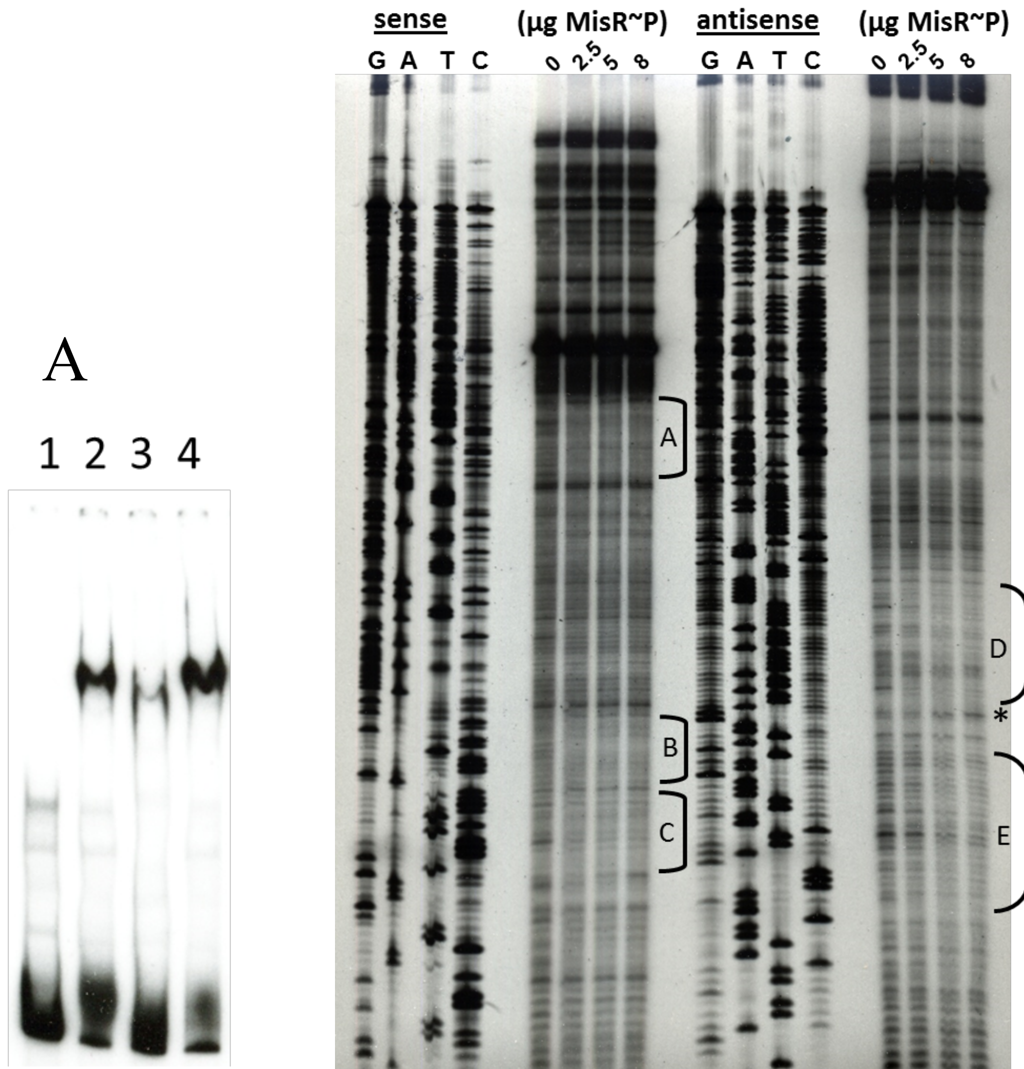


B

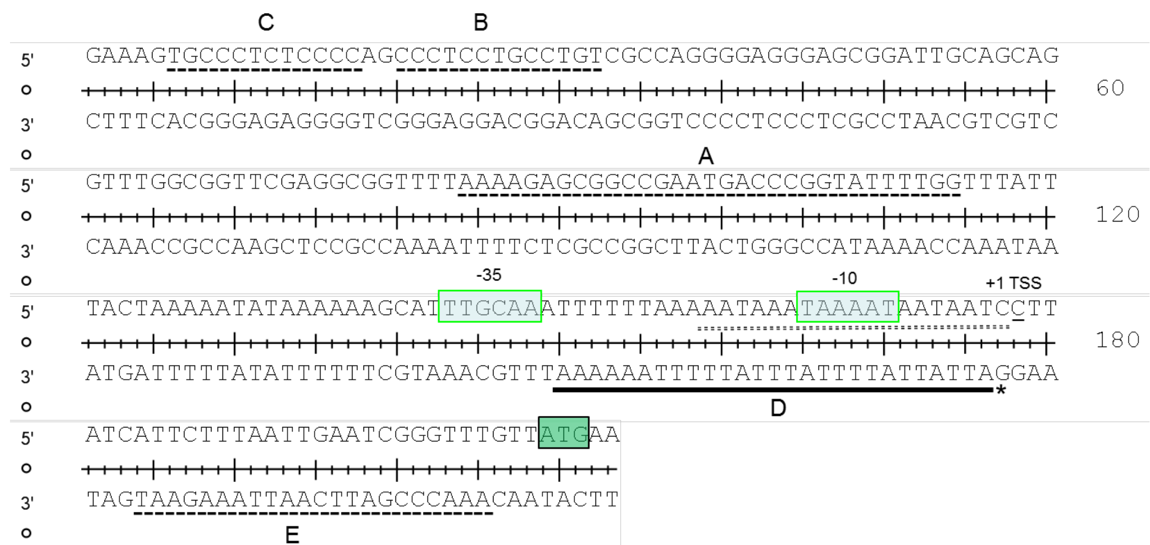


Appendix Figure 3: MisR/MisS does not respond to Mg^{2+} concentration. Gonococci encoding the *misRS-lacZ* translational fusion [constructed using primers 5'misRS-lacZ and 3'misRS-lacZ and the pLES94 vector; see Appendix Table 1A and (10)] were grown as described in Appendix Figure 1 in supplemented GC broth containing various amounts of $MgCl_2$. At T=4.5 hours, cultures were split; one subculture received the divalent cation chelator EDTA (5 mM final concentration) while the other subculture received an equal volume of carrier (ddH_2O). Beta-galactosidase assays were performed as previously described [(11); specific activity was calculated as per the manufacturer's instructions (Invitrogen cat# K1455-01)]. **Panel A)** Cells treated with carrier at T=4.5 hours. **Panel B)** Cells treated with 5 mM EDTA at T=4.5 hours. Note that beta-galactosidase activity is not available for many of the samples after the EDTA addition due to cell lysis. Shown are the results of a single experiment (beta-galactosidase activity was measured in triplicate for all cultures).

Appendix Figure 4

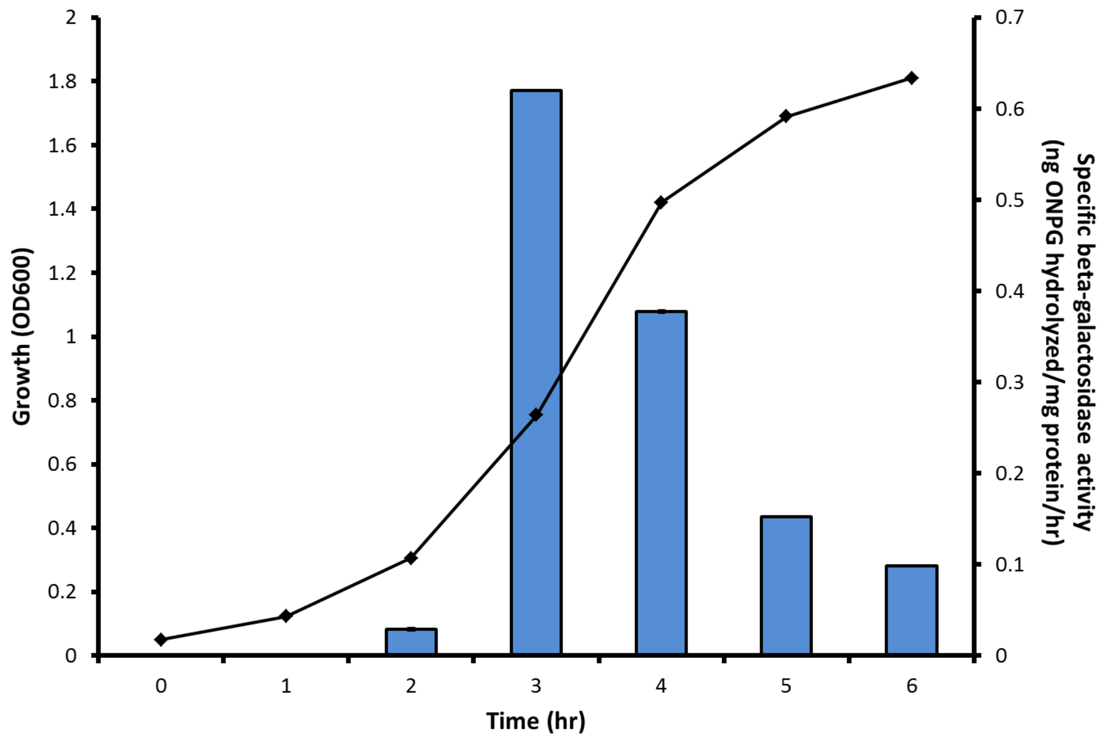


Appendix Figure 4 (continued)



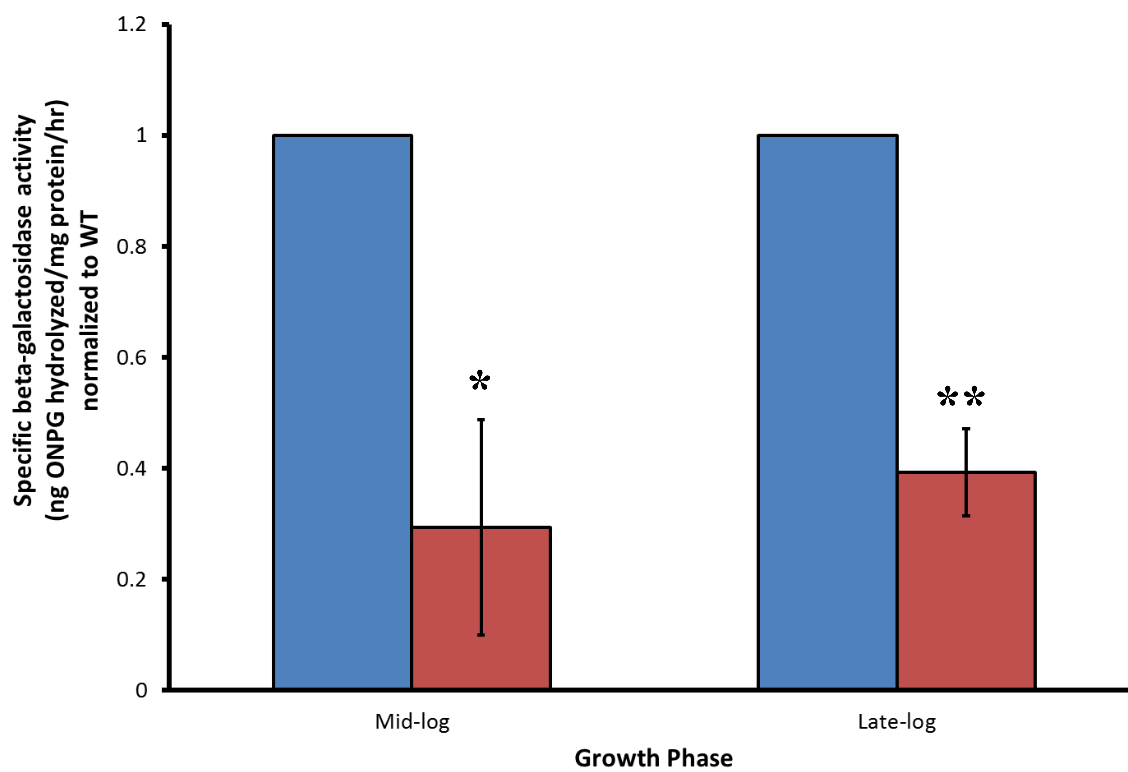
Appendix Figure 4: MisR~P specifically binds to the *tbpBA* promoter at the -10 element. Primers tbpBA_1090F and tbpBA_1090R (Appendix Table 1A) and chromosomal DNA were used to amplify a 297 bp product corresponding to the promoter region (-221 to +75) of the *tbpBA* operon from *N. gonorrhoeae* strain FA 1090 A23a. Phosphorylation of MisR-His$_{6x}$ by acetyl phosphate, electrophoretic mobility shift assays (EMSA), and DNaseI footprinting were performed as described previously (12). **Panel A)** Lane 1: 2 ng hot probe alone; lane 2: hot probe + 2.5 µg MisR~P; lane 3: hot probe + 1 µg cold specific competitor (cold probe) + 2.5 µg MisR~P; lane 4: hot probe + 1 µg cold nonspecific competitor (*rnpB*—amplified using primers RnpB1F and RnpB1R) + 2.5 µg MisR~P. Note that the addition of cold specific competitor (cold probe), but not cold nonspecific competitor (*rnpB*) greatly reduces the amount of MisR~P shifted hot probe. **Panel B)** DNase I footprinting using EMSA reactions described in panel A. Areas of protection are shown by black brackets. A hypersensitivity site is shown by an asterisk. **Panel C)** Sequence corresponding to the footprint in panel B. MisR~P protected sites are indicated on the appropriate strand by dashed lines (weak binding) and a black bar (strong binding). The Fur binding site (13) is shown as a double dashed line. Promoter elements are indicated. EMSA and footprinting was performed with excellent technical assistance from Virginia A. Stringer.

Appendix Figure 5



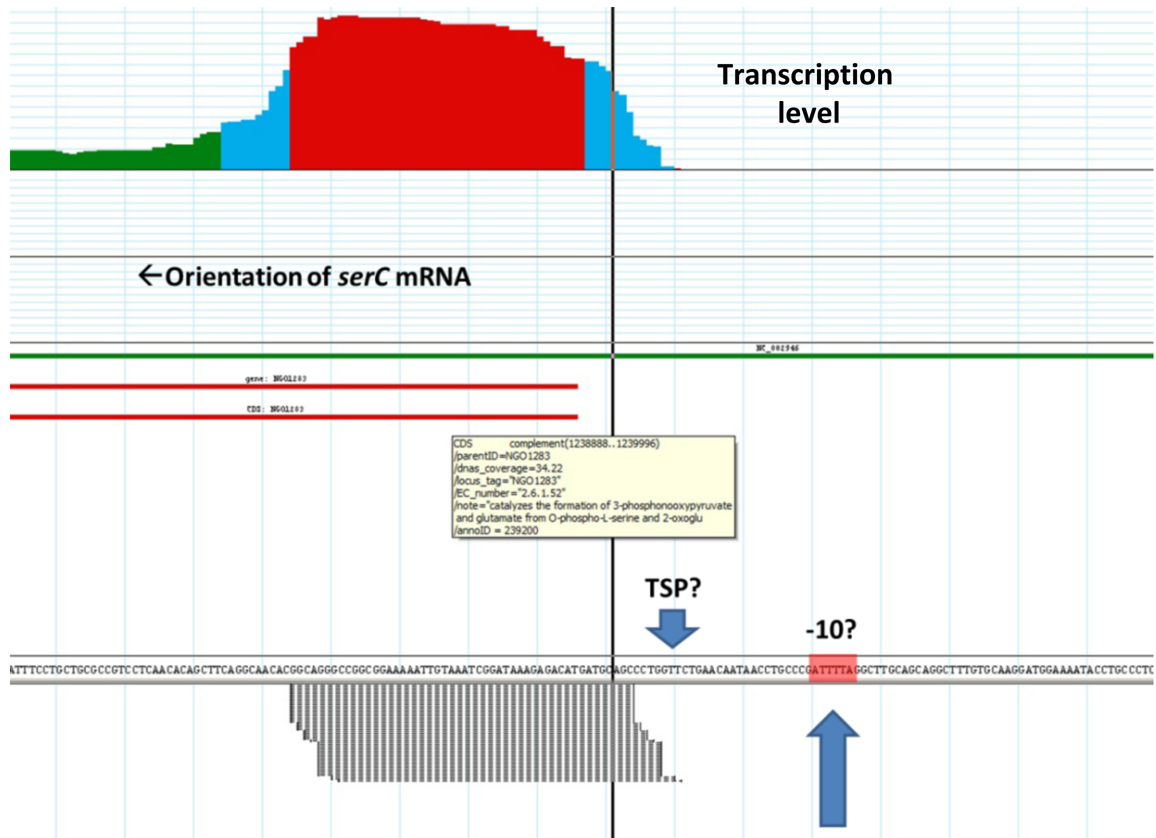
Appendix Figure 5: The internal promoter of the *serC-lptA-nfnB* operon proximal to *lptA* is functional. A 323 bp sequence encompassing the proximal promoter of *lptA* was amplified using primers 5'lptA-Z and 3'lptA-Z (Appendix Table 1A) and used to generate a translational fusion of *lptA* to a truncated, promoterless *lacZ* gene in pLES94 (10). Beta-galactosidase assays were performed as described in Appendix Figure 3 on gonococci grown as described in Appendix Figure 1. Shown is a representative example of three independent experiments measured in triplicate. Error bars represent standard deviation from the mean of three replicate beta-galactosidase reactions.

Appendix Figure 6



Appendix Figure 6: Loss of *misR* results in decreased expression from *lptA-lacZ*. Strains FA19 *lptA-lacZ* (blue) and FA19 *misR::kan*, *lptA-lacZ* (red) were grown in broth and assayed for specific beta-galactosidase activity as described in Appendix Figure 3. The fold difference from the WT specific beta-galactosidase activity is shown. These data are from three independent experiments measured in triplicate. Error bars represent standard deviation from the mean. Statistical significance was analyzed by Student's t-test (*$p \leq 0.01$, **$p \leq 0.001$).

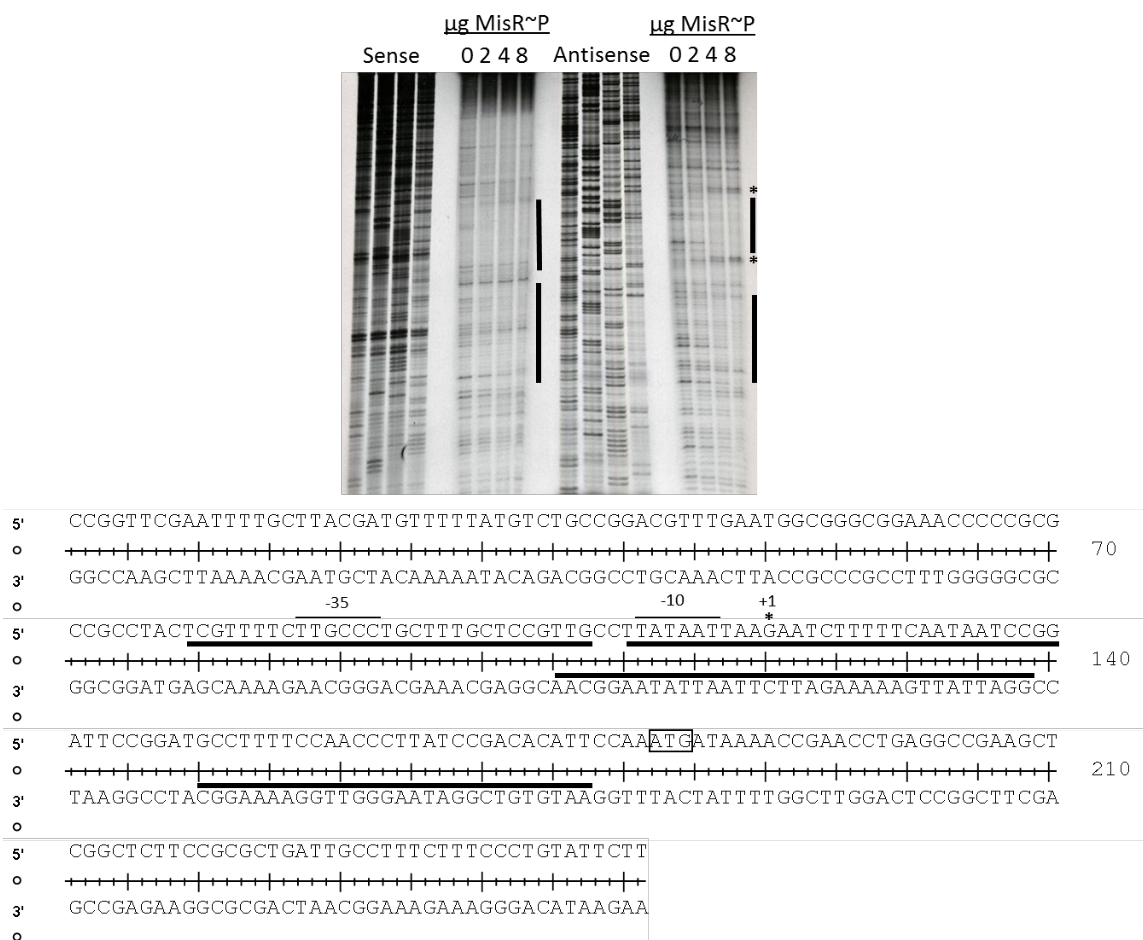
Appendix Figure 7



Appendix Figure 7: Putative location of the *serC* promoter as determined by RNA-Seq analysis. Sequencing reads from the MisR RNA-Seq experiment [see Methods in (13)] were visualized using SeqMan Pro software (DNASTAR) with the expert help of Kim M. Gernert (Emory University BimCore). The transcription level (top) shows the number of sequencing reads at each base pair (green, transcribed areas that do not exceed 100 sequencing reads; red, transcribed areas that exceed 100 sequencing reads at the corresponding sequence; blue indicates that the nucleotides were sequenced on one strand only). The sense sequence of the proposed *serC* promoter is:

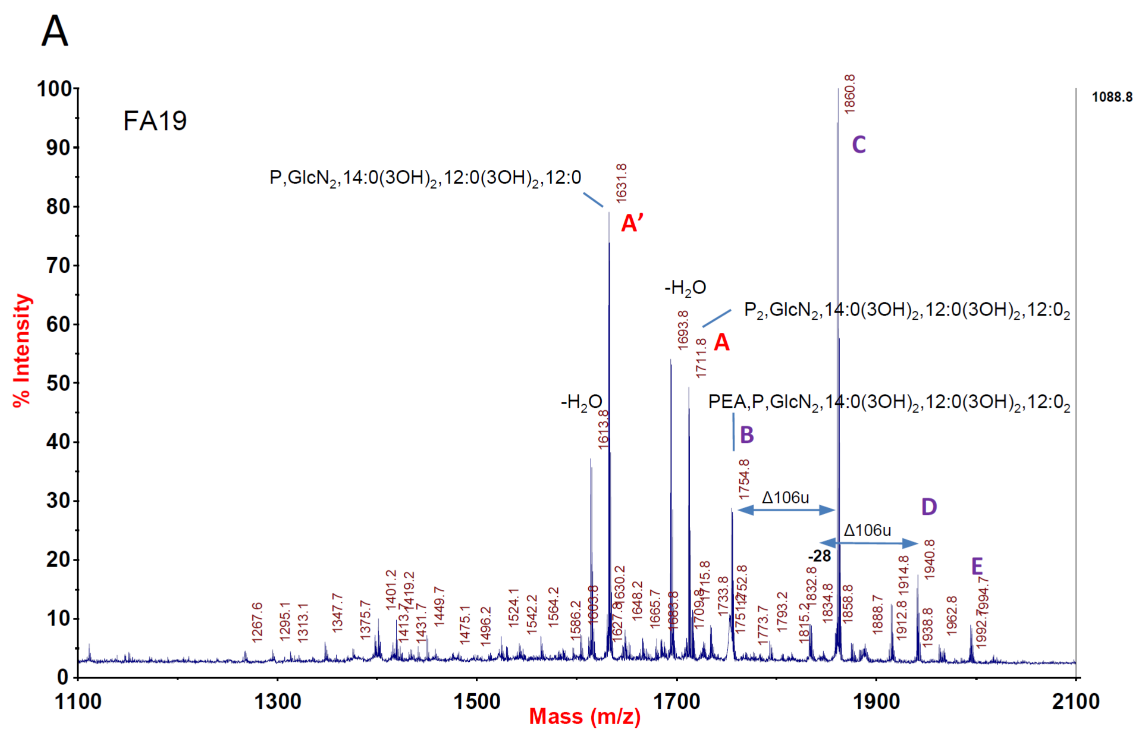
-35 -10 (start codon)
TTGCACAAAGCCTGCTGCAAGCCTAAAAT…34 nt…**ATG**

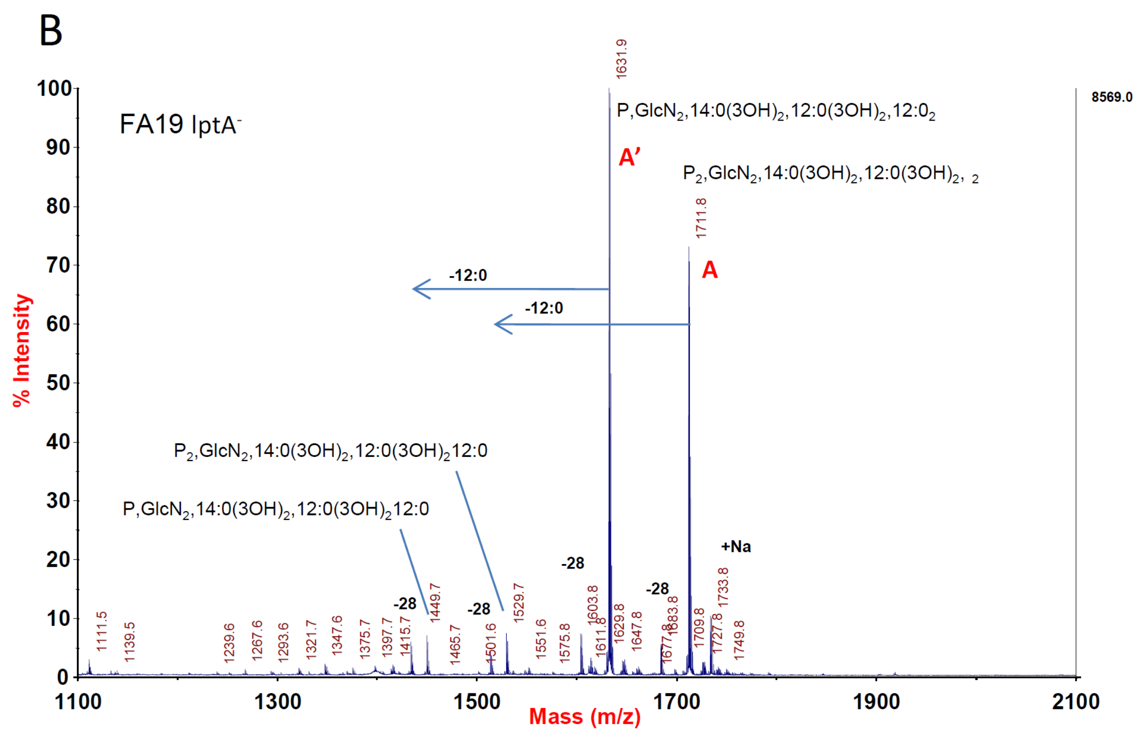
Appendix Figure 8



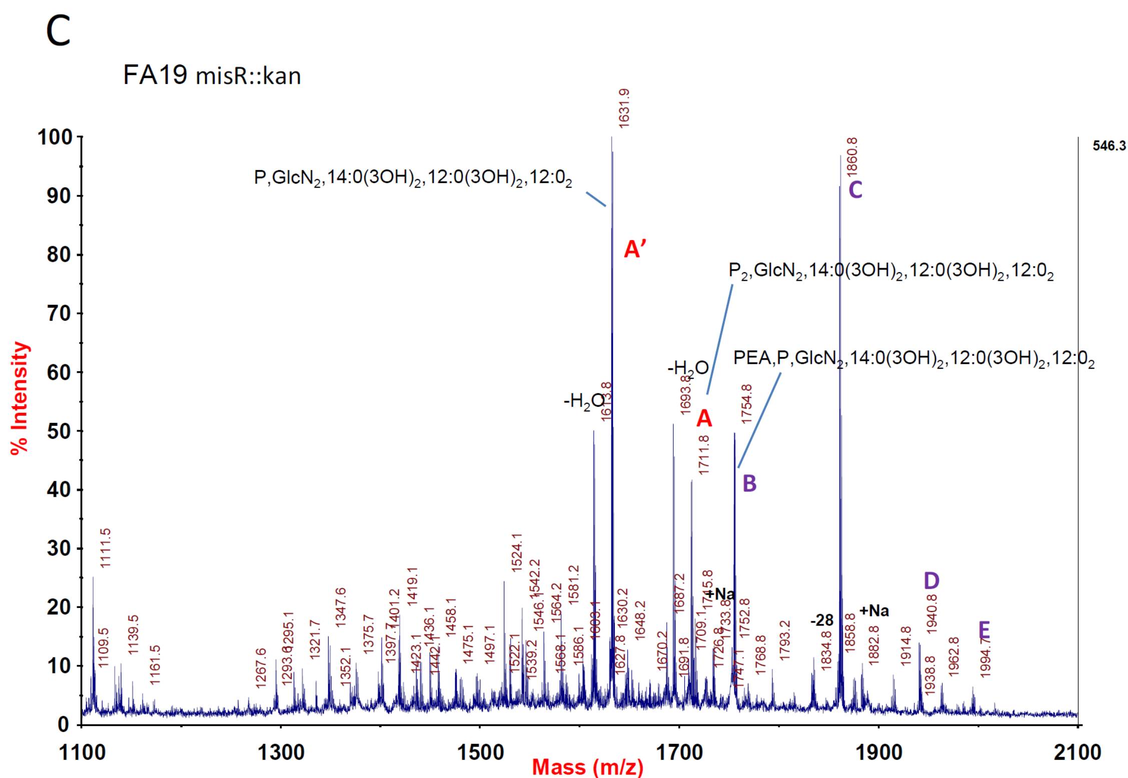
Appendix Figure 8: MisR~P occludes the *lptA* proximal promoter and 5' UTR as determined by DNaseI footprinting. Phosphorylation of MisR-His$_{6x}$ by acetyl phosphate and footprinting assays were performed as described previously (12). Primers LptA11 and LptA7_R (Appendix Table 1A) were used to generate the radioactive PCR product. (Top) regions of protection from DNase I cleavage are shown by black bars. Asterisks denote hypersensitive sites. (Bottom) shown below is the sequence of the PCR product used in the footprinting assay. Black bars indicate nucleotides protected by MisR~P binding on each strand. Also shown are the *lptA* proximal promoter elements described in (14). Footprinting was performed with excellent technical assistance from Virginia A. Stringer.

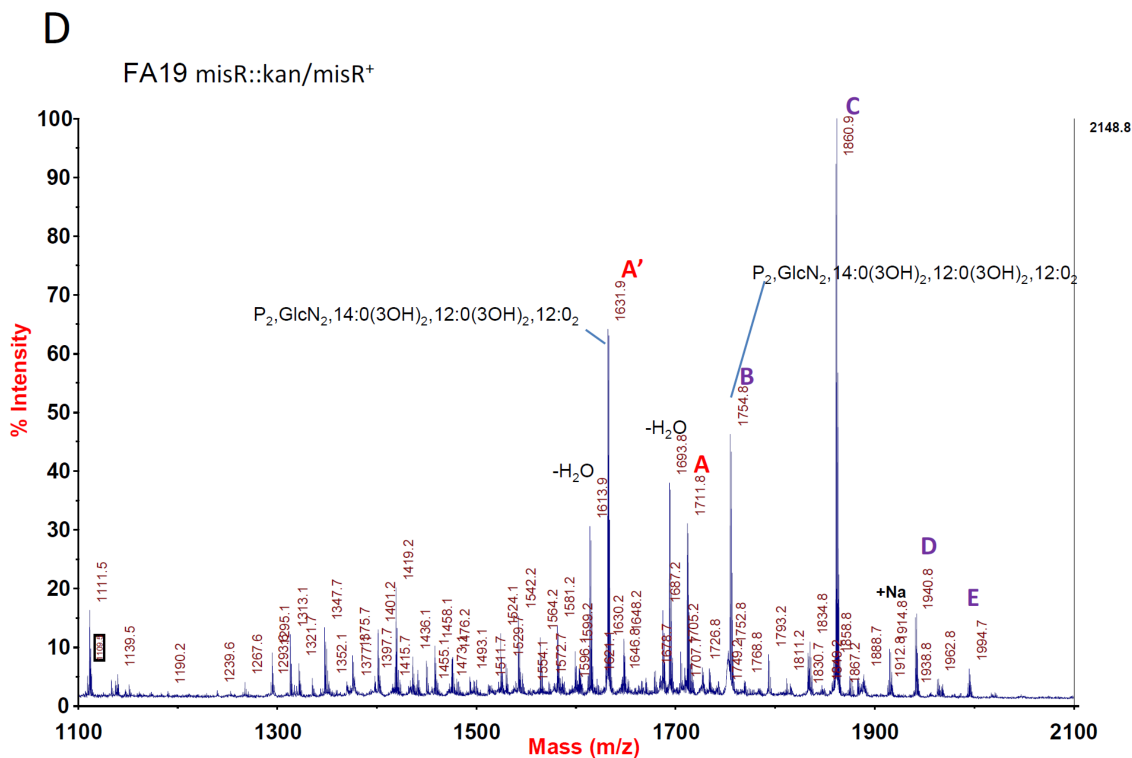
Appendix Figure 9





Appendix Figure 9 (continued)

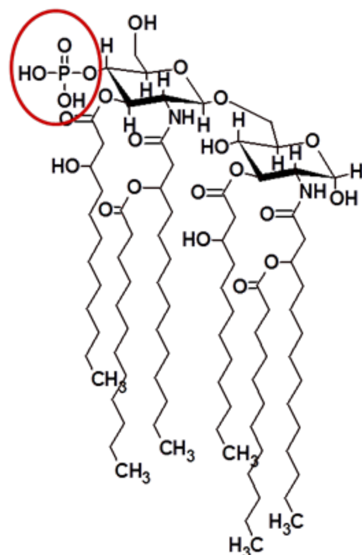




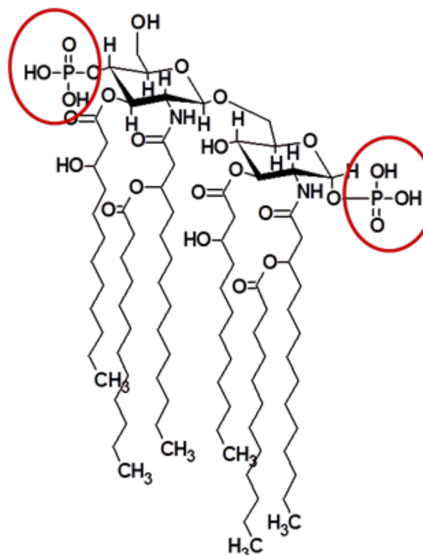
Appendix Figure 9 (continued)

E

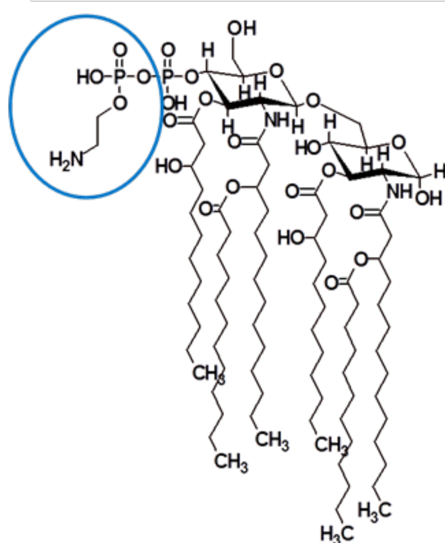
Species A' (1631.8 Da)



Species A (1711.8 Da)

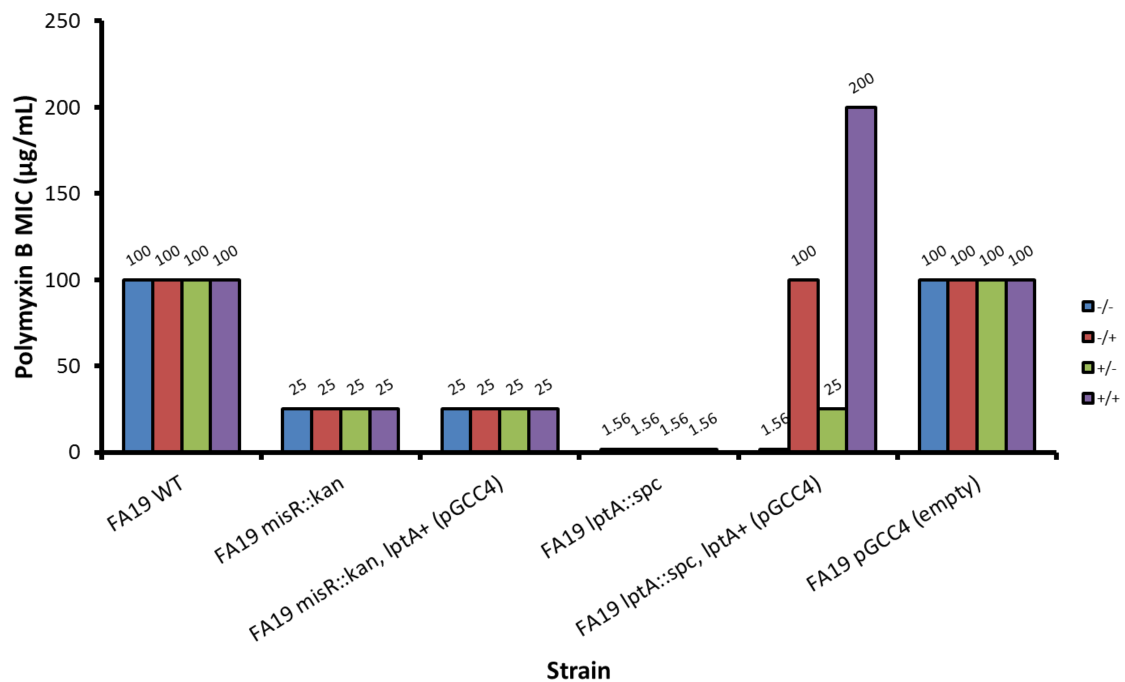


Species B (1754.8 Da)



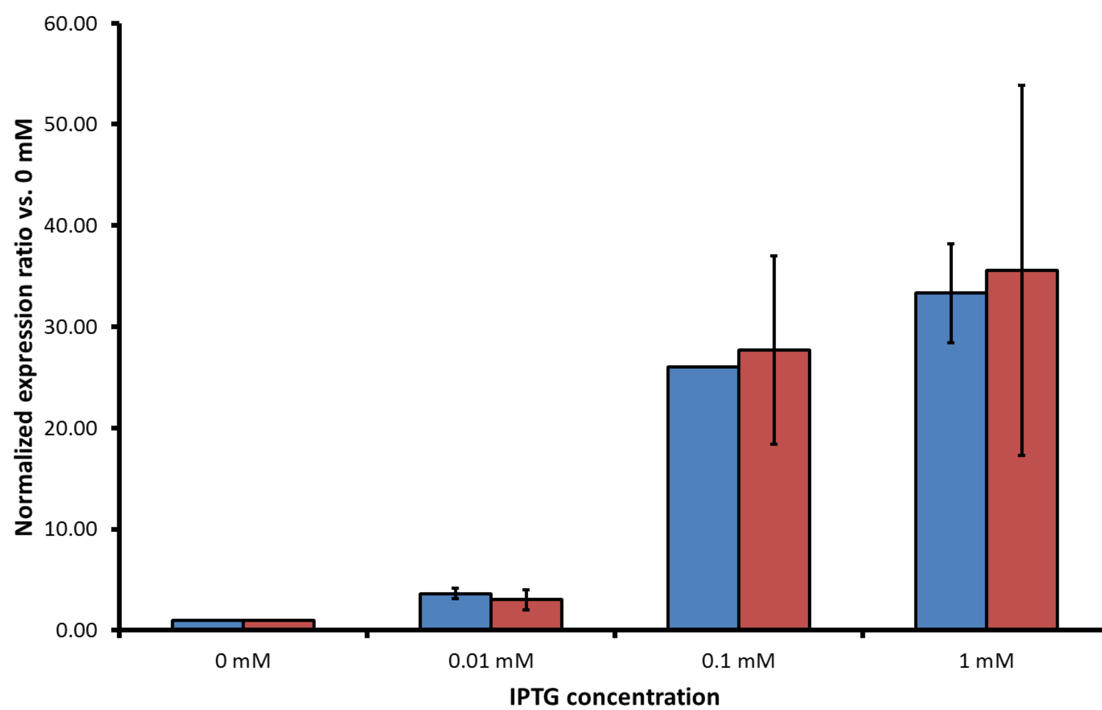
Appendix Figure 9: Loss of MisR does not impact decoration of gonococcal lipid A with the positively-charged small molecule, phosphoethanolamine (PEA). Overnight cultures of FA19 WT, FA19 *misR::kan*, FA19 *misR::kan*/*misR*$^+$ (pGCC4), and FA19 *lptA::spc* (~12 L per strain) were grown O/N at 37°C [in supplemented GC broth containing 1 mM IPTG (for induction of the complement allele of *misR*) and 10 mM $MgCl_2$], pelleted, washed three times in 1x PBS, formalin fixed, and sent to the University of Georgia Complex Carbohydrate Research Center in Athens, GA for lipid A purification and mass spectrometry analysis (lipid A purification and MALDI-MS analysis was performed by Artur Muszyński in the laboratory of Russell W. Carlson). All strains **(panels A, C, and D)** except for FA19 *lptA::spc* **(panel B)** produced the PEA-modified lipid A species B. Note that species C, D, and E (of unknown chemical structure) were also absent from the FA19 *lptA::spc* lipid A spectra, and may require LptA function as a prerequisite step in order to be produced. The chemical structures of species A, A', and B can be seen in **panel E**.

Appendix Figure 10



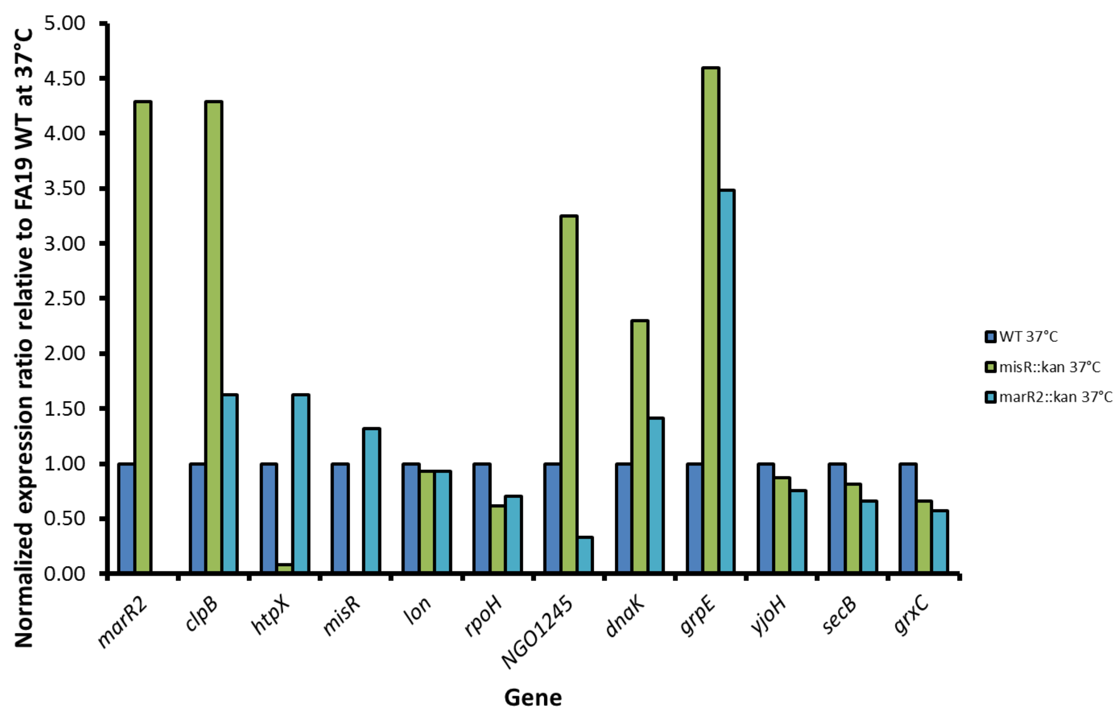
Appendix Figure 10: Overexpression of *lptA* at a second site does not complement the PMB-sensitive phenotype of FA19 *misR::kan* gonococci. Strains were tested for their resistance to PMB in the presence or absence of IPTG, which is an inducer of the *lptA* complement allele encoded at a second site in the chromosome between *lctP* and *aspC* (7). The legend shows whether or not IPTG was present (+) or absent (-) in the pregrowth media/MIC assay media, respectively. Note that the pGCC4-*lptA* construct (5) is highly responsive to IPTG induction [see MICs for strain FA19 *lptA::spc*, *lptA*$^{+}$ (pGCC4)]. Shown are the modal MIC values from three independent experiments.

Appendix Figure 11



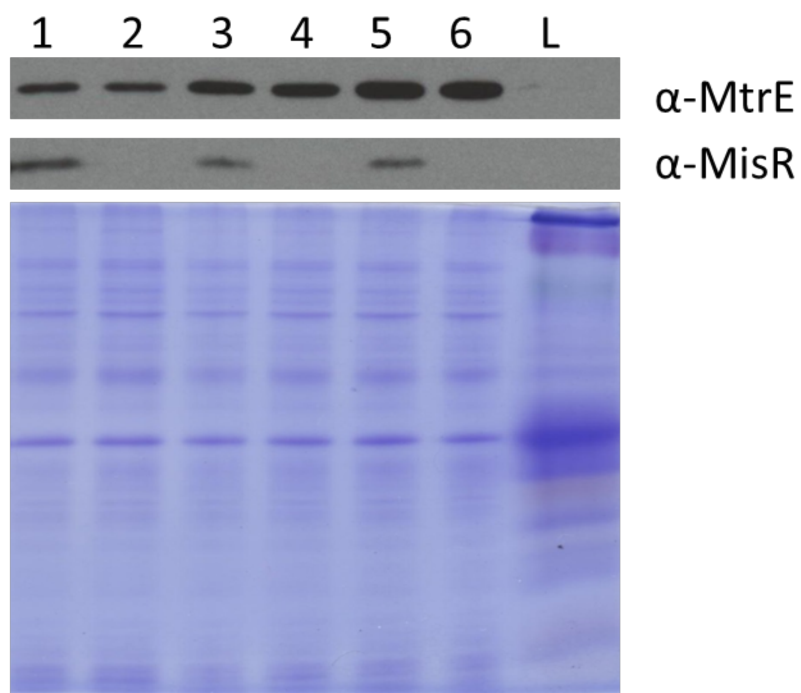
Appendix Figure 11: The pGCC4-*dsbD* construct allows IPTG-inducible production of *dsbD* transcript. *dsbD* was amplified from strain FA19 WT using primers dsbD_FPacI and dsbD_RPmeI (Appendix Table 1A) to generate an 1842 bp product including the *dsbD* ribosome binding site and complete open reading frame but excluding the *dsbD* promoter (15). This product was cloned into pGCC4 to generate pGCC4-*dsbD*. Two separate transformants [FA19 *misR::kan*, *dsbD*$^{+}$ (pGCC4) #1, blue; FA19 *misR::kan*, *dsbD*$^{+}$ (pGCC4) #2, red] were grown in supplemented GC broth containing 10 mM $MgCl_2$ and IPTG at 0, 0.01, 0.1, or 1 mM. Cells were harvested at mid-log, RNA purified by RNeasy (Qiagen) and Turbo DNA*free* (Ambion) treatment, and cDNA generated using a QuantiTect Reverse Transcriptase Kit (Qiagen). Levels of *dsbD* transcription were determined by qPCR using the cDNA as template and primer sets dsbD_qRT_F/dsbD_qRT_R (test) and 16Smai-RTF/16Smai-RTR (16S rRNA reference). Normalized expression was calculated by the $2^{-\Delta\Delta Ct}$ method (16). Shown are the results of two independent experiments (transformant #1 and transformant #2). Error bars represent standard deviation from the mean of duplicate measurements.

Appendix Figure 12



Appendix Figure 12: MisR and MarR2 impact expression of gonococcal stress response genes at 37°C. Broth grown cultures of FA19 WT (1st column), FA19 *misR::kan* (2nd column), and FA19 *marR2::kan* (3rd column) were grown as described for Appendix Figure 1. Cells were harvested at mid-log phase. Cell pellets were processed to purify RNA and qRT-PCR was performed and analyzed as described in Appendix Figure 11. See Appendix Table 1A for primers used during qPCR. Expression ratios are normalized to FA19 WT (value of "1" for all genes). Shown are the results of a single experiment.

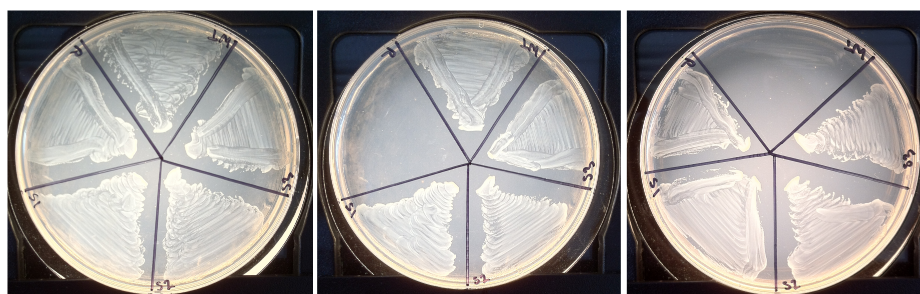
Appendix Figure 13



Appendix Figure 13: Loss of MisR does not impact levels of the MtrCDE outer membrane channel protein, MtrE. Lane 1:FA19 WT; lane 2: FA19 *misR::kan*; lane 3: JF1; lane 4: JF1 *misR::kan*; lane 5: KH15; lane 6: KH15 *misR::kan*. Mid-log phase gonococci from broth cultures grown as described in Appendix Figure 1 were harvested and whole cell lysates prepared in 1x Z buffer (0.06 M Na_2HPO_4, 0.04 M NaH_2PO_4, 0.01 M KCl, 0.001 M $MgSO_4 \cdot 6H_2O$) by freeze-thawing in a dry ice/ethanol bath. A 12% SDS-PAGE gel (with 5% stacking gel) was run in duplicate, with levels of protein normalized by Nanodrop prior to boiling for 10 minutes in 2x SDS loading dye. One gel was Coomassie stained to show that wells were loaded with an equivalent amount of protein. The other gel was transferred to a nitrocellulose membrane, which was blocked O/N at 4°C using 3% BSA (MtrE blot) or 4% non-fat dried milk (MisR blot) in 1x TST buffer [0.01 M Trizma base, 0.150 M NaCl, 0.05% Tween-20 (vol/vol)]. Blocked membranes were washed 3 times in 1x TST and probed with primary antibody O/N at 4°C for MtrE or MisR using 1:10,000 rabbit polyclonal antisera (antisera were generously provided by Ann Jerse and Yih-Ling Tzeng respectively) diluted in 1x TST. α-MtrE antisera was generated using amino acids 110-120 of MtrE [RQGSLSGGNVS; (17)]. α-MisR antisera was generated using purified MisR-His_{6x} protein as described in (12). Blots were washed 3 times in 1x TST and incubated with 1:2500 goat anti-rabbit IgG-HRP (horse radish peroxidase) conjugate secondary antibody (Thermo Scientific product# 32460) diluted in 1x TST for 1 hour at room temperature. Blots were given a final wash 3 times in 1x TST before development with a 1:1 luminol/peroxide solution (Thermo Scientific product# 32209). Bands were visualized by exposure of the membranes to film O/N.

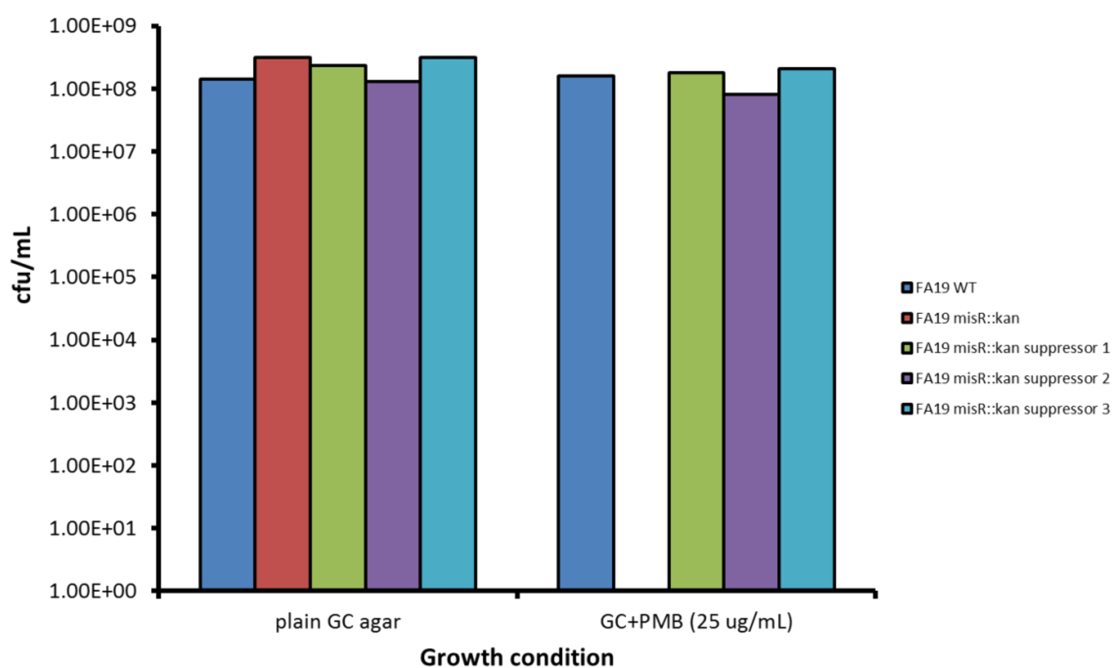
Appendix Figure 14

A



plain GC agar | **GC agar + PMB (25 ug/mL)** | **GC agar + Kan (50 ug/mL)**

B



Appendix Figure 14: Growth of FA19 *misR::kan* PMB-resistant suppressor mutants on non-selective vs. selective agar. Panel A) 48 hr growth of the following strains: WT=FA19 WT; R=FA19 *misR::kan*; S1, S2, S3=suppressor mutants 1-3. **Panel B)** Efficiency of plating assay showing the phenotypic change in the suppressor mutants vs. their parent strain, FA19 *misR::kan*. Strains were grown O/N on GC agar prior to serial dilution and plating for cfu/mL on plain GC agar or GC agar supplemented with PMB (25 ug/mL). The FA19 *misR::kan* parent strain did not grow on PMB-containing media at any dilution. In contrast, the FA19 WT and FA19 *misR::kan* PMB-resistant suppressor mutants all grew lawns at the 10^{-2} and 10^{-4} dilutions. Shown are the raw cfu/mL counts on plain GC agar and GC agar containing 25 ug/mL PMB when 100 uL of the 10^{-6} dilution were spread plated. These results are from a single experiment.

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