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NEISSERIA GONORRHOEAE REGULATORY NETWORKS
INVOLVED IN THE RESPONSE TO HOST CELL CONTACT

presented by

YING DU

has been accepted towards fulfillment
of the requirements for the

Ph.D. degree in Microbiology & Molecular Genetics

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July 19, 2006
Date

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NEISSERIA GONORRHOEAE REGULATORY NETWORKS INVOLVED IN THE RESPONSE TO HOST CELL CONTACT

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology and Molecular Genetics

2006

ABSTRACT

NEISSERIA GONORRHOEAE REGULATORY NETWORKS INVOLVED IN THE RESPONSE TO HOST CELL CONTACT

By

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Neisseria gonorrhoeae is a Gram-negative diplococcus, which causes gonorrhea in humans and poses a significant public health threat worldwide. The adhesion of gonococci to host cells is the critical first step of a gonococcal infection. However, our understanding of the interaction between this pathogen and the host is very limited.

In this dissertation, DNA microarrays, transposon mutagenesis, real time PCR and a cell culture model of infection are employed to study the regulatory networks of *N. gonorrhoeae* upon host cell contact. The underlying theme of this dissertation is the hypothesis that the initial attachment to the host would transmit signals into the bacterium, resulting the modulation of gene expression required for its subsequent survival and amplification in the host.

In this dissertation, we show that gonococcal adherence to host cells indeed results in changes of gene expression in the bacterium and this response is in part mediated by the transcriptional regulator, RpoH, whose induction is necessary for the subsequent invasion step. Furthermore, three of genes induced upon adherence were identified as RpoH-dependent, as depletion of RpoH from the bacteria abolished their induction. In particular, knocking out one of these genes, *NG0376*, encoding a putative

rotamase in *N. gonorrhoeae*, compromised their ability to invade human epithelial cells in culture. This result indicates that signaling is occurring across the bacterial membrane.

In addition, many of genes induced upon host contact are independent of RpoH regulation. One of those genes, *NG0340*, encoding cysteine synthetase, was found to be important in both adherence and invasion. Overall, the identification of RpoH-independent genes indicates a complex regulatory network of *N. gonorrhoeae* is involved in this response. Hence, characterization of this regulatory network will be essential to our understanding of gonococci-host interaction.

In addition to the characterization of the regulatory network in *N. gonorrhoeae*, this dissertation describes the identification of a cell division gene, *zipA*, whose gene product essentially requires the Signal Recognition Particle system for transport to the cytoplasmic membrane.

To my grandmother, Xiangzhen Hu

To my dear husband, Qianchuan He

ACKNOWLEDGMENTS

I would like to express my sincere thanks to my mentor, Dr. Cindy Arvidson. I wish to thank her for letting me work on this wonderful and challenging project. I feel very grateful and honored to be her student. Without her insightful guidance, continuous encouragement and infinite patience, I would not be able to accomplish this dissertation successfully. She was always there to share with me the difficulties or excitements of my research and I cherish each single moment of them. Her invaluable scientific contribution and experience has greatly inspired me to be an independent scientific researcher.

I am deeply grateful to my committee members, Dr. Michael Bagdasarian, Dr. Martha Mulks, Dr. Vincent Young, and Dr. Shengyang He for serving their precious time on my committee. I wish to thank them for their invaluable suggestions and comments on my research and help me to think through questions and think big.

I also owe lots of thanks to the current and former members of the Arvidson laboratory for their help and support. It has always been fun and pleasure to work with all of them in this wonderful laboratory. I wish to thank Roxie Mason and Cody Frasz for their warmhearted help in the early years of my research. I wish to thank Johnanson Lenz, Audrey Butcher and James Tumbrink for their excellent technical assistance in my research. I also wish to thank Kevin Smith, Jason Jens and Mary Fantacone for their thoughtful discussion of science and critical reading of my writings. I also wish to thank Dr. Dennis Arvidson for his advice and help in my research.

I would like to thank Dr. Robert Britton for his invaluable advice in the analysis of the array data and the use of the array scanner and software in his laboratory. I wish to

thank the Research Technology Support Facility at Michigan State University for their excellent technical assistances and services.

I would like to thank the department of Microbiology and Molecular Genetics, the BioSci program and the graduate school for their financial support. I also wish to thank my friends at Michigan State University for their help in many ways.

Finally, I wish to thank my husband and my family for their unwavering support and love throughout these years. I am deeply indebted to my parents. They raised me with endless love, patience and care and it is for this I am sincerely grateful. A special thanks goes to my dear sister, who is always my good listener and supporter. I also wish to express my sincere gratitude to my grandmother, who has always been my emotional support and from whom I have learned to be grateful and joyful in everyday life. Last, but not the least, I wish to thank my wonderful husband, Qianchuan He, for his deep love, care, understanding and support. Without him by my side in this long journey, I would NOT be WHO I AM today.

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Neisseria gonorrhoeae: the causative agent of gonorrhea

Neisseria gonorrhoeae (GC, gonococcus) is a Gram-negative diplococcus, causing one of the oldest documented venereal diseases, gonorrhea. Although descriptions of this disease had appeared in writings years earlier, it was the Greek physician, Galen, who first named it gonorrhea (latin for "flow of seeds"). In 1879, *N. gonorrhoeae* was first described as the causative agent of gonorrhea by a German physician, Albert Neisser (183). The genus *Neisseria* includes 12 species and biovars (193) and only two species, *N. gonorrhoeae* and *N. meningitidis,* are important human pathogens. The latter is significant as a causative agent of septicemia and meningitis in humans.

N. gonorrhoeae is an obligate human pathogen and only transmitted by intimate contacts with an infected person. This pathogen usually colonizes the mucous membrane of the urethra in males and the endocervix in females. However it can also colonize the mucous membranes of the eyes, throat, and rectum. Symptoms of gonoccocal infections differ by gender and the site of infection. Men are more likely to develop acute symptoms that are typically seen as purulent discharges and dysuria; while many infected women remain asymptomatic. The acute symptoms of endocervical infection are vaginal discharge and dysuria. Infections in the rectum and pharynx are rarely symptomatic. In women, gonoccoci may ascend into the fallopian tubes resulting in salpingitis, which may lead to the development of pelvic inflammatory disease (PID), a major problem that can lead to infertility or ectopic pregnancy (253). During vaginal birth, newborns can get gonococcal infections of the conjunctiva from an infected mother and this can lead to blindness (203, 307). Although most of the time gonococci cause localized inflammation,

the bacteria can occasionally spread through the blood stream to other organs, causing disseminated gonococcal infections (DGI), which can result in purulent arthritis, endocarditis and meningitis (347, 417).

Accurate detection of *N. gonorrhoeae* in patients is key to treatment and requires highly sensitive and specific diagnostic methods. Traditionally, culturing *N. gonorrhoeae* on selective media has been the gold standard, because of its high sensitivity and specificity (193). Moreover this culture-based method also allows for the subsequent determination of antibiotic susceptibilities. This is essential in treatment, considering that *N. gonorrhoeae* rapidly develops resistance to antibiotics (235, 337, 389). In order to distinguish *N. gonorrhoeae* from other related *Neisseria* spp, biochemical tests are used to test their ability to ferment carbohydrates, reduce nitrate, or produce polysaccharide from sucrose, *etc.* (192-194, 365). Nonculture-based diagnostic techniques have recently been developed to improve the accuracy of detection, such as the antibody-based enzyme immunoassays (EIA), the hybridization-based nucleic acid hybridization test and the DNA/RNA-based nucleic acid amplification test (NAAT) (196).

Epidemiology of *N. gonorrhoeae:* prevention and control

According to the World Health Organization (WHO), more than 62 million cases of gonorrhea occur each year, posing a huge threat to public health worldwide (6). However, this number is likely underestimated, since the diagnosis and reporting of gonococcal infections are often incomplete due to asymptomatic carriage among patients. Upwards of sixty percent of infected women and twenty percent of infected men are asymptomatic carriers, composing a large reservoir of gonococci in the population (389).

There are more incidents of gonococcal infection in developing countries with South Asia, South-East Asia, Africa and South America reporting the highest rates every year (5).

In the United States, *N. gonorrhoeae* is a leading cause of bacterial sexually transmitted disease, second only to *Chlamydia trachomatis*. Although the incidence of disease has significantly declined since the 1980s, this trend has reversed since 1998 with more than 300,000 cases reported in the United States in 2003 (1). As gonorrhea can significantly enhance the transmission of the human immunodeficiency virus (HIV) (58, 202), the importance of gonorrhea as a human pathogen has increased along with the prevalence of HIV worldwide.

Due to the poor immunogenicity or the antigenic and phase diversities of its surface proteins, an effective vaccine against *N. gonorrhoeae* is still beyond reach (54, 150, 308). Today, antibiotics are the only effective choice for treating gonococcal infections. Historically, penicillins were the most widely used antibiotics to treat gonorrhea; however, penicillin-resistant gonococcal strains quickly appeared globally. In 1998, the proportion of penicillin-resistant gonococci reached more than 60% in South and South-East Asia (4, 405) and in some Asian countries, resistance to penicillin can be as high as 96.8% (316). Because of the high prevalence of the penicillin-resistant strains, penicillins are now only used where they are still effective (4). Tetracyclines and newer macrolides such as azithromycin are not recommended for treatment, due to the high spread of the resistant strains or side-effects (389). Spectinomycin and quinolones have been withdrawn in many parts of the world because of resistance or side-effects (4, 389, 405). The third generation cephalosporins (ceftriaxone and cefixime) are currently the

preferred choice of treatment for gonorrhea (4), but there has been a decline in susceptibility to these drugs in recent years (5).

At present, the most effective way to control the transmission of gonorrhea is education, aggressive detection, and follow-up screening of possible carriers (27, 386) and the key elements of disease control are "prevention through promotion of safer sexual practices and the availability of health care services "(4).

Virulence factors of *N. gonorrhoeae*

N. gonorrhoeae attaches to and passes through the mucosal epithelial cells into the subepithelial space, causing a localized infection, although occasionally it can spread to other tissues and organs. To better prevent and control gonococcal disease, it is essential to understand the biology of this pathogen. Over decades, extensive studies have been done towards this aim and as a result, a variety of virulence factors have been discovered and characterized, most of which are located in the outer membrane or secreted to the extracellular space of *N. gonorrhoeae*.

Pili The N-methylphenylalanine (Type IV) pili on the surface of *N. gonorrhoeae* are one of the most important virulence factors in a gonococcal infection (187, 380, 382). Pili are involved in a broad range of functions including adherence to host cells (291, 324, 380, 400), DNA transformation (42, 354), and twitching motility (250, 436, 437). Non-piliated gonococci are non-adhesive to host epithelial cells and lose their ability to establish infection in the host (380). Gonococcal isolates are usually divided into 4 phenotypic groups, type I-IV, which is partially based on the presence of pili on their surfaces. Gonococci recovered from patients are usually of type I or II, which

are highly piliated, adhesive and virulent (48, 187). When these piliated clinical isolates are maintained on laboratory media, they gradually lose their pili after several serial passages (186, 187).

Gonococcal pili are helical hair like structures composed of one major structural subunit, pilin, a 17-21 kDa protein encoded by the gene *pilE* (251). In *N. gonorrhoeae* strain MS11, there are two identical functional *pilE* genes, while there is only one functional *pilE* gene in most clinical isolates (251, 349). Stabler and colleagues recently showed that the *pilE* gene is only present in the pathogenic *Neisseria* species, *N. gonorrhoeae* and *N. meningitidis* (372). The amino acid sequence of pilin can be divided into three regions, a conserved region (C), a semi-variable region (SV) and a highly variable region containing two conserved cysteine residues (HV) (251, 252, 350). Using site-directed antibody probes and immunoassays, researchers have shown that the C region is essential in pilus assembly and the other two regions contribute to the diversities of pilin structure (99, 288).

Besides the *pilE* expression locus, there are also several *pilS* loci scattered on the gonococcal chromosome (251, 372). These loci have sequences homologous to the *pilE* gene, in particular, the SV and HV regions but lacking important 5' prmoter region and the start codon required for the initation of transcription and translation. These *pilS* loci form a sequence reservoir allowing the transfer of varied sequences into the *pilE* gene through homologous recombination (136, 137, 252, 349). *N. gonorrhoeae* can obtain the *pilS* sequences either from the same strain (intragenic) or from the lysed gonococci within a population (intergenic) (354). It is likely that the latter might be more common *in vivo*, since gonococcus is spontaneously lysable, releasing its DNA into the

surrounding environment (149) and it is also naturally transformable, taking up exogenous DNA at high frequencies (369). This leads to extensive antigenic variation, due to expression of varing pilin proteins on the bacteria in a population. In fact, it is because of the highly diverse pilin structure that the vaccine against pilin is not practical in use, since antibodies against pilin from one strain lack broad cross reactivity to other gonococcal strains (41). Additionally, gonococcal pili are subject to phase variation, resulting in the "on" or "off" of pilin expression. Pilin phase and antigenic variation are closely related as a result of RecA-dependent homologous recombination (137, 349, 350). Recombination between *pilS* and *pilE* loci results in the expression of a mosaic *pilE* gene leading to production of antigenically variable pilins. Recombination leading to a deletion in the *pilE* gene, causing a frame-shift mutation and abortion of translation, results in phase variation. Additionally, the expression of the pilin gene may also be controlled at the transcriptional level, as some non-piliated strains still contain the functional *pilE* gene and the intact promoter region (349).

Pilus biogenesis has been extensively studied. Although pili are composed of one major subunit, pilin, several proteins are engaged in the assembly of the pilus, including PilD-PilF-PilG-PilT and PilQ-PilP (81, 104, 158, 206, 397). It has recently been shown that these proteins have high homologies to those components involved in the Type II secretion system of Gram-negative bacteria, raising an interesting question that pilus assembly might be closely related to the protein secretion system (284, 309).

The gonococcal pilus assembly process is mainly composed of three steps: the assembly of the fiber in the periplasm, the translocation of the fiber onto the cell surface and the final stablization of the pili structure on the surface (438). At each step, different

proteins are required. For instance, the prepilin peptidase PilD cleaves the leader peptide from prepilins so that the pilins can later form the fiber processed by PilF and PilG (104, 397). Then the assembled fiber passes through the pore formed by PilQ subunits to the cell surface (63, 80, 81, 438), where they continue to form dynamic and stable fibers. During this whole process, the pilus retraction mediated by PilT, an ATPase, is required to balance fiber growth (436, 437). A recent report showed that the PilT-mediated pilus retraction is in fact regulated by PilC protein in *N. gonorrhoeae* (259).

Besides the essential role as a primary adhesin, pili are also critical in DNA transformation (106, 436), allowing gonococci to obtain foreign genetic material and retain genetic diversity (330, 357, 438). In non-piliated gonococci, DNA transformation efficiency decreases dramatically, which is 10,000 times lower than piliated gonococci (355, 369). Although the DNA transformation process in *N. gonorrhoeae* is still not completely understood, a recent paper reports that the functional pilus assembly apparatus is required for DNA uptake (223).

Pili also play an important role in signaling pathways in infected epithelial cells. Purified gonococcal pili are shown to cause Ca^{2+} influx in epithelial cells and result in rearrangement of the cytoskeleton in the cell membrane (22, 180). Additionally, part of these signaling pathways are caused by the twitching motility powered by the pilus retraction, as it can create tension on the cell surface, which can be sensed by host cells (250) . Indeed, it has been shown that the mechanical force generated by the pilus retraction can promote the arrangement of the cytoskeleton in the cell membrane, influxes of Ca^{2+} into the cytosol, and the formation of the cortical plaques that are enriched in proteins necessary for the subsequent cellular responses in host cells (246).

Furthermore, pilus retraction is required for the activation of PI-3 kinase/Akt (PKB) pathway (210) and changes of expression of genes necessary for the subsequent cellular responses in host cells (165). Although pili are the primary adhesins of *N. gonorrhoeae*, our knowledge about their host receptor is still elusive. CD46, a host cell surface protein, has been hypothesized as a receptor for pili (182), however, several studies indicate the existance of other different, yet currently unknown receptor(s), as gonococcal pili can interact with epithelial cells in a CD46-independent manner (190, 249, 395). Recently, Edwards and colleagues identified I-domain-containing (IDC) integrins as receptors for gonococcal pili on urethral epithelial cells (89).

Opa Opa (Opacity associated proteins, Protein II, PII) are a group of major outer membrane proteins of *N. gonorrhoeae* that confer an opaque quality to colonies grown on agar plates and visualized under a microscope (157, 379). Because of their ability to interact with polymorphonuclear leukocytes (PMNs), they were once named Leukocyte Association Proteins (LAPs) (382). Opa proteins play critical roles in gonococcal pathogenesis, facilitating adherence to and invasion of epithelial cells and inducing the expression of the pro-inflammatory cytokines/chemokines (114, 125, 231, 419).

Like the diverse gonococcal pili, Opa proteins are also subject to antigenic and phase variation. However, the mechanism is different from that of pilin antigenic variation. *N. gonorrhoeae* has as many as 11 *opa* genes scattered on its chromosome, named as *opa*A-K (31, 200). Each *opa* gene is constitutively expressed and encodes a different Opa protein differing in the variable domains (31). *Opa* genes also undergo extensive "on" and "off" phase variation, the frequency of which is as high as 10^{-2} to 10^{-3}

per colony-forming unit per generation (237). This *opa* phase variation is caused by the slipped-strand mispairing (SSM) mechanism independent of RecA-mediated homologous recombination (268). The SSM mechanism is associated with short DNA coding repeats (CR) CTCTT present in the 5' coding region of *opa* genes. During DNA replication and repair, the number of these CR repeats can be easily changed, leading to frame-shift mutations and the "on" or "off" of the expression of *opa* genes (268, 373). As a result, one single *N. gonorrhoeae* strain is able to express varying combinations of serval Opa variants scattered on its surface at the same time (237) .

Opa proteins are important in mediating the tighter association between gonococci and host cells (14, 232). Opa^+ gonococcal strains have been shown to bind to and invade human epithelial cells (363, 419, 426, 427). In clinical observations, the strains recovered from patients are usually Opa^+ (168, 171, 414).

The identification of host Opa receptors come from studies using Chinese Hamster Ovary (CHO) cells or other animal cells, which were transfected with genes encoding different human cell surface molecules and were then analyzed for adhesion and invasion of Opa^+ gonococci (61, 415, 416). As a result, two major groups of Opa receptors were discovered, although different Opa variants have distinct affinities for these receptors (61, 200, 415, 416). The first group of Opa receptors is Heparan Sulfate ProteoGlycan (HSPG), whose discovery was based on the speculation that the interaction of Opa proteins and its receptor might involve electrostatic interactions, as adding of negative charged DNA or sulfated polysaccharide can significantly affect gonococcal adhesion (379). Proteoglycans are an important family of macromolecules of human epithelial cells and are composed of a protein core covalently attached by one or more

glycosaminoglycan side chains. These molecules can be found in the cytosol, on the surface or in the extracellular matrix of the cell. The syndecan group is a major group of HSPGs, which contain negative-charged heparansulphates and chondroitin sulphates in the side chains and are located on the cell membrane (323). Only syndecan-1 and syndecan-4 seem to interact with Opa proteins and mediate the entry of *N. gonorrhoeae* into epithelial cells (103). However, not every Opa variant can interact with HSPG, with OpaA strongly binding to this receptor followed by OpaC and OpaH (411, 412). HSPGs are important molecules on the human cell membrane, as they can interact with many growth factors and ligands and serve as growth factor receptors involved many cell signaling pathways (72).

The second type of Opa receptor belongs to the CD66 family, also known as carcinoembryonic antigen (CEA). This receptor family has immunoglobulin-like domains (415) and many Opa variants can bind to the N-domain of CD66 molecules, although the affinity may vary (416). CD66 proteins are broadly expressed in various cells, including leukocytes, endothelial cells, and epithelial cells. Studies have shown that Opa^+ gonococci can specifically bind to CD66 proteins on these cells (116, 124, 270, 363, 415, 422).

Porin Porin (Protein I, PI) proteins are the most abundant proteins on the outer membrane of gonococci, and are encoded by the *por* gene (121). *Por* is essential in *N. gonorrhoeae*, as it has not been possible to construct a null mutant thus far. In *N. gonorrhoeae*, there are two *por* alleles, *por*IA and *por*IB, encoding PIA and PIB, and only one of them is expressed in a single gonococcal strain (121, 176). In several studies of tracking the transmission of gonorrhea, gonococcal strains bearing PIA have shown a

strong correlation with the occurrence of DGI (121, 176, 194, 305). It has also been shown that certain mutations in the porin proteins make the bacterium more resistant to antibiotics (52, 162, 285, 319).

PIA is a 34 kDa protein, slightly smaller than PIB (35.5 kDa), however, the two proteins share over 80% similarity in their amino acid sequences (176). The major difference between PIA and PIB proteins resides in the regions exposed on the cell surface, in which PIA only has a small portion exposed outside and is therefore less susceptible to exogenous protease cleavage (176, 410).

Gonococcal porin proteins are homologs of *E. coli* OmpC, OmpF and PhoE porins (121, 258). The porin proteins can form voltage-gated hydrophilic channels through the outer membrane, which might allow the nutrients to enter the bacterium (121, 410). These channels can also be easily translocated into the membranes of cultured human cells (121). During a gonococcal infection, gonococcal porins have been observed to be released from the bacterial membrane and translocated into the host cell membrane, resulting in the influx of Ca^{2+} into the cytoplasm and apoptosis in the target (HeLa) cells (266). A recent study showed that gonococcal porins can be specifically translocated into the mitochondrial membranes and induces apoptosis in host cells (265, 267). However, this porin-induced apoptosis might be tissue specific, as in other cells, such as transformed urethral epithelial cells or primary cells derived from the male urethra, porins were shown to repress apoptosis (34). In addition, the porin-induced Ca^{2+} influx is necessary for the pili-induced Ca^{2+} influx (22).

Besides their roles in gonococcal pathogenesis, porins are also potential candidates for vaccines. Porins are conserved outer membrane proteins on the gonococcal

surface and don't undergo the extensive variation observed for Opa and pilin. Additionally, porins are highly immunogenic and can induce a strong immune response in the host (55, 445). However, the interaction of porin proteins, LOS and Protein III weakens this immune response by significantly masking the porin binding sites from host bactericidal antibodies (37).

Protein III Protein III (PIII, or Reduction modifiable proteins, Rmp) is located on the outer membrane and tightly associated with porins (PI) on the gonococcal surface (238). The gene encoding PIII is exclusively present in the pathogenic *Neisseriae* species, *N. gonorrhoeae* and *N. menigitidis* (435). PIII is 236 amino acids in length, with a cleavable signal peptide of 22 amino acids and the primary structure of this protein is very similar to that of OmpA in *E. coli* (119, 120), a protein tightly associated with LPS and important in conjugation (345, 346). When PIII is subject to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the migrating band of this protein under reducing conditions is 8,000 kDa larger than the size predicted from its sequence (238). Because of its unusual migration pattern on SDS-PAGE, PIII is also called Reduction modifiable protein (Rmp). Blake and colleagues showed that this unusual migration pattern is due to the two pairs of disulfide bonds formed in this protein. In their experiments, PIII was first chemically cleaved into two fragments so that the disulfide bonds could not be formed. These two fragments were then subject to gel electrophoresis. As a result, no difference in migration was observed in the presence or absence of the reducing agent (38). Proteolytic analyses showed that PIII is resistant to protease digestion in intact gonococci, indicating that most of the protein is not exposed to the outside of the cell (36). Indeed, DNA sequence analysis and surface peptide mapping

experiments showed that only a very small portion of PIII was exposed to the surface (119, 120, 177, 229).

Compared to other gonococcal surface molecules, which undergo extensive phase and antigenic variation, PIII is highly conserved in *N. gonorrhoeae* and has very little variability in its structure. This unique characteristic had once triggered interests of exploring of this protein as a potential gonococcal vaccine (177, 228). In 1986, Rice and colleagues found that the antibodies directly against PIII were able to block the killing of gonococcus from immune serum. This fact was further substantiated by an experiment in which anti-PIII antibodies can directly affect the efficiency of insertion of the complement membrane attack complex (318). The anti-PIII antibodies block the binding of antibody complexes to the gonococcal surface, in particular to the binding sites of porin proteins by occupying those sites. Therefore, anti-PIII antibodies allow gonococci to evade the host immune response, instead of activating it. This was supported by the observation of the declining efficiency of the PI vaccine contaminated with PIII in human volunteers (175, 299).

LOS Lipopolysaccharide (LPS) has long been known as a major virulence factor in many bacterial pathogens (152, 217, 282). This is also true in a gonococcal infection, as it triggers an intense inflammatory response in the subepithelial space of the mucous membrane including the activation of the complement complex and attraction and lysis of phagocytes (234). Gonococcal LPS has also been shown to play a role in gonococcal adhesion and invasion of epithelial cells (90, 368).

Compared to the LPS molecules found in most Gram-negative bacteria, the gonococcal LOS (lipooligosaccharide) lacks the typical O-antigen. Instead, three

branches of oligosaccharides are attached into the Lipid A backbone and the length and sugar composition of these oligosaccharide branches differs from strain to strain (112, 128). In *N. gonorrhoeae*, a single strain can display several distinct LOS structures on its surface (16, 233). In 1994, Gotschlich first identified a locus involved in LOS biosynthesis on the gonococcal genome, which includes the five genes, *lgtA-E*, encoding enzymes responsible for adding different sugars to the end of the LOS structure. Three of these genes have poly-G repeats in their coding regions, which indicate the potential for phase variation by the slipped-strand mispairing mechanism (118). This phase variation was confirmed in later studies (68, 441). In addition to the *lgt* locus, other genes involved in LOS biosynthesis were identified and characterized in the following years (24, 82, 451), and many of these genes also bear poly-nucleotide repeats in their coding regions, indicating that they are prone to phase variation by slipped-strand misrepair.

Gonococcal LOS can be further modified by sialylation. The bacteria themselves do not provide the source for sialylation. Instead, they use host CMP-*N*-acetylneuraminic acid (CMP-NANA) as a donor to add a sialic acid to the LOS moiety (177, 234). The enzyme responsible for sialylation, a α-2,3-sialyl-transferase, is subject to extensive phase variation, which leads to the "on" and "off" of sialylation of LOS in *N. gonorrhoeae* (289). The sialylated LOS is important in the pathogenesis, as it helps gonococci to resist serum killing as a result of mimicking the human cell membrane glycosphingolipids (15, 234). However, some studies show that the sialylated LOS can reduce gonococcal ability to invade epithelial cells (343). In fact, it has been hypothesized that during different infection stages, gonococcus can control LOS sialylation accordingly. For instance, in the early infection stages, the bacteria might need

the unsialylated LOS to facilitate the bacterial entry into the host; while during the later phase of infection, the sialylated LOS might be required to evade the immune response from the host (74, 368).

IgA1 Protease IgA1 protease has been identified as a virulence factor in several pathogens, including *Haemophilus influenzae, N. gonorrhoeae, N. meningitidis* and *Streptococcus sanguis* (189, 263, 264, 297, 298). It has been shown that the gene encoding this enzyme exists exclusively in pathogenic, but not commensal species of *Neisseria* (263, 435). IgA1 protease is a secreted enzyme, which can cleave human immunologlobulin A1 (IgA1), the main immunologlobulin secreted on mucosal surfaces. *N. gonorrhoeae* possesses two distinct types of IgA1 protease: type I and II, and each of them cleaves a specific site of human IgA1 molecule (262).

In *N. gonorrhoeae,* the secretion of IgA1 protease may be the simplest, as the protein itself is enough for its secretion into the extracellular space (141), also referred to as " autotransporter". The mature IgA1 protease is derived from a 169 kDa precursor, which is composed of five distinct domains: the N-terminal signal peptide, the central "protease" domain, the C-terminal "helper" domain and two domains α and γ (301). During the export process, the signal peptide at the N-terminus will first guide the protein to pass through the inner membrane through the SecYEG translocon. (301). Cleavage of this signal peptide by a leader peptidase releases the protein to the periplasm. The C-terminal "helper" domain then associates with the outer membrane and forms a pore to allow the remainder of the protein to pass through the outer membrane. Enzyme activity analysis shows that the IgAI protease activity can detected at this step. Autocleavage will then occur to release the protease domain into the outer space. As a result, the "helper"

domain will be retained in the outer membrane. Then, the peptides in the outer space will continue to develop the active form and will be further cleaved to yield the active protease and two peptides. In the end, the mature active secreted protease has a molecular mass of 106 kDa (301).

The human IgA1 molecule is the main substrate for IgA1 protease. However, the presence of consensus sequence of the specific cleavage sites in relevant human proteins indicate that gonococcal IgA1 protease may have other potential substrates (302). This was later confirmed by the finding that the type II IgA1 protease could cleave lysosomal associated membrane protein 1 (Lamp1), a major integral membrane glycoprotein of lysosomes in epithelial cells (20, 145, 163, 216). As a result, the cleavage of Lamp1 can significantly facilitate gonococci to enter epithelial cells and promote their survival in those cells (216). IgA1 protease was also found to cleave the tumor necrosis factor alpha (TNFα) receptor II to inhibit the TNFα-mediated apoptosis in human monocytic cells (26).

N. gonorrhoeae - host interaction

N. gonorrhoeae is an obligate human pathogen with no intermediate animal reservoirs. There are very few animal models available for gonococcal studies, although a mouse model was recently developed and is yielding interesting data (172). Our knowledge of the *N. gonorrhoeae* - host interaction is primarily from studies using tissue culture and organ culture models derived from tissues normally infected by gonococci (48, 144, 240, 241, 244, 360, 363, 419). Currently, there are several cell lines used in studies of gonococcal infection. HEC-1-B, a human epithelial line derived from a cervical

carcinoma has been used to study adhesion and invasion by *N. gonorrhoeae* and *N. meningitidis* (60, 363). A431, an epithelial cell line derived from an epidermal carcinoma (110), is particularly useful in that they are easy to grow and handle in the laboratory and excellent for observation under the microscope. ME180 cells and T84 cell are two other epithelial cell lines used in gonococcal adhesion and invasion studies (179, 247, 422).

N. gonorrhoeae initiates infection via the primary adhesin, type IV pili and forms microcolonies on the surface of the host cell. Following the initial attachment, Opa proteins and other outer membrane proteins mediate an intimate association between the gonococcus and host cells. In fact, this association is so close that some studies suggest that the gonococcal outer membranes are actually fused with the host cell membranes (14, 261, 360). It is also at this step that the microcolonies start to disperse and the bacteria start to lose pili on their surfaces. Losing pili is a beneficial step, as pili can inhibit the subsequent invasion step (232, 360). Furthermore, the association between gonococci and host cells results in the formation of cortical plaques underneath the contact site, site of entry into the cells, and the loss of microvilli from the surface of nonciliated cells (123, 111). The invasion step is mediated by actin-dependent endocytosis, as this step can be disrupted by the anti-actin drug cytochalasin D (CCD) (248, 261, 320, 360). Using Hec-1-B cells as a culture model, Shaw and coworkers showed that the number of invaded gonococci usually increased after 4-hour of incubation (360). Once inside the epithelial cells, gonococci are usually seen in vacuoles, however, other studies have also found gonococci in the cytosol of the epithelial cells (14, 240, 360, 422). Gonococci then exit from the basal side of the cell by exocytosis into

the subepithelial space of the mucosa, where an inflammatory response is elicited (240, 261).

Overall, the interaction between *N. gonorrhoeae* and the host is a dynamic process involving multiple factors from both sides. Gonococcal adhesion transmits signals to the host cells, which leads to cytoskeletal rearrangements, uptake of the pathogen, and production and release of inflammatory cytokines from the host mucosal epithelial cells (123, 248, 277, 278, 430). Extensive studies on gonococcal infection over the past two decades have greatly increased our understanding of signaling pathways happening inside host cells, in particular, the pilus and Opa-mediated signaling pathways.

Pilus-mediated attachment is the essential first step in gonococcal infection. The binding of gonococcal pili to host epithelial cells results in the influx of Ca^{2+} within the target cells (20, 180). This Ca^{2+} influx happens very rapidly in host cells and can be detected within 10 minutes after the initial adhesion (261). Consequently, the transient increase of Ca^{2+} in the cytosol of the target cell results in the subsequent signaling cascades, as Ca^{2+} is an important signal in the target cells (181, 248). Additionally, the Ca^{2+} influx can increase the distribution of the Lamp1 molecules on the host cell membrane by triggering the lysosome exocytosis so that more Lamp1 can be cleaved by the secreted gonococcal IgA1 protease thus promoting the survival of gonococci in epithelial cells (20). Recent experiments show that the porin-mediated Ca^{2+} influx might be required for the pilus-mediated Ca^{2+} influx (22).

Opa-mediated signaling pathways are mediated by two distinct groups of Opa receptors on host cell membranes, HSPG and CD66 (discussed in the previous section). The HSPG-Opa interaction results in distinct signaling cascades in different cell lines and

leads to the activation of the phosphatidylcholine (PC)-dependent phospholipase C (PLC), acidic sphingomyelinase (ASM) and protein kinase C (86, 122, 114). In contrast, Opa-CD66 interaction results in the activation of the Src family kinases, which can in turn activate the Rho-dependent GTPase Rac1 (147). As a result, the activation of these kinases can eventually induce the expression of the transcription factor nuclear factor kappa B (NF-κB) and the dimeric sequence-specific transcription factor activator protein 1 (AP-1), which subsequently up-regulate the expression of several pro-inflammatory cytokines, IL-8, IL-6 and TNF-α through the activation of the JNK kinase pathway (277, 278). The induction of NF-κB and AP-1 happens very quickly, as the NF-κB complex could be transported into the nucleus within 10 minutes after infection (240).

Compared to our understanding of the signaling pathways within host cells, little is known about the signaling pathways happening inside the gonococcus during this interaction. Does the attachment to host cells transmit signals to the bacterium? If so, what are the regulatory networks involved? Once attached to host cells, gonococci undergo several changes including the formation and dispersal of microcolonies, disappearance of pili from the surface and the translocation of porin into the target cell membrane. These changes are highly organized and coordinated, indicating the involvement of regulatory networks. Thus identifying and dissecting these networks is very important for us to understand gonococcal pathogenesis.

Regulatory networks of *N. gonorrhoeae*

When a pathogen enters a new host it faces a variety of new challenges, involving acquisition of nutrients, colonization, competing for space with existing flora and

circumventing the immune response of the host. In order to adapt to the new environment, pathogens have developed many strategies, including modulating gene expression at the transcriptional, translational, or post-translational levels (66, 67, 151, 154, 317, 336, 340). Rapidly turning genes on or off as required may be the most efficient way to do this as it controls gene expression at the starting point. In fact, many pathogens regulate virulence genes to achieve a selective advantage by enabling them to rapidly adapt to changing environments. For example, *Yersinia pestis* up-regulates the expression of a set of genes encoding type-III-secreted virulence factors upon host contact; the products of these genes are then injected into the cytoplasm of the host cell, causing cytoskeletal changes and interfering with phagocytosis (398). In *Helicobacter pylori*, nearly 10% of its open reading frames (ORFs) are differentially regulated when the pathogen is transferred to an acidic environment reflecting a complex, coordinated regulatory network involved in adaptation to the harsh environment in the stomach (13).

The availability of completed *N. gonorrhoeae* genome sequence provides us opportunities to study its regulatory systems (http://www.uohsc.edu). In the annotated gonococcal genome (43), there are about 50 putative transcription factors (Table 1-1). Compared to about 150 transcription factors identified in *E. coli* (396), *N. gonorrhoeae* apparently has far fewer transcription regulators, which might be because *N. gonorrhoeae* has very limited niches to reside, unlike *E. coli*, which can inhabit a broad range of hosts and environments. Thus, some of transcription factors encoded in the gonococcal genome are likely involved in the ability of the pathogen to survive and multiply in the host and are interesting to study.

Based on amino acid sequence homologies to well-characterized regulators in other bacteria, gonococcal regulators can be grouped into several families, including the AraC, DeoR, GntR, LysR, and MarR regulatory families (Table 1-1). Most of these families contain the helix-turn-helix (HTH) motif and can directly bind to DNA and quickly regulate gene expression in response to differing environmental conditions (108, 338). In *N. gonorrhoeae*, regulatory systems involving regulation of iron acquisition, the redox response, and control of antimicrobial efflux have been described (29, 138, 159, 188, 207, 224, 348, 402).

Iron is essential for bacterial growth, as iron is an important cofactor for many enzymes such as catalase, peroxidase, cytochromes and ribonucleotide reductase (221). *N. gonorrhoeae* encodes several proteins that can scavenge iron from the environment and the Fur (Ferric uptake regulation) protein is a global regulator that regulates the expression of most of iron-uptake systems (29, 220, 348, 393). The gene encoding Fur was first detected in *N. gonorrhoeae* by Southern blot using an *E. coli fur* DNA fragment as probe to hybridize gonococcal genomic DNA. This gene was then cloned and shown to complement an *E. coli fur* mutant, confirming that the gonococcal *fur* gene encodes a functional Fur protein (29). In 1996, Thomas *et al* directly characterized the function of the gonococcal Fur regulator in *N. gonorrhoeae* (393), demonstrating that the Fur regulator regulates several iron-uptake genes, *frpB, tbpA* and *tbpB*. Two-dimensional gel electrophoresis and DNA binding experiments later indicated that the gonococcal *fur* regulon may includes proteins of a broad range, which are not only involved in iron-uptake, but also involved in protein secretion and DNA recombination (348, 393). In addition to Fur, a MerR-like regulator was recently identified in microarray analysis as a

regulator of iron-uptake systems, suggesting that the regulation of this system is controlled by several regulators (85).

N. gonorrhoeae lives in an environment which is rich in antimicrobial fatty acids (FAs), bile salts, hormones and antimicrobial peptides that are toxic to the bacteria, however, gonococci have developed several mechanisms to resist these antibacterial molecules. This makes the current antibiotic treatments challenging. Mtr (Multiple transferable resistance) and Far (Fatty acid resistance) efflux pumps are two such systems that help *N. gonorrhoeae* to resist antimicrobial agents. The Mtr system is composed of three cell envelope proteins (MtrC, MtrD and MtrE) that confer the resistance to hydrophobic agents (HAs), such as erythromycin and Triton X-100 (138). The Far system is composed of a membrane protein FarA and cytoplasmic transporter protein FarB, which mediate the resistance to long-chained fatty acids and depend on MtrE to export these agents. However, these two systems act independently to mediate the resistance to host-derived agaents (208). The regulation of these two systems is mediated by the same transcription repressor protein, MtrR (Multiple transferable resistance Regulator) (138, 139, 207, 224). MtrR binds the DNA sequences between *mtrR* and *mtrC* and represses the expression of the *mtrCDE* operon (138, 159, 224). Mutations in *mtrR* can lead to an increase in the resistance of gonococci to HAs including Triton X-100, erythromycin and penicillin (359, 413, 446). MtrR can also activate expression of the *farAB* encoded efflux pump by repressing the expression of the *farAB* repressor, FarR (207). In addition to the two repressors, MtrR and FarR, MtrA (Multiple transferable resistance Activator) was recently discovered as an activator of the *mtrCDE* encoded efflux pump, indicating that the regulation of gonococcal resistance to antimicrobial molecules is controlled by both

positive and negative regulatory mechanisms (327). Recently, another efflux system (MacAB) was identified in *N. gonorrhoeae*, although its function remains to be determined (326).

During a gonococcal infection, reactive oxygen species (ROS) are an important weapon of phagocytes of the host against gonococci. The ROS include hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\cdot}$), and superoxide anion ($O_2^{\cdot-}$), which are generated in the lysosomes of the phagocytic cells of the host. They are highly toxic and can damage macromolecules in the bacterium such as DNA, lipids and proteins. Gonococci are always associated with activated polymorphonuclear leukocytes on the inflamed mucosal membrane. To manage the ROS encountered, gonococci possess catalase and peroxidase, which can convert the encountered ROS into harmless products (O_2 and H_2O) (17, 174, 449). Recent studies show that the regulation of this ROS response system in *N. gonorrhoeae* is mediated by two important regulators, OxyR and NmlR. OxyR represses expression of the gene encoding catalase (*kat*), while NmlR can up-regulate the expression of several genes (*adhC, copA* and *trxB*) encoding dehydrogenases and reductases (188, 402).

There are four pairs of putative two-component regulatory systems in the annotated *N. gonorrhoeae* genome (43). Two-component regulatory systems are composed of a "sensor kinase/His-kinase" located in the cell membrane and a "response regulator" in the cytoplasm (57, 92). Two-component regulatory systems are important in bacterial pathogens, because the sensor kinase is able to sense an external environmental stimulus or signal and then transmit it to the "response regulator" in the cytoplasm via a phospho relay (92, 375, 376). As a result, the corresponding response regulator can

directly bind to target genes to regulate gene expression or cell behaviors (25, 28). In the *E. coli* K-12 genome, genes encoding more than 30 pairs of two-component systems have been identified (257, 286). These regulatory systems are involved in a wide range of cellular functions, including responses to the osmotic pressure, nitrogen availability, glutamate metabolism and pathogenesis (KEGG database) (95, 129, 236, 312). Our understanding of two-component systems of *N. gonorrhoeae* is comparatively limited. The only characterized two-component system of *N. gonorrhoeae* is the NarQ-NarP system, which is involved in nitrite reduction under oxygen limiting conditions (219, 432). Studies have shown that NarQ-NarP is able to initiate transcription of *aniA*, a gene encoding the nitrite reductase AniA, which is essential for anaerobic respiration in gonococci (219, 243, 432).

Many bacteria use multiple sigma factors to control gene expression in response to a variety of environmental stimuli. The sigma factor is the subunit of the RNA polymerase and confers promoter specificity. There are at least seven sigma factors in *E. coli* (51, 222). However, *N. gonorrhoeae* appears to possess relative few sigma factors. Western blot using antibodies against *E. coli* or *Salmonella enterica* serovar Typhimurium sigma factors first indicated the presence of multiple sigma factors in *N. gonorrhoeae*, which are later identified as σ^{70} (RpoD), σ^{E} (RpoE), and σ^{32} (RpoH) (43, 191, 204). σ^{70} (RpoD) is the primary "house-keeping" sigma factor, highly conserved among Gram-negative bacteria and responsible for the expression of most housekeeping genes. In other bacteria, σ^{E} (RpoE), σ^{32} (RpoH), and σ^{54} (RpoN) have been shown to be important in regulating a variety of genes including heat shock, nitrogen assimilation, and bacterial pathogenesis (45, 283, 392, 423). The sigma factor σ^{54} (RpoN) regulates

expression of a variety of genes involved in nitrogen assimilation in *E. coli* (43, 184, 399). Although there is an *rpoN* homolog in *N. gonorrhoeae*, it has been shown to be non-functional (assessed by the transcriptional and translational analysis of *rpoN* and its gene product RpoN) (204). This is likely due to a deletion in the *rpoN* gene, which removes the region encoding the essential HTH DNA binding motif (204). In *E. coli*, the extreme heat shock sigma factor, σ^E (encoded by *rpoE*) responds to misfolded extra-cytoplasmic proteins (135), which is important for the maintenance of membrane and periplasm homeostasis. Additionally, in *S. typhimurium*, σ^E also regulates the expression of virulence genes (166). σ^{32} (encoded by *rpoH*) is another heat shock sigma factor, which is partially regulated by σ^E in *E. coli*. Although *N. gonorrhoeae* has homologs of both *rpoE* and *rpoH* on its chromosome, their functions remain to be identified. Interestingly, recent work in our lab shows that the gonococcal *rpoE* homolog may be different from that in other bacteria, as it does not regulate the gene expression of *rpoH* in the standard laboratory media or upon host cell contact (84). Moreover, our study also shows that the gonococcal σ^{32} (RpoH) is involved in gonococcal infection, as several genes induced upon host cells contact are regulated by σ^{32} (RpoH) (Details in Chapter 4 and 5).

Signal transduction and the Signal Recognition Particle (SRP) system in bacteria

Bacteria, particularly pathogens, have evolved many ways to modulate the production of proteins in order to adapt to changing environments. This modulation occurs at many levels, including transcription, translation, protein folding and protein localization. In *N. gonorrhoeae*, many of the important proteins identified so far are

involved in interaction with host cells and are found in the outer membrane (OM) such as pili (380), Opa (379) and porin (176). However, our knowledge about cytoplasmic membrane (CM) proteins is very limited, although this class of proteins may also play critical roles in pathogenesis. CM proteins include many proteins with a variety of functions, such as transporting nutrients, excluding antimicrobial agents and some basic housekeeping functions, such as energy generation, cell division and protein translocation. Moreover, CM proteins also contain proteins important in signal transduction, which is necessary for the organism to sense its environment and respond to signals.

In bacteria, there are at least two conserved pathways to transport proteins to the cytoplasmic membrane: the general secretory pathway (GSP) and the signal recognition particle (SRP) pathway (303, 309). The major functional difference between these two systems is that the GSP is a posttranslational targeting system that transports fully synthesized proteins to and across the cytoplasmic membrane and the SRP functions cotranslationally to target a subset of cytoplasmic membrane proteins to the membrane.

The SRP system was first identified in eukaryotes and later identified in prokaryotes, and is highly conserved across species. The well-characterized eukaryotic SRP system has six proteins (SRP 9, 14, 19, 54, 68 and 72) and one RNA molecule (7S RNA) (226, 433). By contrast, the bacterial SRP system is much simpler with two proteins called Ffh ("Fifty-four homolog" of eukaryotic SRP54), and FtsY, the SRP receptor (SR) and a 4.5S RNA, all of which are essential for cell growth (49, 113, 294).

In the model for SRP targeting in Gram-negative bacteria, the SRP complex (Ffh/4.5S RNA) first binds to the newly synthesized signal peptide as it emerges from the

ribosome. This nascent peptide/SRP/ribosome complex is then targeted to the cytoplasmic membrane through the interaction between the SRP complex and SR. The role of 4.5S RNA in the SRP system is not only to increase the interaction between Ffh and the signal sequence of the nascent polypeptide, but also to increase the interaction between Ffh and FtsY (450). The binding and hydrolysis of GTP by Ffh and/or FtsY results in the dissociation of SRP complex from the Ribosome-nascent-chain complex (RNC) and transfer the RNC to the translocon (SecYEG) located in the cytoplasmic membrane, where translation resumes and the newly synthesized peptide crosses through the cytoplasmic membrane cotranslationally (292, 306). The SRP system is beneficial to the bacterium, likely because this system primarily targets CM proteins, thus preventing the aggregation of these hydrophobic proteins in the cytoplasm (73, 230, 358, 407).

Orthologs of SRP components have been identified in *N. gonorrhoeae* (19, 102). The gonococcal SRP system has some unique characteristics that are being studied in our laboratory. For instance, gonococcal 4.5S RNA can directly stimulate the GTPase activity of gonococcal FtsY (PilA), but not that of gonococcal Ffh. Moreover, the promoter sequences of genes encoding several SRP-dependent proteins can specifically interact with gonococcal FtsY (PilA) and stimulate its GTPase activity (102). The role of DNA binding in SRP funtion in *N. gonorrhoeae* is still to be determined.

Scope of dissertation

N. gonorrhoeae is a public health problem worldwide. To better prevent and control diseases caused by this organism, we must improve our understanding of gonococcal biology. Towards this goal, this dissertation focuses on the study and

characterization of regulatory networks of *N. gonorrhoeae*. DNA microarrays and transposon mutagenesis were developed and used to examine gene expression in *N. gonorrhoeae* in a tissue culture model of infection. In addition, this dissertation also describes the identification and characterization of an essential Signal Recognition Particle (SRP) targeted protein in *N. gonorrhoeae*.

Chapter two of this dissertation describes the characterization of a gonococcal gene whose product requires the SRP system for transporting to the cytoplasmic membrane. This gene was initially identified in a genetic screen designed to isolate potential cytoplasmic membrane proteins, based on the fact that overproduction of any of three SRP components would be lethal to the bacterium. This gene was later shown to encode a functional cell division protein, ZipA, in *N. gonorrhoeae*, as that can complement an *E. coli* conditional *zipA* mutant. This observation is significant in that it is the first ZipA homolog identified in a non-rod-shaped organism and its essential role in cell division may in part explain why SRP components are essential in bacteria.

Chapter three describes the development and construction of a gonococcal expression-capable clone set and an associated DNA microarray that were critical for the later studies. The constructed expression-capable clone set represents 83% (1672 out of 2250) protein-coding ORFs of the gonococcal genome including 42 ORFs of the gonococcal genetical island of *N. gonorrhoeae* strain MS11 (78). The construction of this clone set was based on a lambda phage-based site-specific recombination system, which provides multiple choices for expression and analysis of genes of interest (420). PCR products used to construct the clone set were spotted on glass slides to generate an *N. gonorrhoeae* DNA microarray. This microarray represents 2035 ORFs of *N. gonorrhoeae*

genome. The availability of this array allows us to examine the transcriptional profiles of *N. gonorrhoeae* at the genome wide level in a single experiment.

In chapter four, we discuss the identification of genes differentially regulated in gonococci upon host cell contact using the DNA microarray and a cell culture model of infection. The findings show that host cell contact results in changes of gene expression and this response is in part mediated by a heat shock sigma factor, RpoH. Studies also show that while induction of *rpoH* expression is not necessary for adherence of gonococci to epithelial cells, it is important for the subsequent invasion step, as gonococci depleted for *rpoH* invade cells less efficiently than a wild-type strain.

Chapter five describes the identification of additional genes regulated by RpoH. Several different approaches were used here, including DNA arrays, real-time PCR, transposon mutagenesis and a cell culture model of infection. In this work three genes induced upon adherence were identified as RpoH-dependent for this induction, as the expression of these genes was no longer induced upon host cell contact when RpoH was depleted. Additionally, one RpoH-independent gene was identified as important in both adhesion and invasion steps. Furthermore, the observation that only a few of genes differentially regulated upon host cell contact are regulated by RpoH indicates that more than one regulator is involved in the response to host cell contact.

In chapter six, the dissertation is summarized and suggestions for future research to expand upon the current knowledge of gonococcal regulatory networks during infection are discussed.

Table 1-1. Transcriptional regulators in *Neisseria gonorrhoeae* FA1090 and in related *Neisseria meningitidis* serogroup A Z2491 (NMA) and serogroup B MC58 (NMB)

GC ID	NMA ID	NMB ID	Name		Gene product
Transcription: Transcription factors					
NG0199	NMA0825	NMB0617	*rho*		Transcription termination factor rho
NG0256	NMA0885	NMB0683	*nusB*		NusB protein (transcription termination factor)
NG0262	None	NMB0689	*greB*		Transcription elongation factor
NG0288	NMA0917	NMB0712	*rpoH*		RNA polymerase sigma-32 factor
NG0899	NMA1643	NMB1430	*greA*		Transcription elongation factor (GreA)
NG0999	NMA1737	NMB1538	*rpoD*		RNA polymerase sigma factor RpoD
NG1766	NMA0049	NMB0217	*rpoN*		Sigma factor 54
NG1856	NMA0147	NMB0126	*nusG*		Transcription antitermination protein
NG1944	NMA0230	NMB2144	*rpoE*		ECF-family RNA polymerase sigma factor
Regulatory functions: No subcategory					
NG0025	NMA0578	NMB1878			Possible AraC family transcription regulator
NG0058	NMA0613	NMB1843			MarR family transcriptional regulator
NG0128	NMA0701	None	*wrbA*		Trp repressor binding protein, WrbA
NG0152	NMA1632	NMB1420	*fis*		Factor for inversion stimulation protein, Fis
NG0267	NMA0897	NMB0694	*folI*		FolC transcriptional regulator
NG0393	NMA1020	NMB0810			TetR-family transcriptional regulator
NG0602	NMA1517	NMB1303	*nmlR*		MerR-family transcriptional regulator NmlR
NG0671	NMA1559	NMB1347	*suhB*		Extragenic suppressor protein (inositol monophosphatase)
NG0692	NMA1437	NMB1049			LysR-family transcriptional regulator
NG0718	NMA1605	NMB1389	*hexR*		RpiR/HexR family transcriptional regulator
NG0797	NMA1375	NMB1204			Transcriptional regulator
NG0990	NMA1729	NMB1529	*araC*		AraC-family transcriptional regulator
NG1053	NMA1689	NMB1479	*oraA*	*recX*	RecX-family transcriptional regulator
NG1064	NMA1698	NMB1493	*cstA*		Carbon starvation protein (CstA)
NG1116	None	NMB0910	*prtR*		Transcription regulatory protein, PrtR
NG1185	None	None	*asrR*		ArsR-family transcriptional regulator
NG1219	NMA1749	NMB1561	*deoR*	*glpR*	DeoR-family transcriptional regulator; glycerol-3-phosphate regulon repressor
NG1221	NMA1751	NMB1563	*gntR*	*mdcY*	GntR-family transcriptional regulator
NG1244	NMA1774	NMB1585			MarR-family transcriptional regulator
NG1250	NMA1783	NMB1591	*mtrA*		Transcriptional regulator MtrA
NG1294	NMA1905	NMB1650	*lrp*		Leucine-responsive regulatory protein (Lrp family)
NG1360	NMA1965	NMB1711			GntR-family transcriptional regulator
NG1366	NMA1971	NMB1717	*acrR*	*mtrR*	*MtrCDE* transcriptional regulator, repressor
NG1401	None	None	*nosR*		Regulatory protein NosR
NG1402	NMA0761	NMB0577	*nosR*		Regulatory protein NosR
NG1407	NMA0756	NMB0573	*asnC*		A*snC*-family transcriptional regulator
NG1427	NMA0738	NMB0556			Transcriptional regulator, repressor
NG1474	NMA0601	NMB1856	*crgA*		LysR-family transcriptional regulator
NG1562	NMA2087	NMB0398	*arsR*		ArsR-family transcriptional regulator
NG1578	NMA2106	NMB0381	*cysB*		Cysteine transcriptional regulatory protein,

					activator (CysB)
NG1579	NMA2107	NMB0380			Transcriptional regulator, Anr/Fnr family
NG1630	None	None			Lambda repressor-like protein cI
NG1652	NMA1544	NMB0900			Probable antirepressor protein - phage associated
NG1706	NMA2197	NMB0290	*lysR*		LysR-family transcriptional regulator
NG1779	NMA0064	NMB0205	*fur*		Ferric uptake regulation protein Fur
NG1813	NMA0098	NMB0173	*oxyR*		LysR-family transcriptional regulator
NG2027	NMA0381	NMB2055	*metR*		Methionine biosynthesis transcriptional regulator (MetR)
NG2102	NMA0463	NMB1981	*luxS*		LUXS protein -- autoinducer AI2 synthesis
NG2130	NMA0498	NMB1953	*regF*	*sspA*	Regulator of *pilE* expression; stringent starvation protein A
NG2131	NMA0499	NMB1952	*regG*	*sspB*	Regulator of *pilE* expression (RegG); stringent starvation protein B
NG2161	NMA0529	NMB1924			Possible extragenic suppressor; inositol monophosphatase family protein
Regulatory functions: Two component system					
NG0111	NMA0798	NMB0595	*basR*		DNA-binding component of two-component regulatory system
NG0112	NMA0797	NMB0594	*basS*		Sensor component of two-component regulatory system, BasS
NG0176	NMA0798	NMB0595	*resE, phoP, MisS*		Sensor histidine kinase of two-component system
NG0177	NMA0797	NMB0594	*ompR , phoQ, MisR*		Transcriptional response regulator of two-component system
NG0314	NMA0947	NMB0737	*HprK*		Hpr kinase/phosphatase (two component system)
NG0313	NMA0946	NMB0736			Putative regulatory protein, probable PTS system, nitrogen regulatory IIA protein
NG0752	NMA1419	NMB1250	*narP*		NarP (nitrite/nitrate two-component response transcriptional regulator)
NG0753	NMA1418	NMB1249	*NarQ*		Nitrate/nitrite two-component system sensor protein NarQ
NG1866	NMA0159	NMB0115	*hydG AtoC*	*ntrX*	Probable nitrogen assimilation regulatory protein
NG1867	NMA0160	NMB0114	*atoS*	*hydH*	Probable two-component system sensor kinase

(Table 1-1 cont'd)

Chapter 2

Identification of ZipA, an SRP-dependent protein from *Neisseria gonorrhoeae*

This manuscript has been previously published in Journal of Bacteriology:

Du, Ying and Arvidson, Cindy Grove 2003 Identification of ZipA, a Signal Recognition Particle-Dependent Protein from *Neisseria gonorrhoeae*, *J.Bacteriol.* **185**:2122-2130

ABSTRACT

A genetic screen designed to identify proteins that utilize the signal recognition particle (SRP) for targeting in *Escherichia coli* was used to screen a *Neisseria gonorrhoeae* plasmid library. Six plasmids were identified in this screen and each is predicted to encode one or more putative cytoplasmic membrane (CM) proteins. One of these, pSLO7, has three open reading frames (ORFs), two of which have no similarity to known proteins in GenBank other than sequences from the closely related *N. meningitidis*. Further analyses showed that one of these, SLO7ORF3, encodes a protein that is dependent on the SRP for localization. This gene also appears to be essential in *N. gonorrhoeae* as it was not possible to generate null mutations in the gene. While appearing unique to *Neisseria* at the DNA sequence level, SLO7ORF3 was found to share some features with the cell division gene *zipA* of *E. coli*. These features included similar chromosomal locations (with respect to linked genes) as well as similarities in the predicted protein domain structures. Here, we show that SLO7ORF3 can complement an *E. coli* conditional *zipA* mutant, and therefore encodes a functional ZipA homolog in *N. gonorrhoeae*. This observation is significant in that it is the first ZipA homolog identified in a non-rod-shaped organism. Also interesting is that this is the fourth cell division protein (the others are FtsE, FtsX, and FtsQ) shown to utilize the SRP for localization, which may in part explain why the genes encoding the three SRP components are essential in bacteria.

INTRODUCTION

The prokaryotic signal recognition particle (SRP) is a complex of two proteins, FtsY and Ffh, and a 4.5S RNA that targets a subset of proteins to the cytoplasmic membrane (CM) co-translationally (73, 230, 279, 358, 407). The use of a co-translational targeting system by this subset of proteins is thought to be advantageous in that it avoids their aggregation in the cytoplasm, which might occur if they were targeted post-translationally. All of the SRP components are essential for viability in *Escherichia coli* (49, 113, 294) suggesting that this targeting system is critical for the proper localization of proteins involved in essential cell processes.

This is certainly true for the sexually transmitted pathogen, *Neisseria gonorrhoeae*. The gonococcal FtsY ortholog PilA was shown to be essential to the gonococcus (387) before PilA was shown to be part of the SRP (19). Since *N. gonorrhoeae* is an important human pathogen, the most intensively studied proteins of this bacterium are proteins involved in interactions with host cells. These virulence factors are mostly outer membrane (OM) components such as pili (380), PII or Opa (200), PI or porin (176), LOS (341) and iron utilization proteins (35, 59, 64). A few, such as the IgA1 protease, are secreted (364). Relatively little is known, however, of other membrane-associated proteins in *Neisseria* or their functions, especially those of the CM. CM proteins include many transporters for nutrients as well as enzymes involved in the maturation of outer membrane components. Also included are efflux pumps, which prevent otherwise harmful materials from accumulating within the cytoplasm. This class of proteins can be very important for pathogens as a mechanism to exclude harmful

antimicrobial agents and are important targets for vaccine and drug development. Other CM proteins include those involved in energy generation and conservation, respiration, cell division, and protein translocation. The CM also contains proteins involved in signal transduction, which is necessary for the organism to sense its environment, and components necessary to respond to such signals. One of the goals of our research has been to identify and characterize putative CM proteins of *N. gonorrhoeae*, with the ultimate goal of identifying unique proteins that might be useful as targets for drug development.

Using a screening approach that takes advantage of the fact that the relative levels of each of the components of the prokaryotic SRP are critical for function and survival of the organism (407), we have identified several genes of *N. gonorrhoeae* that encode proteins that utilize the SRP for localization. Sequence analysis of these genes revealed one that is apparently unique to *Neisseria,* having no close matches in the GenBank database. Further examination of this gene, however, suggested that it might be structurally and functionally related to the cell division protein, ZipA, of *E. coli*.

MATERIALS AND METHODS

DNA manipulations. *E. coli* recombinant DNA manipulations were performed as described (333). Cloning vectors used were pET24a (Novagen, Madison, WI), pACYC184, pHSS6 (356), pWSK129 (424) and pWSKlacIOPE1. pWSKlacIOPE1 was constructed by ligating a 2.8 kb *lacIOP*Erm fragment of pHSXErm (351) into the *Not*I site of pWSK29 (424). Restriction enzymes and T4 DNA ligase (New England Biolabs, Beverly, MA) were used according to the manufacturer's recommendations. Polymerase chain reaction (PCR) was done using a GeneAmp 9700 thermocycler (Applied Biosystems, Foster City, CA) and Taq DNA polymerase (Roche Molecular Biochemicals, Indianapolis, IN). Oligonucleotide primers were purchased from Sigma-Genosys (The Woodlands, TX) or the Michigan State University Macromolecular Structure, Sequencing and Synthesis Facility and the sequences are available on request. The QIA quick PCR Purification kit (Qiagen, Valencia, CA) was used to purify PCR products. DNA sequence determination of pSLO plasmids was done by the Core Facility of the Department of Molecular Microbiology and Immunology at Oregon Health Sciences University. All other plasmids were sequenced by the Michigan State University Macromolecular Structure, Sequencing and Synthesis Facility. DNA sequences were analyzed using the Oxford Molecular Group DNA analysis programs MacVector and Omiga (Accelrys, San Diego, CA).

Transposon mutagenesis. Transposon mutagenesis of plasmid DNA was performed by in vitro transposition using EZ::TN™ transposase (Epicentre Technologies, Madison, WI) and the modified transposon TnErmUP contained on the plasmid pMODErmUP (H.

S. Seifert, unpublished). The TnErmUP transposon was prepared by PCR amplification from pMODErmUP using oligonucleotide primers FP-1 and RP-1 (Epicentre). Following the transposition reaction, plasmid DNA was transformed into *E. coli* strain DH5α, and transformants selected for Em^R. The position of the transposon insertion was determined by PCR using oligonucleotide primers homologous to the ends of the transposon (SqFP and SqRP, Epicentre) and to the ends of the pSLO7 insert, followed by restriction analysis.

Southern blot analysis. Chromosomal DNA from *N. gonorrhoeae* transformants was isolated and digested with *Cla*I. Fragments were separated on 1% agarose gels and DNA blotted to nylon membranes as described (19). DNA probes for hybridization were generated by the random priming method with the digoxygenin (DIG) DNA-labelling and detection kit (Roche).

Growth of bacterial strains. *E. coli* strains used were DH5α, BL21λDE3 (378), S17-1λpir, HDB29 (214), N4156::pAra14-FtsY (227), CH3pCH32 and CH5pCH32 (140). *E. coli* were routinely grown in Luria broth supplemented as necessary with ampicillin (Ap) at 100 mg/l, chloramphenicol (Cm) at 20 mg/l, kanamycin (Kn) at 50 mg/l, spectinomycin (Sp) or streptomycin (St) at 25 mg/l, or erythromycin (Em) at 300 mg/l. *N. gonorrhoeae* strain MS11A [P^+tr;](349) was maintained in a humidified 5% CO_2 atmosphere on GC agar (Difco Laboratories, Sparks, MD) with supplements (187). *N. gonorrhoeae* transformation was performed as described (355). Em was used at 3 mg/l for *N. gonorrhoeae*.

Protein isolation and analysis. Bacterial cells were fractionated into periplasm, cytoplasm, cytoplasmic and outer membranes as described (19) and protein concentrations determined by the Bradford method (BioRad Laboratories, Richmond, CA). Proteins were separated on 15% polyacrylamide gels and transferred to nitrocellulose membranes electrophoretically. Detection of His_6-tagged proteins was done using Ni-NTA-HRP (horseradish peroxidase) conjugate (Qiagen) in TBS (Tris-buffered-saline) buffer. Membranes were incubated in 10% (w/v) nonfat dry milk in TBS to block and then with Ni-NTA-HRP in 2% milk. HRP was detected with the chemiluminescent detection agent SuperSignal (Pierce, Rockford, IL) used according to the manufacturers' directions.

Construction of the SLO screen strain. *E. coli ffh* was amplified from genomic DNA by PCR and cloned into pBluescript II SK- to create pBluFfh. pBluFfh was then digested with *Hpa*I to remove an internal 777 bp fragment which was replaced with a 1222 bp fragment encoding resistance to erythromycin (401). This is the same deletion as was used for construction of the *ffh::kan-1* allele (294). The entire *ffh::erm* fragment was then cloned into the suicide plasmid pKAS32 (367), which contains the π-dependent R6K origin of replication (255), an RP4 origin of transfer for conjugation, and the *rpsL* gene, which renders the host strain (S17-1λpir) sensitive to streptomycin (Sm) which is dominant to Sm^R alleles. The resulting strain S17-1λpir/pSFfh::erm was mated with HDB29 (Sm^R) and transconjugants selected for Em^R and Sm^R. pSFfh::erm cannot replicate in HDB29, forcing the *ffh::erm* allele to recombine with the *ffh::kan-1* allele on the chromosome of HDB29. Selection for Sm^R insures that a double crossover event

(replacement of *ffh::kan-1* with *ffh::erm*) and not a single crossover event (integration of pSFfh::erm into the chromosome) occurs. HDB29 also contains an *ffh* gene under the control of P_{trc} on a separate replicon, although if the *ffh::erm* allele were to recombine at this locus, there would be no intact *ffh* gene and these recombinants would not survive, since *ffh* is essential to *E. coli* (294). Multiple Em^R transconjugants were obtained and screened for Kn^S and Cb^S, to insure that the *ffh::kan-1* allele was no longer present and that the entire plasmid, pSFfh::erm had not integrated into the chromosome. The resulting strain was called CGA29 (*ffh::erm* λD69 *Hin*DIII::*lacI*^{Q} P-$_{trc}$*ffh*).

RESULTS

SLO screen of a gonococcal gene bank. Previous studies have shown that multicopy plasmids expressing SRP-dependent *E. coli* proteins confer a lethal phenotype in strains producing limiting amounts of Ffh (407). In *E. coli* strain CGA29, *ffh* is under the control of the IPTG-inducible *trc* promoter. LacI repression of *ffh* is not complete in this strain, and a small amount of Ffh is made in the absence of IPTG. Ulbrandt *et al.* (407) showed that this small amount of Ffh is sufficient for survival of the bacterium under normal conditions. However, if a multicopy plasmid expressing a protein that utilizes the SRP is present in this strain, it can no longer survive unless the level of Ffh in the cell is increased by the addition of IPTG to the growth medium. They termed this effect SLO, for synthetic lethality upon overproduction. Since the SRP proteins from *E. coli* and *Neisseria* are similar, and we have shown that *N. gonorrhoeae* PilA can functionally replace FtsY in *E. coli* (19), we reasoned that gonococcal proteins that utilize the SRP might also confer a SLO phenotype to the conditional Ffh strain in this system.

The *N. gonorrhoeae* strain MS11A gene library pCBB (19) was introduced into CGA29 and transformants selected on plates containing 10 μM IPTG. Transformants were next cultured on duplicate plates with and without IPTG, and examined to identify those with little or no growth under Ffh-limiting conditions. In a screen of 1008 CGA29pCBB transformants, 14 were found to be sensitive to growth under Ffh-limiting conditions. These were divided into two groups based on the severity of their growth defect. Five isolates were moderately affected, with colonies noticably smaller than a vector-only control on plates lacking IPTG. Nine isolates were severely affected, with little or no growth in the absence of IPTG.

Plasmid DNA was isolated from each strain and retransformed into CGA29 to confirm that the SLO phenotype was plasmid-linked. Of the 14 original isolates, 4 were eliminated because the inserts were unstable. The SLO phenotypes of the remaining 10 were the same as the original isolates (6 severe and 4 moderate) and the DNA sequences of each of the plasmids were determined. First, the sequences were scanned for the presence of open reading frames (ORFs) using the DNA analysis programs MacVector and Omiga. The deduced protein sequence for each ORF was then analyzed using PSORT (272), which predicts the cellular localization of a protein. The protein sequences were also used to search the GenBank database using BLAST (10). And finally, the sequences of each insert were used as query in a BLAST search of the annotated *N. gonorrhoeae* strain FA1090 database (http://www.stdgen.lanl.gov).

A summary of the sequence analyses of the 6 severe SLO clones is shown in Table 2-1. These results showed that each of the plasmids encoded at least one putative inner/cytoplasmic membrane protein. Since the bacterial SRP targets a subset of proteins whose final destination is the CM (73, 279, 332, 358, 407), these results indicate that this heterologous screen does identify proteins from a *Neisseria* gene bank that interact with the *E. coli* SRP. Of the 4 plasmids conferring a moderate SLO phenotype, 3 encoded at least one putative CM protein and only 1 appeared to be a false positive, encoding 2 putative cytoplasmic proteins (data not shown). The frequency obtained in this preliminary screen is 0.9% (9 of 1008), similar to the 1-2% estimated by Ulbrandt and co-workers for SRP-dependent proteins in *E. coli* (407). Of the 6 severe SLO clones, one, SLO7, appeared to encode a putative CM protein that was not similar to any sequences in the GenBank database, suggesting it was unique to *Neisseria*.

Table 2-1. Sequence analysis of severe SLO clones.

SLO clone	Insert size	BLAST results	PSORT location
7	2121 bp	AmpD	cytoplasm
		Conserved hypothetical protein (5' 914/945[a])	periplasm
		Unknown[b] ORF (5' 642/1284[a])	CM[c]
16	4307 bp	cytosine permease (5' 883/1224 bp)	CM
		TldD (CsrA supressor)	cytoplasm
		histidine permease	CM
		glutamine permease	CM
24	2953 bp	protein disulfide isomerase (3' 312/492 bp)	CM or OM[d]
		ubiquinone oxidoreductase	CM
		ABC-type exporter	periplasm
48	2273 bp	phosphoglucomutase (5' 463/1383 bp)	cytoplasm
		proline cis/trans isomerase	cytoplasm
		Na^+/H^+ antiporter	CM
53	2366 bp	ABC transporter permease (5' 694/1932 bp)	CM
		DsbC (protein disulfide isomerase)	periplasm
		DNA replication protein (5' 658/2199 bp)	cytoplasm
87	2161 bp	Hypothetical protein	CM or OM
		glutamate permease (5' 618/1213 bp)	CM

[a] partial sequence [b]not in GenBank; [c]cytoplasmic membrane; [d]outer membrane

Analysis of the ORFs of pSLO7. Sequence analysis of the pSLO7 insert showed it contains 3 putative ORFs which we designated ORF1, ORF2, and ORF3 (Figure 2-1). Only the 5' 914 bp of the 945 bp ORF1 are on pSLO7, and this ORF encodes a hypothetical protein with no similarities in the GenBank database, other than a conserved hypothetical protein from *N. meningitidis*. PSORT analysis of the predicted protein sequence of ORF1 suggests it is localizes to the periplasm. ORF2 encodes a protein with 49% identity and 61% similarity to AmpD of *E. coli*. AmpD is thought to be a regulator of β-lactamase induction (403) and is probably involved in the recycling of products of peptidoglycan turnover (160). PSORT analysis of the putative AmpD homolog predicts it to localize to the cytoplasm. ORF3, like ORF1, is only partly on pSLO7 (5' 642 bp of 1284 bp). Also like ORF1, ORF3 appears to encode a protein unique to *Neisseria,* and encodes a protein with no similarities in the GenBank database, aside from hypothetical proteins in *N. meningitidis*. PSORT analysis of the predicted protein encoded by ORF3 suggests that it localizes to the cytoplasmic membrane.

Transposon mutagenesis of pSLO7. In order to determine which of the ORFs of pSLO7 was responsible for the SLO phenotype, each ORF was disrupted by in vitro transposition (117) using the transposon TnErmUP contained on the plasmid pMODErmUP (kindly provided by H. Seifert). pMODErmUP is a modification of pMOD™-2<MCS> (Epicentre) in which the *ermC* gene (418) and a *Neisseria* uptake sequence (115) have been inserted between the mosaic ends (ME) recognized by the EZ::TN™ transposase (Figure 2-1B). Following transformation of the transposition mix, $Kn^R Em^R$ transformants were selected and the positions of the TnErmUP insertions determined by PCR and

restriction analysis (Figure 2-1). Each of these plasmids was then transformed into CGA29 and transformants scored for growth on plates with and without IPTG (Table 2-2). Plasmids with insertions in ORF1 (pSLO7::TnEm #11) or ORF2 (pSLO7::TnEm #3) only grew in the presence of IPTG, i.e., confer a SLO phenotype. However, CGA29 harboring either of the ORF3 insertion plasmids (pSLO7::TnEm #10 or #26) grew well in the presence and absence of IPTG, no longer conferring a SLO phenotype. This indicates that the remaining ORFs on this plasmid, ORF1 and ORF2, do not encode SRP-dependent proteins, and suggests that ORF3 is responsible for the SLO phenotype of pSLO7. To confirm this, since pSLO7 contains a truncated ORF3 gene, the entire predicted ORF3 gene along with associated upstream expression sequences was PCR amplified from MS11A genomic DNA and cloned into pHSS6 (356). The resulting plasmid, pSLO7ORF3, conferred a SLO phenotype when transformed into CGA29 and grown without IPTG (Table 2-2), confirming that ORF3 is responsible for the SLO phenotype.

Effects of SRP-depletion on cellular localization of SLO7ORF3. In order to detect small amounts of the SLO7ORF3 protein in cellular protein extracts, a plasmid was constructed such that an epitope tag (His_6) was added to the C-terminus of the full-length ORF3 protein. We were unable to detect a full length SLO7ORF3-His_6 when overexpressed (data not shown), therefore a truncated version was constructed. The 5' 840 bp of the SLO7ORF3 coding region was PCR amplified from *N. gonorrhoeae* strain MS11A genomic DNA and ligated into pET24a, placing a truncated (280 amino acids) ORF3 with a C-terminal His_6 tag under the control of a T7 promoter.

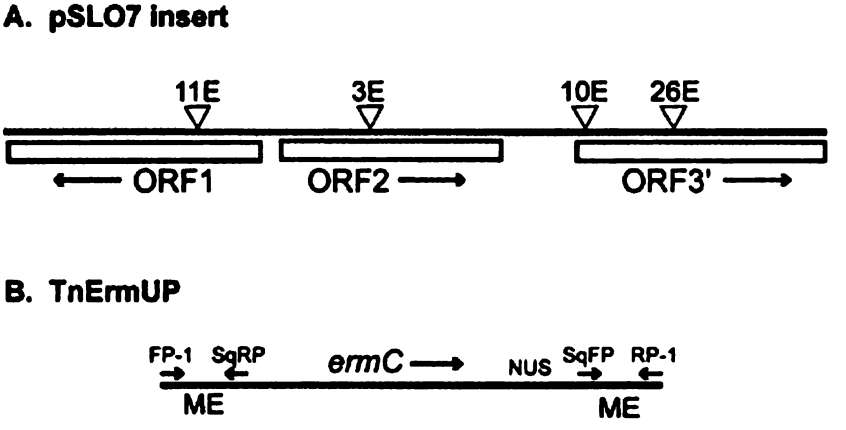


Figure 2-1. Schematic diagram of the pSLO7 insert and the TnErmUP insertions. A. pSLO7 insert. Arrows of ORFs indicate direction of transcription. ▽ indicates position of the TnErmUP (3E, 10E, 11E, 26E) insertions. B. TnErmUP transposon. Small arrows indicate oligonucleotide primers used to prepare the transposon for in vitro transposition (FP-1 and RP-1) and to map transposon insertions (SqRP and SqFP).

Table 2-2. SLO screen of pSLO7::TnErmUP insertion mutants.

Plasmid	Genotype	Growth + IPTG	Growth - IPTG
pHSS7	Vector control	+	+
pSLO7	Wild-type insert	+	-
pSLO7::TnErmUP #3	*orf1*$^+$*orf2*$^-$*orf3*$^+$	+	-
pSLO7::TnErmUP #11	*orf1*$^-$*orf2*$^+$*orf3*$^+$	+	-
pSLO7::TnErmUP #10	*orf1*$^+$*orf2*$^+$*orf3*$^-$	+	+
pSLO7::TnErmUP #26	*orf1*$^+$*orf2*$^+$*orf3*$^-$	+	+
pSLO7ORF3	*orf3*$^+$	+	-

The resulting plasmid, pSLO7ORF3'-His_6, was transformed into *E. coli* strain BL21λDE3 (378) and induced with IPTG. Cells were then fractionated into periplasmic, cytoplasmic, and membrane fractions as described (19). The membrane fraction was extracted with 0.2 % Sarkosyl to separate soluble (CM) and insoluble (OM) proteins (140). Samples were separated by SDS-PAGE and transferred to nitrocellulose, and the ORF3'-His_6 protein was detected using an Ni-NTA-HRP conjugate. ORF3'-His_6 was found in both membrane fractions, but not the cytoplasm or periplasm, suggesting that SLO7ORF3 localizes to the cytoplasmic membrane.

To determine whether the localization of the ORF3'-His_6 protein was dependent on the SRP in *E. coli*, the pSLO7ORF3'-His_6 construct was subcloned onto a derivative of pWSK129 (424) placing an IPTG-inducible *lac*-promoter upstream of ORF3. This construct, pWSKORF3'-His_6, was transformed into the conditional SRP *E. coli* strain,

N4156::pAra14-FtsY (227), in which the production of the SRP receptor, FtsY, is controlled by the *araBAD* promoter. This strain absolutely requires L-arabinose for growth, and culturing in the absence of this sugar results in the depletion of FtsY and eventually kills the cell. However, before the cells die, the depletion of FtsY causes an accumulation in the cytoplasm of proteins that are dependent on the SRP. N4156::pAra14-FtsY/pWSKORF3'-His_6 was grown overnight in medium containing 0.2% L-arabinose. The culture was washed with medium lacking arabinose, and subcultured to medium containing 0.2% glucose with or without arabinose at a starting concentration of ~10^7 CFU/ml. After growth for 7 hr at 37°C, the cultures were harvested and cells fractionated as described above. ORF3'-His_6 protein was detected with Ni-NTA-HRP and the results showed that under conditions of FtsY-depletion (medium lacking L-arabinose) ORF3'-His6 accumulates in the cytoplasm (Figure 2-2), indicating that under these conditions ORF3'-His_6 is not targeting to the membrane as it does under FtsY-replete (w/arabinose) conditions. We conclude from this result that SLO7ORF3 is directly or indirectly dependent on the SRP for CM localization.

ORF3 is essential for *N. gonorrohoeae*. To determine whether any of the genes on pSLO7 were essential to *N. gonorrhoeae*, plasmids containing transposon insertions in each of the putative ORFs were used to transform *N. gonorrhoeae* strain MS11A (349). *Neisseria* are naturally competent for transformation and will take up DNA at high frequencies as long as the DNA contains a *Neisseria* uptake sequence (NUS) (115).

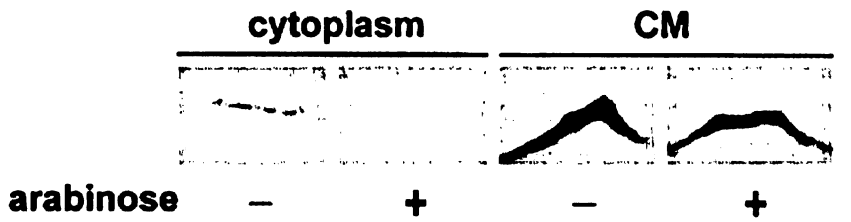


Figure 2-2. Effect of SRP depletion on localization of SLO7ORF3'-His_6 in *E. coli*. N4156::pAra14-FtsY transformed with pSLO7ORF3'-His_6 was grown for 7 h with (FtsY replete) or without (FtsY depleted) arabinose. The cells were fractionated, and 100 μg of cytoplasm or Sarkosyl-extracted membrane (CM) proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. SLO7ORF3'-His_6 was detected with Ni-NTA-HRP, followed by chemiluminescent detection of HRP.

Since there are two NUS in pSLO7 (both in ORF2) as well as one on TnErmUP, it was predicted that the transposon insertion mutant plasmids would be taken up efficiently by MS11A. MS11A was transformed with linearized pSLO7::TnErmUP plasmid DNA and selected for Em^R. If a gene is essential to the gonococcus, it would be predicted that no Em^R transformants would be obtained in such an experiment. Table 2-3 summarizes the results of 3 independent transformation experiments. Experiments 1 and 3 utilized 0.2 μg of DNA, and experiment 2 was done using 0.6 μg of plasmid DNA. In each experiment, high frequencies of transformation were observed for plasmids with insertions in ORF1 and ORF2 (2.1×10^{-5} to 4.6×10^{-4} Em^R/total CFU), but very few Em^R transformants were obtained with plasmids containing insertions in ORF3. This strongly suggested that ORF3 is essential to the gonococcus. pSLO7::TnErmUP#10, which contains an insertion just after the predicted start codon of ORF3, yielded a total of six Em^R transformants, two in experiment #2, and four in experiment #3. In order to determine whether these transformants had a disruption of the ORF3 gene, Southern blot analyses were performed on DNA from 2 of the ORF3 Em^R transformants and one each of the ORF1 and ORF2 Em^R transformants (data not shown). When the TnErmUP transposon was used as a probe, a single band was observed for each of the mutants, as expected. When pSLO7 was used as a probe, two bands were observed in the putative ORF3 mutants, whereas only a single band (the same mobility as that observed with the transposon probe) was observed for the ORF1 and ORF2 mutants. The mobility of this band was slightly slower than the band observed in MS11A (wild-type) consistent with an insertion of 1.3 kb, the size of the transposon. The two bands observed for the putative ORF3 mutants suggested a duplication event, such that these isolates are heterodiploids containing a mutated as

well as a wild-type copy of ORF3. This supports the conclusion that ORF3 is essential to the gonococcus.

Analysis of the putative ORF3 gene product. The ORF3 sequence on the *N. gonorrhoeae* strain FA1090 chromosome is located between *ligA*, the gene for DNA ligase (211) and *ampD*. Comparison to the *ligA* region of the *E. coli* K-12 genome (39) revealed that the cell division gene, *zipA* (140), is found in the same position with respect to *ligA* as ORF3 in *Neisseria*.

The predicted SLO7ORF3 polypeptide (Figure 2-3) is 428 amino acid residues with a calculated molecular weight of 47.5 kDa, somewhat larger than *E. coli* ZipA at 328 residues and 36.4 kDa. *E. coli* ZipA has an hydrophobic N-terminal domain (residues 1-21) with no apparent signal peptidase cleavage site, followed by a stretch of basic residues, which reportedly prevents membrane translocation (12). This is followed by a large apparently cytoplasmic domain with no hydrophobic stretches that are long enough to span the membrane. It has been suggested that ZipA is anchored in the cytoplasmic membrane with the remainder of the protein in the cytoplasm (140). Comparison of the predicted SLO7ORF3 protein sequence and *E. coli* ZipA showed no significant similarities in the primary sequence (using pairwise BLAST). Interestingly, however, these two proteins do appear to share features of their predicted secondary protein structure, including the N-terminal hydrophobic region (residues 1-21), the following basic region (residues 26-50, net charge +6) and the remaining cytoplasmic regions. An interesting feature of *E. coli* ZipA is the unusually high number of proline (31%) and glutamine (23%) residues, which are thought to form a rigid linker that holds the C-

terminal domain in place extended from the membrane anchored domain (21). SLO7ORF3 also has an atypically high number of proline (13%) and glutamine (8%) residues in a similar region of the predicted protein sequence (Figure 2-3). Furthermore, a ClustalW alignment of the amino acid sequences of the C-terminal region of *E. coli* ZipA with the similar region of ORF3 and the putative ZipA of *H. influenzae* (shown in Figure 2-3) shows that there is significant similiarity in this region, leading us to hypothesize that SLO7ORF3 might encode an ortholog of ZipA.

Complementation of an *E. coli* conditional *zipA* mutant. The entire SLO7ORF3 region from *N. gonorrhoeae* strain MS11A was ligated into pET24a placing a full length ORF3 under the control of the T7 promoter. The resulting plasmid, pET-ORF3, was transformed into *E. coli* strain BL21λDE3 (378) and analyzed for protein production upon induction with IPTG. SDS-PAGE analysis showed the induction of a protein of ~50 kDa, similar to the predicted 47.5 kDa size of the ORF protein (data not shown).

In order to determine whether ORF3 could complement a conditional *zipA* mutant strain of *E. coli*, we obtained two strains, CH3(*recA*::Tn10, *zipA*$^{+}$) and CH5(*recA*::Tn10, *zipA*::*aph*), both harboring the plasmid pCH32(*repA*^{ts}*zipA*$^{+}$*ftsZ*$^{+}$) from Piet deBoer (140). The *zipA* allele on the chromosome of CH5 has been insertionally inactivated [*zipA*::*aph*], such that this strain can only grow when harboring pCH32 and at the permissive temperature (30-32°C), since *zipA* is an essential gene. CH3 is a wild-type (*zipA*$^{+}$) control that grows well at the permissive (30-32°C) as well as non-permissive (37-42°C) temperature, even while harboring pCH32.

Figure 2-3. Alignment of the amino acid sequence of putative gonococcal ZipA homolog (*N.g.*) with the *E. coli* (*E.c.*) (21) and *H. influenzae* (*H.i.*) (14) ZipA sequences. The entire coding region of SLO7ORF3 is annotated in the STDGEN database (http://www.stdgen.lanl.gov/) as NG0236. ClustalW alignment was done using the DNA analysis program Omiga. Numbering is sequential for *N.g.* The amino-terminal hydrophobic residues thought to anchor the protein in the CM are double underlined. The positively charged region (residues 26-50) believed to prevent further export of the protein through the CM is indicated by bold underline. Proline (P) and glutamine (Q) residues in a region of high concentration of these residues are indicated in black boxes. Gray boxes indicate residues in the most conserved C-terminal region that are identical or functionally similar in at least two of the proteins indicated, and shows the high degreee of similarity between SLO7ORF3 and these other two ZipA homologs.

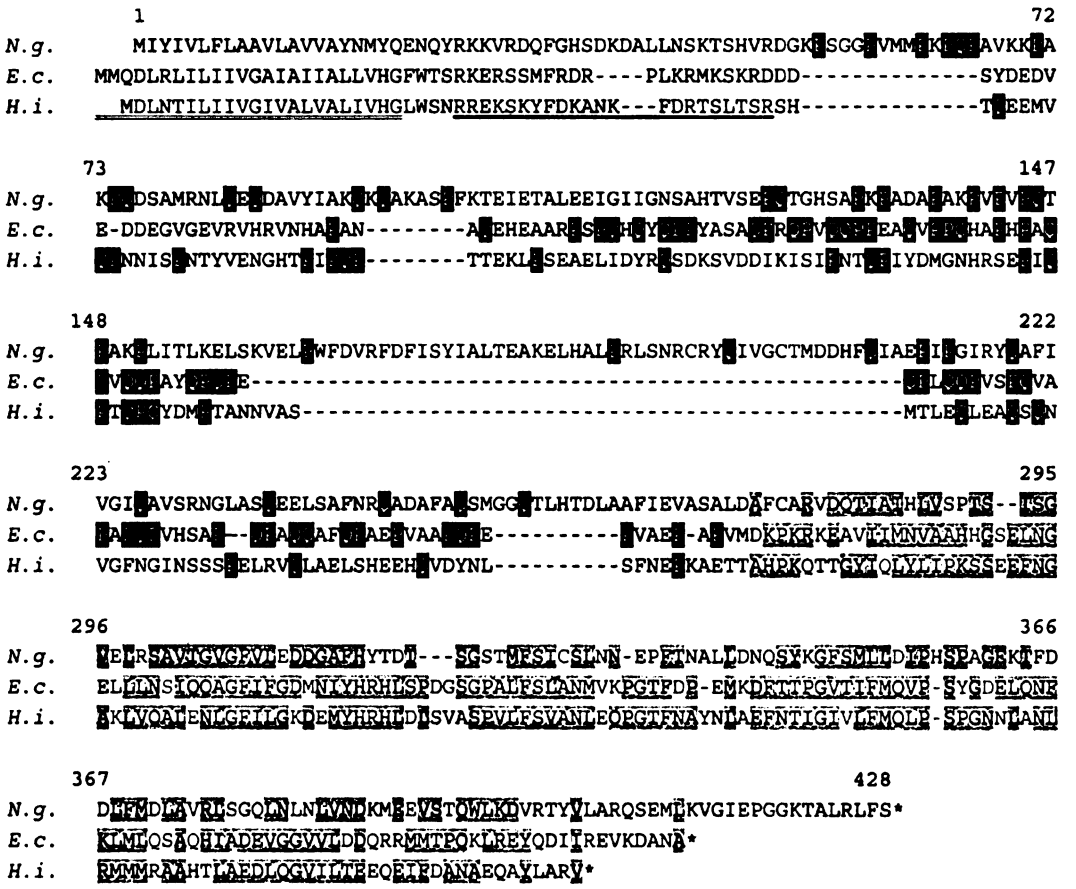


Table 2-3. Transformation of MS11 with pSLO7::TnErmUP insertion mutants.

Plasmid (genotype)	Transformation requency[a]		
	Exp #1	**Exp #2**	**Exp #3**
pSLO7::TnErmUP#3 (*orf2*$^-$)	4.6×10^{-4}	6.3×10^{-5}	7.0×10^{-5}
pSLO7::TnErmUP#11 (*orf1*$^-$)	3.6×10^{-5}	5.1×10^{-5}	2.1×10^{-5}
pSLO7::TnErmUP#10 (*orf3*$^-$)	<1[b] $\times 10^{-7}$	2.4×10^{-7}	3.9×10^{-7}
pSLO7::TnErmUP#26 (*orf3*$^-$)	$<1 \times 10^{-7}$	$<1 \times 10^{-7}$	$<1 \times 10^{-7}$

[a]Frequencies are reported as erythromycin resistant (Em^R) transformants per total number of CFU.

[b]The limit of detection in this assay is 1.0×10^{-7}; a value of $<1 \times 10^{-7}$ indicates that no transformants were obtained.

To construct appropriate plasmids to test whether SLO7ORF3 could complement *zipA* in *E. coli*, ORF3 was subcloned from pET-ORF3 to a plasmid containing a *lac* promoter and the $lacI^Q$ gene, pWSKlacIOPE1 (see Materials and Methods). The entire $lacI^Q$-P*lac*-SLO7ORF3 construct was then ligated into pACYC184. The resulting plasmid, pACYCLacORF3, was used to transform CH3pCH32 and CH5pCH32 (along with pACYC184 as a control). Transformants were then streaked on LBCm plates in duplicate and incubated at 30°C or 37°C (Figure 2-4). At 30°C, all four strains grew well, as expected. At 37°C the wild-type strain CH3pCH32 harboring pACYC184 or pACYCLacORF3 grew well, but CH5pCH32 harboring pACYC184 did not, which was also expected, since at the non-permissive temperature ZipA would be depleted in this strain. However, CH5pCH32 harboring pACYCLacORF3 did grow well at 37°C, the non-permissive temperature for replication of the $zipA^+$ plasmid, demonstrating that SLO7ORF3 can complement a *zipA* null mutation in *E. coli*.

We next asked whether SLO7ORF3 could alleviate the filamentous phenotype of ZipA-depleted cells previously reported (140). Strains CH3pCH32pACYCLacORF3, CH3pCH32pACYC184, CH5pCH32pACYCLacORF3, and CH5pCH32pACYC184 were grown in liquid culture overnight at 30°C, then diluted 1:500 and shifted to 37°C and grown until an optical density (OD) at 600 nm of ~0.2-4 was reached. All strains except CH5pCH32pACYC184 reached this density in about 2 hr. After 6 hr of growth CH5pCH32pACYC184 reached a maximum OD_{600} of just under 0.2, consistent with the inability of this strain to grow at the non-permissive temperature. Samples of each culture were spotted onto glass microscope slides and gram-stained (Figure 2-5).

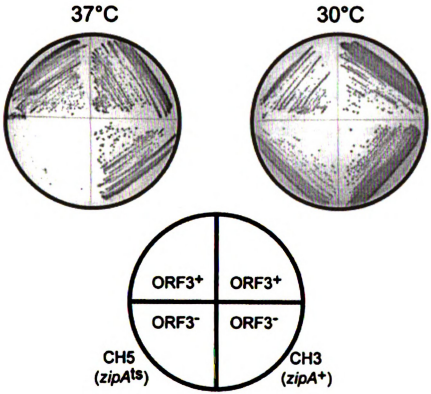


Figure 2-4. *In vivo* complementation of *E. coli zipA* with *N. gonorrhoeae* SLO7ORF3. *E. coli* strains CH3(*zipA*$^{+}$)pCH32(*repA*^{ts}*zipA*$^{+}$*ftsZ*$^{+}$) and CH5 (*zipA*::*aph*)pCH32(*repA*^{ts}*zipA*$^{+}$*ftsZ*$^{+}$) transformed with pACYC184 (ORF3^{-}) or pACYCLacORF3 (ORF3^{+}) were streaked on LB Cm^{20} plates and grown at the permissive (30°C) or non-permissive (37°C) temperature. Plates were photographed using a UVP BioDoc-It system with back lighting.

As expected, the *zipA*$^+$ control strain CH3pCH32 showed normal individual cells, with the occasional pair in the process of dividing. This was observed whether pACYC184 or pACYCLacORF3 was present, suggesting this level of ORF3 expression did not affect cell division in the presence of *E. coli zipA*. CH5pCH32 harboring pACYC184, however, was extremely filamentous. Few, if any, individual cells were observed in any slide (4 slides prepared from 2 different cultures were examined). Interestingly, CH5pCH32 harboring pACYCLacORF3 showed an intermediate phenotype. Some filaments were observed, although none were as long as those of CH5pCH32pACYC184. Numerous individual as well as short filaments of 2-5 cells were visible, indicating that septation did occur, but not as efficiently as in the wild-type. This suggests that while SLO7ORF3 can complement the growth defect caused by ZipA-depletion in *E. coli*, it only partially alleviates the defect in cell division. This may explain our inability to cure these strains of pCH32 (data not shown).

The growth of *E. coli* strain DH5α containing pSLO7ORF3 (ORF3 expressed from its own promoter) had a slightly reduced growth rate, which could be explained by a defect in cell divison. Examination of these cultures by light microscopy showed numerous filamentous cells, suggesting that overexpression of *N. gonorrhoeae* SLO7ORF3 blocks cell division (data not shown) consistent with the phenomenon observed upon overexpression of *zipA* in *E. coli* (140). However, when SLO7ORF3 was overexpressed from an IPTG-inducible promoter (pACYCLacORF3) and pCH32 (*zipA*$^+$*ftsZ*$^+$) was also present, this effect on growth was alleviated, presumably due to the excess FtsZ produced from this plasmid.

Figure 2-5. *N. gonorrhoeae zipA* partially alleviates filamentation phenotype of *E. coli* depleted for ZipA. *E. coli* strains harboring various *zipA* plasmids were cultured overnight at 30°C, then diluted 1:500 and shifted to 37°C and incubated for 2 hr (A-C) or 6 hr (D). Cultures were harvested and concentration adjusted to ~2 x 10^9 CFU/ml. 10 µl was spotted onto a microscope slide and gram-stained.

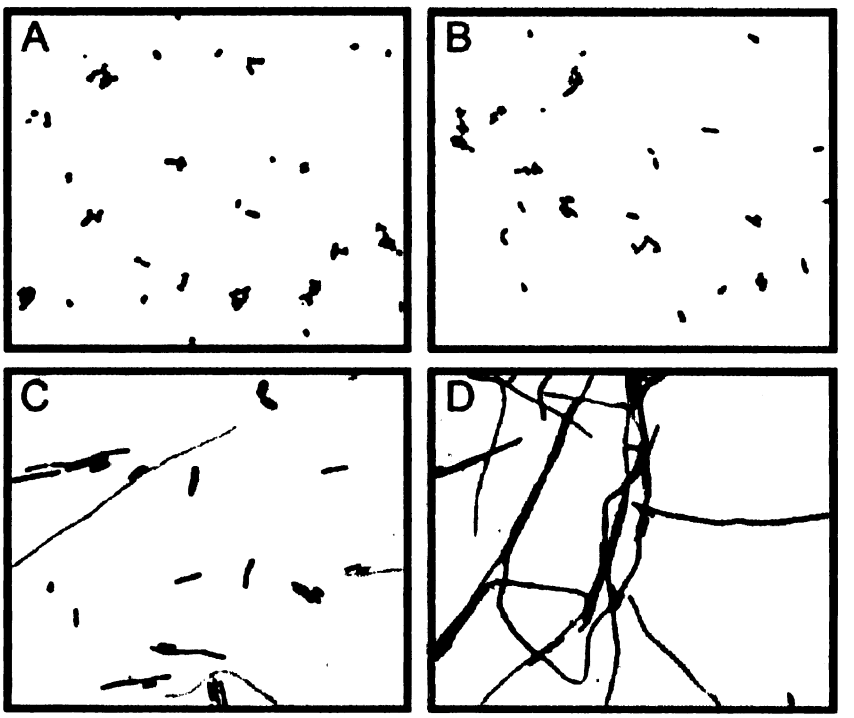
A: CH3(*zipA*$^+$)pCH32(*repA*^{ts}*zipA*$^+$*ftsZ*$^+$) pACYC-ORF3;

B: CH3(*zipA*$^+$)pCH32(*repA*^{ts}*zipA*$^+$*ftsZ*$^+$)pACYC184;

C: CH5(*zipA*::*aph*)pCH32(*repA*^{ts}*zipA*$^+$*ftsZ*$^+$) pACYC-ORF3;

D: CH5(*zipA*::*aph*)pCH32(*repA*^{ts}*zipA*$^+$*ftsZ*$^+$)pACYC184.

Photomicrographs (courtesy of Matti Kiupel) were taken using an Olympus Camedia E-10 digital camera attached to an Olympus BX40 microscope. Images were viewed under oil immersion at a magnification of 1000X.


A
B
C
D

DISCUSSION

The prokaryotic SRP is a ubiquitous protein targeting system that targets a subset of proteins to the cytoplasmic membrane co-translationally (73, 230, 279, 358, 407). All of the components of the SRP appear to be essential in bacteria (49, 113, 161, 273, 294, 434) suggesting that at least one of the proteins dependent on this system for localization is involved in an essential cell process. In this work, we set out to identify proteins from *N. gonorrhoeae* that utilize the SRP in an effort to identify essential CM proteins from this organism. Using a heterologous screening approach in *E. coli*, we identified several genes encoding proteins that appeared to utilize the SRP. Of these, one (SLO7ORF3) was determined to be a functional homolog of ZipA, an essential cell division protein in *E. coli*.

In *E. coli*, there are at least ten components (FtsA, -I, -K, -L, -N, -Q, -W, -Z, YbgQ, and ZipA) involved in the assembly of the septal ring, a membrane-associated cytoskeletal element that directs the formation of the division septum (50), reviewed in (325). In the initial stages of cell division FtsZ self-associates to accumulate at the prospective division site on the inner side of the CM, forming a structure called the Z ring (31), which then acts as a scaffold to which the other cell division proteins are recruited. ZipA is an essential protein that interacts with FtsZ (140), which while not required for Z ring formation, is required for recruitment of additional proteins to the Z ring (20, 29). Although ZipA is essential in *E. coli*, it appears to be less conserved in Gram-negative bacteria, and is the least conserved cell division proteins (325). Putative homologs of ZipA have been identified in several gram-negative bacteria (140, 325) mostly based on sequence homologies between the N-terminal and C-terminal domains of the protein,

both of which are reported to be important for ZipA function (22, 29). Predicted proteins with significant homology to ZipA have not been identified in genome sequences of gram-positive bacteria, archaea, and some gram-negative bacteria, leading to the conclusion that it has either divergently evolved or other proteins serve its function in the cell (325).

While the similarity of *N. gonorrhoeae* SLO7ORF3 to *E. coli* ZipA is low at the amino acid sequence level, there are significant similarities in key domains (Figure 2-3). These include: an N-terminal hydrophobic region (with no signal peptidase cleavage site) which is followed by a basic region (net positive charge of 5-8) which likely functions to anchor the protein in the CM; and central region rich in proline and glutamine residues (140). A ClustalW alignment of this fragment with the *N. gonorrhoeae* ZipA shows 15% identity and 49% similarity at the amino acid level in this region (Figure 2-3). Taken together, these observations and our data indicate that SLO7ORF3 does indeed encode the gonococcal cell division protein, ZipA. To the best of our knowledge, this is the first ZipA homolog identified in a non-rod-shaped bacterium. Thus, it will be interesting to more closely examine the role of ZipA in cell division in *N. gonorrhoeae*.

Of the ten proteins shown to be involved in formation of the division septum in *E. coli*, genes encoding seven of these have been identified in *N. gonorrhoeae*, *ftsZ*, *ftsA*, *ftsQ*, and *ftsI* (101, 332) and *ftsK*, *ftsW* and *ygbQ* (http://www.stdgen.lanl.gov). In addition to these, the *min* genes, which encode three proteins, MinC, MinD, and MinE, which are involved in positioning of the division septum have also been identified and characterized in *N. gonorrhoeae* (380). Studies of the function of these proteins in *N. gonorrhoeae* by Jo-Anne Dillon and coworkers indicates that they play similar roles in

cell division as in *E. coli* (315, 385). Genes encoding FtsE and FtsX have also been identified in *N. gonorrhoeae* (30), although it is not clear if they are involved in cell division in *Neisseria*. FtsL and FtsN are the only key cell division proteins remaining to be identified in *Neisseria*.

Numerous proteins have now been identified that utilize the SRP for targeting in bacteria (73, 230, 279, 358, 407, 408). These include an efflux pump (AcrB), several transport systems (LctP, LacY, KgtP, MalF, MtlA, ProW), and some membrane-associated enzymes (PgsA, MdoH, CdsA), none of which are essential for cell viability. In addition to these, however, are several proteins that may be involved in cell division. These include FtsQ (394, 409), which is essential in formation of the division septum (7, 109, 227, 429), and FtsE and FtsX (407). While most SRP-dependent proteins are polytopic membrane proteins, two SRP-dependent cell division proteins (FtsQ and FtsE) are bitopic, having a single membrane-spanning domain, similar to ZipA.

There are several links between cell division and the SRP. Depletion of Ffh, the signal sequence binding SRP component, or cells expressing a mutated *ffh* gene results in defects in cell division in *E. coli* (294, 335). A point mutation in *ffs*, which encodes the 4.5S SRP RNA, of *Caulobacter crescentus* confers a temperature-sensitive defect in cell division (434). And finally, *ftsY*, which encodes the SRP docking protein, FtsY, in *E. coli* was initially identified as part of an operon (*ftsYEX*) containing genes which are temperature-sensitive for filamentation (*fts*; (113), and FtsY-depleted cells are filamentous, apparently defective in completion of septation during cell division (227). All of these observations can be explained if one or more essential cell division proteins is dependent on the SRP for targeting. Since all of the other cell divison proteins, with the

exception of FtsZ and FtsA, are reported to localize to the CM, it will be interesting to determine whether any of these utilize the SRP.

Chapter 3

Expression capable library for studies of *Neisseria gonorrhoeae*, version 1.0

This manuscript has been previously published in BMC Microbiology:

Thomas Brettin, Michael R. Altherr, Ying Du, Roxie M. Mason, Alexandra Friedrich, Laura Potter, Chris Langford, Thomas J. Keller, Jason Jens, Heather Howie, Nathan J. Weyand, Susan Clary, Kimberly Prichard, Susi Wachocki, Erica Sodergren, Joseph P. Dillard, George Weinstock, Magdalene So, and Cindy Grove Arvidson, 2005, Expression capable library for studies of *Neisseria gonorrhoeae*, version 1.0, *BMC microbol.* **5**:50

Authors' contributions

Thomas Brettin: design of clone set construction, primer design
Michael R. Altherr: design of clone set construction, design and supervision of high-throughput PCR amplification of ORFs and recombination cloning into entry vectors
Ying Du: construction and tests of DNA microarrays, amplification and cloning of several ORFs not obtained in the initial batch cloning
Roxie M. Mason: cataloging of all cloning reactions processed at MSU (Arvidson lab), screening of transformants and preparation of clones for sequencing
Alexandra Friedrich: recombination of selected clones into expression vectors and analysis of proteins produced (Figure 3-1)
Laura Potter: initial project design, screening and cataloging of all cloning reactions processed at OHSU (So lab), screening of transformants and preparation of clones for sequencing
Chris Langford: batch BLAST analysis of sequencing results
Thomas J. Keller: batch BLAST analysis of sequencing results, wrote new program to faciliate analysis of sequence data
Jason Jens: processing of sequence data, batch program design for analysis of subset of clones from Gonococcal Genetic Island
Heather Howie: screening of transformants and preparation of clones for sequencing
Nathan J. Weyand: screening of transformants and preparation of clones for sequencing
Susan Clary: screening of transformants and preparation of clones for sequencing, amplification and cloning of several ORFs not obtained in the initial batch cloning
Kimberly Prichard: primer design
Susi Wachocki: high-throughput PCR amplification of ORFs and cloning into entry vectors
Erica Sodergren: sequencing of clone set
Joseph P. Dillard: contributed data of the Gonococcal Genetic Island prior to publication, participated in project design
George Weinstock: initial project design
Magdalene So: initial project design, supervison of all work performed at OHSU, substantial writing of manuscript
Cindy Grove Arvidson: initial project design, supervison of all work performed at MSU, screening of transformants from LANL, cloning of several ORFs not cloned in initial high-throughput cloning, collated and organized all sequence data, primary writing of manuscript, corresponding author.

ABSTRACT

The sexually transmitted disease, gonorrhea, is a serious health problem in developed as well as in developing countries, for which treatment continues to be a challenge. The recent completion of the genome sequence of the causative agent, *Neisseria gonorrhoeae*, opens up an entirely new set of approaches for studying this organism and the diseases it causes. Here, we describe the initial phases of the construction of an expression-capable clone set representing the protein-coding ORFs of the gonococcal genome using a recombination-based cloning system.

The clone set thus far includes 1672 of the 2250 predicted ORFs of the *N. gonorrhoeae* genome, of which 1393 (83%) are sequence-validated. Included in this set are 48 of the 61 ORFs of the gonococcal genetic island of strain MS11, not present in the sequenced genome of strain FA1090. L-arabinose-inducible glutathione-S-transferase (GST)-fusions were constructed from random clones and each was shown to express a fusion protein of the predicted size following induction, demonstrating the use of the recombination cloning system. PCR amplicons of each ORF used in the cloning reactions were spotted onto glass slides to produce DNA microarrays representing 2035 genes of the gonococcal genome. Pilot experiments indicate that these arrays are suitable for the analysis of global gene expression in gonococci.

This archived set of Gateway® entry clones will facilitate high-throughput genomic and proteomic studies of gonococcal genes using a variety of expression and analysis systems. In addition, the DNA arrays produced will allow us to generate gene expression profiles of gonococci grown in a wide variety of conditions. Together, the

resources produced in this work will facilitate experiments to dissect the molecular mechanisms of gonococcal pathogenesis on a global scale, and ultimately lead to the determination of the functions of unknown genes in the genome.

INTRODUCTION

Neisseria gonorrhoeae (gonococcus), a Gram-negative diplococcus, is one of two pathogenic members of the Neisseriaceae family of bacteria. *N. gonorrhoeae* is the causative agent of the sexually transmitted disease, gonorrhea, one of the oldest documented infectious diseases. Gonorrheal disease has significant morbidity both in the US and worldwide. According to the Centers for Disease Control (6), >350,000 cases of gonorrhea were reported in the United States in 2002. The World Health Organization (www.who.org) estimates that over 19 million cases occur annually in the African continent alone. Treatment of gonorrhea is increasingly problematic due to the high frequency of acquisition of resistance to multiple antibiotics (100, 361) and to the observation that gonococcal infection does not elicit protective immunity (342). Gonorrheal infections, though not usually life-threatening, also enhance the transmission of HIV (202).

N. gonorrhoeae is strictly a human pathogen, with no known animal reservoir. The bacterium has no environmental niche, and cannot survive outside the human host. In adults, *N. gonorrhoeae* is acquired primarily through sexual contact. However, the eyes of newborn infants may be infected by passing through an infected birth canal, resulting in the condition, ophthalmia neonatorum, which can lead to blindness. In most cases, gonococcal infections are limited to the urogenital tract, causing urethritis in men and cervicitis in women. Occasionally, gonococci cross the epithelial barrier to enter the bloodstream causing septicemia, and transit to the joints resulting in arthritis. In women, ascending infections from the endocervix can result in pelvic inflammatory disease,

salpingitis, tubal blockage and infertility. *N. gonorrhoeae* can also establish a carrier state in which apparently healthy individuals harbor culturable and infectious bacteria (98). Carriers are thought be important for disease dissemination. A recent study revealed that the gonococcal carriage rate in women was 6.7% in a major metropolitan area (404).

Due to the importance of *N. gonorrhoeae* to human health, much research effort has focussed on identifying virulence factors and elucidating the biochemical interactions of these factors with the host cell (146, 249, 310), with the goal of developing vaccines and alternative treatments. It is clear, however, that in order to fully understand the capabilities of this organism to cause disease and elude eradication, it will be necessary to ultimately determine the functions of a great deal more of the gene products encoded by the gonococcal genome. The recent genome sequencing makes possible a variety of genomic and proteomic studies of *N. gonorrhoeae*. To facilitate such studies, we have cloned into a bacteriophage lambda-based recombination cloning system (Gateway® (420), Invitrogen, Carlsbad, CA) 1624 of the 2189 predicted ORFs from the genome of *N. gonorrhoeae* strain FA1090 (43), and 48 of the 61 ORFs of the gonococcal genetic island (GGI) of strain MS11 (78, 142). This clone-set allows the generation of transcriptional and translational fusions without the necessity of additional cloning and sequencing. Coupled to the construction of this clone set, DNA microarrays were generated by spotting the insert DNA onto glass slides. Preliminary experiments with the clone set and DNA arrays indicate that this system is suitable for studies of expression of genes from *N. gonorrhoeae* in heterologous systems as well as for the study of global gene expression in this organism.

METHODS

Gene predictions and initial sequence preparation. All ORF IDs for strain FA1090 reference records can be found at the STDGEN *Neisseria gonorrhoeae* annotated genome sequence database (43). All gene sequences were prepared to include the natural start and stop codons. NG0540 and *NG*0634 have internal stop codons in the sequence database, and nothing was done to correct for this. Sequences of the ORFs of the MS11 GGI (142) have been deposited in the GenBank database with the accession number AY803022.

Signal peptide identification. In order to determine whether the ORFs contained signal sequences, the programs PSORT (272) and SignalP (272) were employed. If both programs predicted a cleavable signal peptide for a given ORF sequence, that constituted "high support". The result of the signal peptide analyses showed 149 sequences with high support for a signal peptide. For those gene nucleotide sequences with a high support signal peptide, the nucleotide sequence representing the signal peptide was removed prior to cloning primer design. When both programs predicted different cleavage sites, the cleavage site that represented the shorter signal peptide was chosen. The rationale for this choice was that it would be better to include a bit of the signal peptide in the PCR product than to exclude a bit of the mature protein in the PCR product.

Primer Design. In the first cycle, the forward and reverse primers were fixed at the same length. This was due to the ease at which Primer3 (329) could be used. The input file was all gene sequences for which no signal peptide sequence was detected (see above). For the second cycle, the prim.aux file was manually inspected. This file contained

information about successful primer picks where only a left or right primer could be picked. The strategy was to combine primers of different length. The potential for primer-dimer formation using this strategy was also assessed.

***N.g.* ORF cloning.** Primers for each of the genes were purchased from Illumina, Inc (San Diego, CA) and were designed to add sequences corresponding to part of the *attB* site necessary for recombination into the Gateway® entry vector, pDONR221 (Invitrogen). Primary amplification was done using genomic DNA at a concentration of ~10 ng/reaction and gene specific primers at 2.5 μM. Reaction mix contained dNTPs, reaction buffer, $MgCl_2$ and Taq polymerase as recommended by the manufacturer (Roche). Reactions conditions for primary PCR were as follows: denaturation at 94°C for 10 min; 10 cycles of 94°C 30 sec, 50°C 1 min, 74°C 1-5 min (depending on length of predicted product); 20 cycles of 94°C 30 sec, 55°C 1 min, 74°C 1-5 min (depending on length of predicted product); and a final extension at 74°C for 10 min. Following the primary amplification with the primer set, products were diluted 1:100 and 1 μl used as template for a secondary amplification using a pair of primers corresponding to the partial *attB* site common to all of the amplicons, and including additional sequences to generate a complete *attB* site for the cloning reaction. Reactions conditions for secondary PCR were as follows: denaturation at 94°C for 1 min; 5 cycles of 94°C 15 sec, 45°C 30 sec, 68°C 2 min; 15 cycles of 94°C 15 sec, 55°C 30 sec, 68°C 2 min. 5 μl of the 50 μl PCR was removed and used for cloning, and the remainder used for agarose gel analysis and printing of DNA arrays (*N.g.* array version 1.0, see below). Cloning reactions were performed according to the manufacturer's instructions (Invitrogen), transformed into *E.*

coli strain DH5α, and transformants selected for kanamycin resistance. Individual transformants were picked into wells of 96-well plates containing 100 μL broth containing kanamycin (50 mg/l) and the same toothpick then used to place a small amount of bacteria directly into another plate containing a PCR cocktail. PCR was done using the M13 universal primers, which flank the *att* sites of the entry vector, pDONR221. A product of 350 bp was observed if no insert was present, providing an internal control for the PCR reactions. Individual clones were identified, stocked in duplicate, and grown for DNA isolation for sequencing.

Sequencing. Sequencing runs from each end of the insert of each of the clones was determined to verify the ORF inserted. Complete sequence verification, (ie. both strands completely across the insert) was not done as it was determined to be impractical. DNA sequencing reactions were performed at the Baylor College of Medicine HGSC. Additional File 1 is a zipped file containing each of the sequence reads as .exp files generated using PREGAP4, and can be opened and read using word processing software. The file is separated into folders labelled SeqPlate #, which refers to sequencing plate number (1-21), and corresponds to the SP# designation in the list of clones in Additional File 2. SP18 sequencing reactions were done twice (SeqPlate 18-1, SeqPlate 18-2) as a sequencing reaction control, and the reactions of SP5 were analyzed three times (SeqPlate 5-1, SeqPlate 5-2, SeqPlate 5-3) as controls. SP20 does not exist, SP22 was not sent for sequencing, and the quality of SP17 sequence was too poor to be readable. The naming of the individual read files is as follows: BGACA(project code) # (1, 2, or 3 = reaction) D or F (primer D = forward, F = reverse) # (box, not same as SP) # (01-96;

well, 1 = A1, 2 = B1, 9 = A2, and so on) A or B (run). For example: BGACA3D1701A (found in SeqPlate 1 file) corresponds to SP1 well A1 sequenced with the forward primer (5' end of ORF), result from the third reaction and the first gel.

Gene expression analysis. pDONR221 derivatives chosen from the clone set were recombined with the destination vector, pDEST15 (Invitrogen), an N-terminal GST fusion vector. Plasmid DNA from the pDONR221 derivatives was isolated and incubated with pDEST15 in a LR recombination reaction performed according to the manufacturer's instructions. The recombination mixes were transformed into *E. coli* DH5α and transformants were selected on LB plates containing 50 mg/l Carbenicillin (Cb^{50}). To test the possibility of false positive clones, the overexpression clones were tested for growth on chloramphenicol (20 mg/l), on which expression recombinants should not grow since the chloramphenicol resistance gene of pDEST15 is replaced by the insert. The resulting plasmids were purified and transformed into *E. coli* BL21-AI (Invitrogen), which expresses T7 RNA polymerase from the *araBAD* promotor, and transformants were selected on LB Cb^{50} plates. BL21-AI strains containing the pDEST15 derivatives were grown overnight and used to inoculate fresh LB medium containing Cb^{50} to an OD_{600} of 0.05. Expression of the GST-fusion proteins in *E. coli* BL21-AI was induced at an OD_{600} of 0.5 by addition of L-arabinose to a final concentration of 0.2%. Aliquots were removed after 2 hr and total proteins electropheresed on 10% polyacrylamide SDS gels (201).

DNA microarray construction. PCR amplicons remaining from the cloning reactions (described above) were concentrated and then spotted in duplicate onto TeleChem SuperAmine glass slides (TeleChem International, Inc., Sunnyvale, CA) using a GeneMachines Omnigrid 100 (GeneMachines, Inc., San Carlos, CA) with 16 TeleChem Chipmaker 3 pins at the Genome Technology Support Facility (GTSF) at Michigan State University. Preparation of probes and hybridization conditions were as described (84). Hybridized microarray slides were scanned using a GenePix 4000B scanner (Axon Instruments, Union City, CA) and images were processed and analyzed using GenePix version 4.1 software.

Availability of clone set. The master library was used to generate a limited set of stock plates, which are stored at various locations as reference stocks. An additional set is stored at Michigan State University and is regarded as the working copy, which is the sole source of material for distribution. Interested parties should contact Cindy Arvidson (corresponding author) to arrange for a copy of the clone set. The clones will only be available as an intact set, individual clones or groups of clones are not available.

Availability of microarrays. DNA arrays will be generated by re-amplification of the amplicons used for the recombination-cloning reactions using primers recognizing the common sequences at the ends of the amplicons at the MSU GTSF. Interested parties should contact Cindy Arvidson (corresponding author) to request slides, which will be produced upon request.

RESULTS

Design of oligonucleotide primers. The goal of this project was to create a plasmid library representing the annotated ORFs of *N. gonorrhoeae*. The Gateway® Cloning System from Invitrogen (420) was selected for several reasons. First, Gateway® uses a recombination-based cloning method which has the added benefit that once an archival clone is sequence-validated, subsequent recombinants (ie. into expression vectors) do not need to be sequenced. Second, the initial clones lack transcriptional machinery such that the cloned ORFs are not expressed, thus avoiding problems from lethality due to troublesome gene products. Third, there are several expression and epitope-tagging vector options for the subsequent study of proteins encoded by the cloned ORFs, allowing a variety of approaches to study their functions. The high efficiency also lends itself to high throughput approaches that are suitable for automation.

A total of 2071 unique primer pairs were successfully designed for the 2189 annotated ORFs of the FA1090 genome (43) and 61 ORFs of the GGI (78, 142). These primers were gene specific, and their termini contain sequences for recombination cloning into the entry vector, pDONR221 (Invitrogen). All primers were designed such that the final recombination product yielded the native start codon at the 5' end of the gene, including the 206 of the 229 predicted ORFs with alternative (non-ATG) start codons. Since nearly half of the genes of this group (110/229) are annotated (that is encode putative proteins with significant similarity to proteins of known function), it is very possible that they are functional genes in the gonococcus and were thus included in the clone set design. Whether or not they encode functional proteins will ultimately depend on the results of future expression and mutation studies. The 23 ORFs of this

subset not included were less than 400 bp in length and considered too small for the Gateway® system (see below). For the 149 ORFs encoding predicted proteins with an amino-terminal signal sequence (as identified by PSORT (272) and SignalP (281)), sequences encoding the signal sequence were removed and an ATG start codon placed at the 5' end of the remaining coding sequence. This was done to reduce problems of expression of hydrophobic signal sequences and to facilitate future expression and targeting studies for such recombinant proteins.

The primer design strategy was iterative, starting with an annealing temperature range of 62°-72°C and primer length set to 18 nt. All ORFs were included in the first iteration, and those ORFs for which a primer pair was not selected were subjected to subsequent iterations. In each subsequent iteration the primer length parameter was increased by one, up to a maximum primer length of 34 nt. Primers larger than this were not designed in part due to cost and convenience of oligo synthesis in our 96-well format. Next, the annealing temperature range was expanded 5°C in both the positive and negative directions and each primer size from 18 to 34 nt was tried again. No further iterations were attempted after the annealing temperature range exceeded 47°-87°C. Failing to meet either of these criteria resulted in a primer pair not being designed for the ORF. There were a total of 177 ORFs for which no primers were designed (see Additional file 2), 174 from FA1090 and 3 from the GGI. Most of these ORFs (165/177 = 93%) were less than 500 bp in length, and were not included since the Gateway® system is reportedly less efficient for cloning fragments of this small size.

To each of the gene specific primer sequences for the 5' ends of the ORFs was added a 21 nt sequence including a consensus ribosome binding site (Shine-Dalgarno

sequence). To each of the gene specific primer sequences for the 3' ends of the ORFs was added 20 nt corresponding to the 3' end of the *attB2* site necessary for recombination into pDONR221. These were the primary PCR primers, the sequences of which are available on request. A single pair of primers was then designed for a secondary amplification to generate gene specific products containing the *attB* sites for the recombinatory cloning step. The 5' secondary primer contained the 24 nt *attB1* tail and the 21 nt sequence (Shine-Dalgarno) common to all of the primary 5' primer sequences. The 3' secondary primer contained the remaining 10 nt of the *attB2* site and the 20 nt *attB2* sequence common to all of the primary 3' primer sequences. The *attB* sequences were as recommended in the Gateway® manual.

PCR amplification of ORFs. The first round of PCR, using gene specific primers, included a total of 2071 different primer pairs. Plates 1 and 2 were organized as pilot reactions and contained primers designed to amplify products ranging from 143 bp to 3485 bp in length. Genomic DNA from *N. gonorrhoeae* strain FA1090 was used as a template for the reactions. Agarose gel analysis of the amplicons showed that 173 of the 192 reactions yielded a product of the expected size, an efficiency of 90%. Plates 3-21 were then arranged with increasing size of expected product, with plate 3 containing the smallest products and plate 21 the largest. Several primer pairs (from plates 1 and 2) were included in plates 3-21 as internal controls. Plate 22 was an additional control plate, with one half of the plate (rows A-D) duplicated on the other half (rows E-H). Plate 23 corresponded to the genes of the GGI present in strain MS11 (78, 142), and MS11 genomic DNA was used as the template for this plate of reactions. 5 μl of each reaction

from the first round of PCR of each plate were run on agarose gels and scored for production of a product and whether it was of the expected size. The results showed 89% of the reactions to produce a product of the correct size.

Following the primary amplification with gene specific primers, all products (in the original 96-well format) were diluted 1:100 and an aliquot subjected to a secondary amplification using a pair of primers corresponding to the sequences common to all of the amplicons, and including additional sequences to generate a complete *attB* site for the cloning reaction. Aliquots of the secondary amplification were then used directly in the recombination reaction to generate entry clones.

Construction of the library. Secondary amplicons were inserted into pDONR221 by *in vitro* recombination between the *attB* sites introduced at the ends of the amplicons and the *attP* sequences of the vector, maintaining the 96-well format arrangement. Cloning reactions were then transformed into *E. coli* strain DH5α and a portion of the transformation mix plated on LB plates containing kanamycin. Individual transformants were screened by PCR to determine the presence of and size of the insert. For the first round of screening, four independent transformants from each reaction were screened, maintaining the original 96-well format. A product of 350 bp was observed if no insert was present, a positive clone was identified as having a product 350 bp larger than the size of the corresponding primary PCR product.

For the initial set of 2147 transformation reactions, clones corresponding to 1165 genes were identified in the first four transformants screened, an efficiency of 54%. This efficiency varied greatly with the predicted size of insert, the smallest inserts (plate 3)

were 83% positive in the first four screened and the next largest inserts (plate 20) had 17% positive in the first four screened. Plate 21, which had the largest inserts, only yielded 3 transformants as positive after several rounds of screening. Additional transformants for those clones not identified in the first round of screening were individually cultured, screened by PCR, and positive clones frozen down as they were identified. This approach yielded an additional 354 clones.

Following the initial rounds of screening, a list of missing clones was generated and the amplification and cloning steps repeated, optimizing several parameters and analyzing on an individual basis. This approach yielded an additional 283 clones, for a total of 1802 which were subsequently sequenced. Arrangement of the clones in plates for the master set and for sequencing was on an "as identified" basis, such that they are not arranged as in the original 96-well format. Each of the 21 plates contain viable clones in up to 95 of the 96 available positions, with position H12 (and additional wells on some plates) left empty for controls, providing a unique identity for several of the plates.

Sequence verification of the clones. Transformants identified as having an insert of the predicted size were grown in 96-well plates and DNA isolated for sequence analysis. DNA isolation and sequencing was done by at the Human Genome Sequencing Center at Baylor College of Medicine (HGSC) using the same set of primers used to screen transformants for insert size. Sequence reads were posted onto an HGSC website and subsequently downloaded by FTP. DNA reads were processed initially using the STADEN DNA analysis software package (3). Binary files were converted into .exp files using PREGAP4 to generate text files for each individual sequence read, with sequence

quality cutoffs. These data are provided in Additional File 1 (AF1 exp sequence files.zip), and a description of the labelling scheme for the files is in the Methods section.

The data were next analyzed by BLAST (11) against the *N. gonorrhoeae* genome sequence database (43). Clones expected to contain inserts from the GGI, not present in FA1090, were analyzed by BLAST 2 (390) using sequence of the individual GGI ORFs ((142), GenBank accession number AY803022). BLAST results were then manually tabulated in a file containing the expected gene for each archived clone. Of the 1802 sequenced clones, 58 were expected to be duplicates, leaving 1744 unique clones expected in the clone set. 1550 of the sequences were readable and corresponded to a predicted ORF from *N. gonorrhoeae*, 1399 of them unique, corresponding to 151 duplicates. Some duplicates were expected, and the remainder likely due to cross contamination from neighboring wells. Of those sequence validated, 55 were not in positions predicted. Most of these were due to human error, such as obvious well transpositions and numbering transpositions. 26 of these, however, had inserts in a backwards orientation and were incomplete. These clones will not be usable in subsequent recombination reactions using the Gateway® system. Together, these data indicate that at present we have a collection of 1672 individual clones from *N. gonorrhoeae*, 48 of which are from the GGI, and 83% of which have been sequence validated. A list of genes in the clone set with sequencing result information can be found in Additional File 2 (AF2 NG clone set seq status.xls).

Overexpression of randomly chosen ORFs. In order to examine the flexibility of using the clone set to construct various derivatives for which the Gateway® system was designed, three randomly selected pDONR221 derivatives were used to create inducible

glutathione-S-transferase (GST) fusions. Plasmids containing ORFs *NG*1490 (*aspS,* encodes aspartyl-tRNA synthetase), *NG*1561 (*xthA,* encodes exodeoxyribonuclease III), and *NG*1641 (*pivNG,* encodes a pilin gene inverting homolog, PivNG) are predicted to encode native proteins of 9.6, 29.0, and 36.2 kDa respectively. Plasmid DNA of pDONR221 derivatives containing these three genes were recombined with the destination vector pDEST15, an N-terminal GST fusion vector. The resulting recombinants were then transformed into *E. coli* BL21-AI for expression analysis. SDS-PAGE analysis of the proteins after a 2 hr induction with 0.2% L-arabinose is shown in Figure 3-1. The results show high levels of induction for each of the fusion proteins, with the sizes as predicted (GST adds 29 kDa).

DNA microarray production. Since a very small portion of the secondary amplicons were used for the cloning reactions (5 µl of a 50 µl reaction), the remaining products were used to produce a set of DNA arrays for gene expression analysis. Gel electrophoresis (data not shown) indicated that the efficiency after the secondary amplification was 81%, representing 1681 ORFs of a total of 2071. To generate a more complete DNA array, a second set of primers (360) were designed to amplify internal portions of those ORFs not visible following the secondary PCR. Primers to amplify sequences of two small RNAs were also designed: *NG*0892.1, *ffs*, encodes the 4.5S RNA component of the gonococcal signal recognition particle (102); and *NG*0880.1, *tmRNA*, encodes an RNA that tags abnormal proteins in the cell arising from stalled ribosomes and targets them for proteolysis (133). Gel analysis of the amplicons produced from the internal PCR primers showed 294 of the 362 to produce products of the expected size, for

an efficiency of 82%. Together, the cloning amplicons (1681) and internal ORF and RNA amplicons (294) represent a minimum of 1975 ORFs of the *N. gonorrhoeae* genome. This is a minimal estimate since all products (regardles of gel result) were to be spotted, and some products might be present, but at amounts too low to be visualized. DNA samples were processed and spotted onto glass slides as described in Methods. As a first test, the arrays were hybridized with a Cy3-labelled random nonamer oligonucleotide (Qiagen). A scan of the slide at 532 nm showed spots at the appropriate positions where DNA had been spotted, and blank spots at the buffer control spots. The next test was to hybridize the arrays with labelled genomic DNA. Total DNA from *N. gonorrhoeae* strain MS11 was digested with *Rsa*I and labelled by including Cy3-dCTP in a random-primed Klenow DNA polymerase reaction (Roche Applied Science, Indianapolis, IN). DNA arrays were hybridized and then scanned at 532 nm. Valid hybridization signals (at least one standard deviation above background) were detected for 98% of the spots expected to contain DNA, with those of the MS11-specific island comparable in intensity to those of FA1090 amplified DNA. Overall, the results showed valid signals for 2035 individual genes, with several in duplicate. The fact that this number is higher than expected based on agarose gel analysis of the amplicons before spotting (see above) indicates that some of the reactions produced a product, but at amounts too low to be visualized by ethidium bromide (EtBr) staining. Thus, these arrays represent 90% (2035/2250) of the predicted ORFs of *N. gonorrhoeae* (including 58 ORFs of the GGI; (78), (142)) and 98% of those for which primers were designed.

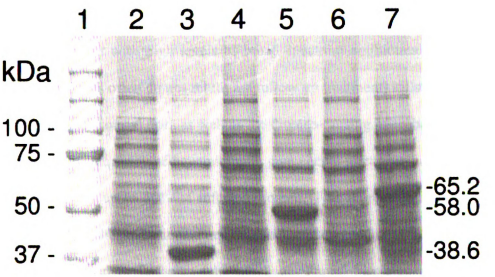


Figure 3-1. SDS-PAGE analysis of NG ORF-GST fusions. Equivalent amounts of total protein was boiled in sample buffer and electrophoresed on 10% polyacrylamide gels. Gels were stained with Coomassie Blue. Lane 1: MW markers; lane 2: NG1490(AspS)-GST, uninduced; lane 3: NG1490 induced (38.6 kDa); lane 4: NG1561(XthA)-GST uninduced; lane 5: NG1561 induced (58.0 kDa); lane 6: NG1641(PivNG)-GST uninduced; lane 7: NG1641 induced (65.2 kDa).

DISCUSSION

Despite the advent of antibiotics in the 1940's, disease due to infection with *N. gonorrhoeae* remains a major health problem worldwide. The reasons for this are multi-fold. First, resistance to antibiotics by *N. gonorrhoeae* continues to rise (6, 100, 361). In addition, treatment with high levels of broad spectrum antibiotics (which is frequently done since patients often do not return for follow up treatment) kills many bacteria of the (often beneficial) normal flora as well as the disease-causing microbe. Second, there is an incredibly high frequency of asymptomatic gonococcal infection, occurring in 5-10% of infected men and up to 50% of infected women. This represents a major reservoir for transmission of the infection. Furthermore, undiagnosed and untreated gonococcal salpingitis can lead to fallopian tube blockage. Partial blockage can result in ectopic pregnancy, which can be life threatening, and complete blockage of the fallopian tubes often leads to infertility. Third, development of a vaccine to protect against gonorrhea has been seriously hampered by the observation that gonococcal infection does not elicit protective immunity (342). Patients can be reinfected following treatment, and can even be infected by multiple strains at a given time. Thus, alternative treatments and preventative strategies for gonococcal infection are sorely needed.

As a first step in the identification of such alternative treatments and preventatives, it will be necessary to more thoroughly understand the biology of the gonococcus and the molecular mechanisms involved in its interactions with the host environment. Much of the studies to date have focused primarily on identifying the molecules on the surface of the bacterium that directly interact with the host, and the toxic moieties involved in damage to host cells. Many of the molecules identified are

outer membrane (OM) components (97, 311, 331, 380, 411), and iron utilization proteins (reviewed in (344)). A few are secreted (364), or shed in blebs (126, 128, 245). There has also been significant work in the identification of eukaryotic host cell receptors for gonococcal surface proteins (reviewed in (250)).

Interactions between gonococci and epithelial cells are beginning to be unraveled. Chen and Clark showed that contact with Hec-1-B human endocervical epithelial cells increases gonococcal infectiveness and that the process involves, in part, *de novo* protein synthesis by the bacterium (249). The gonococcal type IV pilus (Tfp) and Opa proteins promote attachment, invasion and trans-epithelial trafficking. The mechanisms underlying Tfp- and Opa-mediated virulence are not yet understood, but these surface structures modulate a series of events in the infected epithelial cell, among them Ca^{2+} fluxes (20, 180), cortical rearrangements (246), and receptor phosphorylation (62, 124, 209). Tfp retraction enhances the activation of stress-responsive kinases and the transcription of cytoprotective genes in the infected cell (165), and triggers the infected cell to produce a molecule that alters bacterial motility behavior (210). Finally, binding of gonococci to primary urethral cells up-regulates anti-apoptotic factors (33). These and other observations indicate that gonococcal infection requires the active participation of both the bacterium and the host cell.

The recent completion of the annotated genome sequence of *N. gonorrhoeae* (43, 321), coupled with the development of high throughput methods for the analysis of gene expression and function, provide an opportunity to significantly advance the study of gonococcal biology and pathogenesis. Like many sequenced genomes, nearly half (44%) of the genes of the annotated gonococcal genome encode hypothetical proteins of

unknown function. Furthermore, many of the annotations are based on homologies at the nucleotide and/or amino acid level, and the actual function of the gonococcal proteins have not been demonstrated. In order to realize the full potential of information gleaned from the genome sequence of this (and any) organism, it will be necessary to assign functions to all of the genes of the genome. The newly emerging fields of functional genomics and proteomics offer much promise towards achieving the goal of eventual assignment of functions for each and every gene in a given organism.

In this work, we describe the initial phases of the construction of an expression-capable clone set representing the annotated ORFs of the gonococcal genome using a recombination-based cloning system. The advantages of the system used for this set are numerous. 1) The original sequences in the clone set contain only the ORFs, not the gene expression sequences, thus avoiding the issue of expression-related lethality of the recombinants. 2) The clones can be transferred to a number of expression systems (prokaryotic and eukaryotic), allowing the regulation of genes for overproduction of proteins, or the production of proteins out of the context of the particular environments so as to study their functions. 3) The clones can also be transferred to vectors that result in epitope fusions, such as hexa-histidine, GST, green fluorescent protein (GFP), Lumio™, etc., to the proteins of interest. Protein fusions are useful in localizing proteins (within the bacterium or infected cell), in determining protein-interacting partners, in allowing smaller step purification protocols for structural and activity studies, and for antibody production. This ability has been demonstrated by constructing IPTG-inducible GST-fusions from three random clones from this set (Figure 3-1). 4) The clone set is also catalogued in such a way that individual clones of interest are easily identified and

recovered from the clone bank (see Additional file 2). 5) Entry clones can also be used to create knockouts by *in vitro* transposition (83, 84) or shuttle mutagenesis (355) followed by transformation into naturally competent gonococci (115). This system is also ammenable to automation, thus increasing the potential output and consistency in the data obtained.

The *N. gonorrhoeae* clone set thus far includes 1672 of the 2250 predicted ORFs of the genome (43), of which 83% are sequence-validated. Included in this set are 48 of the 61 ORFs of the MS11 GGI (78, 142). While this clone set is not yet complete, we believe these initial efforts have resulted in generating a valuable resource for the *Neisseria* research community. It is hoped that others in the community will share compatible reagents and add to the clone set, making it more comprehensive over time.

Coupled to the clone set construction, a PCR-amplicon based DNA microarray was generated. DNA microarrays are a powerful tool that allow one to measure relative transcript levels for essentially each gene of the genome simultaneously. These DNA arrays represent 2035 ORFs of the *N. gonorrhoeae* genome: 1977 from strain FA1090 (77) and 58 from the MS11 GGI (78), comprising 90% of the genes of the genome. Preliminary studies show that these arrays are suitable for examining global gene expression in *N. gonorrhoeae*.

Many bacterial pathogens are known to respond to changes in their physical environment, often integrating responses to several environmental signals via complex regulatory networks to control expression of a variety of genes (65, 79, 225, 293, 366). The examples of regulatory systems characterized in gonococci are few, with the best characterized being the response to iron availability (348, 431) and antimicrobial

compounds (139, 359). The advent of microarray technologies has opened avenues of research on global gene expression in both prokaryotes and eukaryotes, providing opportunities for studying a variety of organisms, including such genetically intractable microbes as *Trepanema pallidum* (242) and *Chlamydia trachomatis* (280). Thus, the use of DNA arrays will allow us to more fully explore the response of *N. gonorrhoeae* to environmental signals at the gene expression level. Since genes are typically only transcribed when the gene product function is required, expression profiles and cluster analyses will allow us to begin to determine the functions of unknown genes in the genome. Thus far, use of these DNA arrays has led to the identification of a regulator involved in the modulation of gonococcal gene expression upon adherence to epithelial cells in (84).

In summary, the tools described in this work represent a resource which will facilitate experiments to dissect the molecular mechanisms of gonococcal pathogenesis on a global scale. Combining these tools with the gonococcal infection models [tissue culture (360), organ culture (239-241), and the mouse model (170)], will allow us to make significant advances in the study of this important pathogen, thus providing us with the knowledge necessary to design therapeutics with which to treat and prevent gonococcal disease.

Chapter 4

Global gene expression and the role of sigma factors in *Neisseria gonorrhoeae* in interactions with epithelial cells

This manuscript has been previously published in Infection and Immunity.

Du,Y. Lenz, J. and C.G. Arvidson. 2005 Global Gene Expression and the Role of Sigma Factors in *Neisseria gonorrhoeae* in Interactions with Epithelial Cells. *Infect. Immun.* **73**:4834-4845

Arvidson, CG performed the Southern blot experiment on MS11 genomic DNA.
Lenz, J, performed the heat shock experiment.

ABSTRACT

Like many bacterial pathogens, *Neisseria gonorrhoeae* must adapt to environmental changes in order to successfully colonize and proliferate in a new host. Modulation of gene expression in response to environmental signals is an efficient mechanism used by bacteria to achieve this goal. Using DNA microarrays and a tissue culture model for gonococcal infection, we examined global changes in gene expression in *N. gonorrhoeae* in response to adherence to host cells. Among those genes induced upon adherence to human epithelial cells in culture was *rpoH*, which encodes a homolog of the heat shock sigma factor, σ^{32} (RpoH), as well as genes of the RpoH regulon, *groEL* and *groES*. Attempts to construct an *rpoH* null mutant in *N. gonorrhoeae* were unsuccessful, suggesting that RpoH is essential for viability of *N. gonorrhoeae*. The extra-cytoplasmic sigma factor, RpoE (σ^{E}), while known to regulate *rpoH* in other bacteria, was found not to be necessary for the up-regulation of *rpoH* in gonococci upon adherence to host cells. To examine the role of RpoH in host cell interactions, a *N. gonorrhoeae* strain conditionally expressing *rpoH* was constructed. The results of our experiments showed that while induction of *rpoH* expression is not necessary for adherence of gonococci to epithelial cells, it is important for the subsequent invasion step, as gonococci depleted for *rpoH* invade cells 2-3 fold less efficiently than a wild-type strain. Taken together, these results indicate that σ^{32}, but not σ^{E}, is important for the response of gonococci in the initial steps of an infection.

INTRODUCTION

Neisseria gonorrhoeae (gonococcus, GC), an obligate human pathogen, is the causative agent of the sexually transmitted disease (STD) gonorrhea. Gonococcal disease is prevalent world-wide, with ~500,000 reported cases each year in the US alone. It is estimated that at least as many additional cases go unreported. While aggressive safe sex practice campaigns significantly reduced the incidence of several STDs in the 1980's and 1990's, the last five years have shown a gradual increase in gonorrhea as well as another bacterial STD, syphilis (Centers for Disease Control and Prevention, http://www.cdc.gov/std/stats/toc2002.htm). There are several challenges in the treatment of gonococcal disease, including continuing acquisition of antibiotic resistance (2, 100, 361), a high incidence of asymptomatic infection (especially in women), and the observation that gonococcal infection does not elicit protective immunity (343). Patients can be reinfected following treatment, and can even be infected by multiple strains at a given time. Thus, understanding the biology of this organism and how it senses and responds to its environment will be key in the development of alternative treatments and preventative strategies for gonococcal infection.

Gonorrhea is typically a disease of the urogenital tract, although the bacterium can also infect tissues of the pharynx, rectum, and the conjunctiva (which can lead to blindness in newborns who are infected during birth). The initial step of a gonococcal infection is colonization of mucosal tissues. Following adherence to mucosal epithelial cells, the bacteria then enter the cells and traverse across them to exit to the subepithelial space where they elicit an acute inflammatory response, resulting in the symptoms

characteristic of gonorrhea. In some cases, the organism will ascend from the site of infection into the fallopian tubes resulting in gonococcal salpingitis, a leading cause of infertility in women. It will also occasionally spread to other tissues in both men and women resulting in a full-blown bacteremia, termed disseminated gonococcal infection (DGI), a major sequelae of which is arthritis.

The colonization of mucosal tissues by *N. gonorrhoeae* is a critical first step in a gonococcal infection. Adherence to cells of the mucosal epithelia is essential to prevent the bacterium from being washed away by urine (in the male urethra) or vaginal fluid (in the female genital tract). Colonization of the host epithelia by gonococci is a complex process involving multiple components of both the bacterial and host cell. The incoming bacterium first binds to epithelial cells via interactions between specific receptors on the bacterial and host cell surfaces. The primary adhesins on the gonococcal cell surface are the type IV pili (380, 384), although there are additional surface structures of the bacterium that can participate in adherence (97, 127, 176, 194, 232, 233, 341, 419, 428). Binding of gonococcal pili to host cells results in the induction of signal transduction pathways in the eukaryotic cell that affect multiple cellular processes (20, 181, 209, 276, 278, 296). Adherence of piliated gonococci to epithelial cells also results in a variety of rearrangements in the components of the cellular cytoskeleton (249)] as well as significant changes in host cell gene expression (295). However, our understanding of gonococcal infection with respect to the specific response of *N. gonorrhoeae* at the level of gene expression is still incomplete.

Our hypothesis is that the initial contact between gonococci and host epithelial cells signals the bacterium to modulate the expression of genes that will allow it to

colonize and proliferate in the new host. In this work, we sought to identify genes that are triggered by the adherence of gonococci to host cells and to identify regulatory phenomena that are involved in this response. Using a tissue culture model of infection and gonococcal DNA microarrays, we examined changes in patterns of gene expression in the gonococcus in response to contact with human epithelial cells in culture. Here we report that *rpoH*, which encodes RpoH, a homolog of the heat shock sigma factor, σ^{32}, as well as genes expected to be controlled by RpoH, are induced after host cell contact. Further studies show that *rpoH* is essential to *N. gonorrhoeae* and is important for the invasion of, but not adherence to human epithelial cells. These data therefore suggest a role for the heat shock regulon in gonococcal pathogenesis.

MATERIALS AND METHODS

Growth and construction of bacterial strains. *E. coli* strain DH5α was used for all recombinant DNA manipulations (334) and was grown in Luria broth (LB) supplemented as necessary with ampicillin (Ap) at 100 mg/l, chloramphenicol (Cm) at 20 mg/l, kanamycin (Kn) at 50 mg/l, or erythromycin (Em) at 300 mg/l.

N. gonorrhoeae strain MS11 (P^+ Tr) (349) was grown in a humidified 5% CO_2 atmosphere in GC medium (Difco Laboratories, Sparks, MD) with supplements (187). When necessary, antibiotics (Em and Cm) were added at 3 mg/l and 6 mg/l respectively. *N. gonorrhoeae* transformation and genomic DNA isolation were performed as described (349, 355). Gonococcal strains used in these studies were predominantly Opa^- and P^+ as determined by colony microscopy.

DNA manipulations. Cloning vectors used were pHSS6 (356), pDONR221 (Invitrogen, Carlsbad, CA), pET24a (Novagen, Madison, WI) and pKH35 (142). Restriction enzymes, T4 DNA ligase (New England Biolabs, Beverly, MA) and Gateway™ PCR cloning kits (Invitrogen) were used according to the manufacturer's recommendations. Polymerase chain reaction (PCR) was done with Taq DNA polymerase (91) in a GeneAmp 9700 thermocycler (Applied Biosystems, Foster City, CA). When necessary, amplification products were purified using the QIAquick PCR Purification kit (Qiagen, Valencia, CA). Oligonucleotide primers were synthesized at the Michigan State University Macromolecular Structure, Sequencing and Synthesis Facility (MSU GTSF, sequences available on request). DNA sequence determination was done by the MSU GTSF and analyzed using the Oxford Molecular Group DNA analysis program Omiga (Accelrys,

San Diego, CA). Transposon mutagenesis was performed by in vitro transposition using EZ::TN™ transposase (Epicentre Technologies, Madison, WI) and the modified transposon TnErmUP as described (83). The position of transposon insertions was determined by PCR using primers homologous to the ends of transposon (SqFP and SqRP; Epicentre) and to the ends of the gene in question and corroborated by restriction analysis.

Cell culture. Human epithelial cell line A431 (ATCC CRL 1555) was grown at 37°C in a humidified 5% CO_2 atmosphere in Dulbecco modified Eagle medium (DMEM, Gibco/Invitrogen) supplemented with 5% fetal calf serum (FCS, Gibco). Adhesion and invasion assays were performed as described (19, 419). Briefly, gonococci grown on GC agar (16-20 h cultures) were swabbed into GC broth and concentration determined spectrophotometrically. Bacteria were diluted in DMEM/5% FCS to appropriate concentrations, and the bacterial suspension added to A431 cells grown to 50-90% confluency in 24-well or 100 mM tissue culture plates as indicated in the text. Serial dilutions of each bacterial inoculum were diluted and plated on GC agar plates to determine the actual number colony forming units (CFU) added. Infected cells were incubated at 37°C w/5% CO_2 for 3 h (for adherence assays) or 7 h (for invasion assays). Following incubation, the samples were divided into two sets (for adherence assays) or three sets (for invasion assays). For the first set (total CFU), bacteria in the supernatant were transferred to a sterile tube. Cells (with adherent bacteria) were lifted with saponin (1% in GCB) and this suspension added to the bacteria from the supernatant, and serial dilutions were plated onto GC agar. This represents the total number of CFU in the well

at the end of the experiment, a necessary control since gonococci multiply in the cell culture media thoughout the experiment, and adherence is measured as a function of total number of bacteria at the end of the experiment. For the second set (cell-associated CFU), infected cells were washed with PBS to remove non-adherent bacteria and cells (with adherent bacteria) lifted with 1% saponin in GC broth. Serial dilutions were plated to determine cell associated CFU. The ratio of cell-associated CFU to total CFU at the end of the experiment was defined as the adhesion frequency. For invasion assays, a third set of wells were washed to remove non-adherent bacteria (as above) and fresh DMEM/5% FCS containing 50 mg/l gentamicin (Gm^{50}) added to each well to kill extracellular bacteria. Following incubation at 37°C w/5% CO_2 for 1 h, Gm-containing media was removed, cells were washed with PBS, and cells lifted with 1% saponin in GC broth. Serial dilutions were plated to determine intracellular CFU. The ratio of Gm^R CFU to cell-associated CFU at the end of the experiment was defined as the invasion frequency.

RNA isolation. RNA was isolated from adherent bacteria or bacteria grown in the absence of cells using Trizol reagent (Invitrogen). RNA was quantified spectrophotometrically, and quality assessed by agarose gel electrophoresis.

Construction of GC DNA arrays. DNA microarrays were made by amplification of 2043 ORFs of the *N. gonorrhoeae* genome, 1985 from strain FA1090 (43, 77) and 58 from the gonococcal genetic island (GGI) of *N. gonorrhoeae* strain MS11 (78, 142). Sizes of the amplicons spotted ranged from 143 bp to 3485 bp in length, and corresponded to the predicted ORF of each gene. An additional 362 amplicons

corresponding to internal ORF sequences were generated for genes with insufficient DNA observed upon agarose gel electrophoresis of the initial PCR. PCR amplicons were spotted in duplicate onto SuperAmine glass slides (TeleChem International Inc., Sunnyvale, CA) at the MSU GTSF. The presence of DNA on the arrays was validated by hybridization with a Cy3-labelled random nonamer oligonucleotide (Qiagen). A scan of the slide at 532 nm showed spots at the appropriate positions where DNA had been spotted, and blank spots at the buffer control spots (data not shown). DNA hybridization of the arrays with MS11 genomic DNA showed valid hybridization signals (above background) for 98% of the genes spotted on the slides, and indicated that these arrays would be suitable for the analysis of gene expression in *N. gonorrhoeae* strain MS11. (A manuscript detailing the construction and use of the arrays has been submitted for publication.)

Microarray hybridization. 10 μg of total bacterial RNA was labeled using the CyScribe first-strand labelling kit (Amersham Biosciences, Piscataway, NJ) which employs random primed reverse transcription in the presence of Cy3 or Cy5-dCTP. Cy3 and Cy5 labeled cDNA were purified on QIAquick Cleanup columns (Qiagen) and concentrated using Microcon YM-30 microcentrifugation units (Millipore, Billerica, MA).

DNA array slides were pre-hybridized at 42°C in prehybridization buffer (5X SSC, 0.1% SDS, 1% BSA). For hybridization, Cy3- and Cy5-labelled probes were mixed (10 μl each) with 1 μl 1 mg/ml sheared, sonicated herring sperm DNA and denatured by boiling for 5 min, followed by chilling on ice. 20 μl 4X hybridization buffer (Amersham) and 40 μl formamide were added to the denatured DNA and the entire hybridization mix

was pipetted onto the prehybridized array slide. Following hybridization overnight at 42°C, the coverslips were removed by gently immersing in the first wash (1X SSC; 0.2% SDS). Washes were: once in 1X SSC; 0.2% SDS at 42°C, twice in 0.1X SSC; 0.2% SDS at room temperature, once in 0.1X SSC. Slides were then dried and scanned using a GenePix 400 scanner (Axon Instruments, Union City, CA) and images were processed and analyzed using GenePix version 4.1 software.

DNA array data analysis. Data from four independent experiments (biological replicates) including one in which the dyes were swapped, were normalized to eliminate labelling artifacts (intensity based normalization) and expression ratios determined. In these four hybridization experiments, 75-85% of the spots on the slide had at least 40% of the pixels >1 standard deviation above background in at least one channel (535 or 632 nm wavelength). Spots not above this cutoff were not included in the normalization or outlier analyses. Outliers were identified by iterative outlier analysis in which three successive rounds of analysis are done to determine genes with ratios ($\log_2$ transformed) greater than 2.5 standard deviations from the mean (47). Confidence limits of the data were calculated including a standard error factor (distance from gene's average value to the nearest 2.5 standard deviation cutoff) and the number of standard deviations separating the spot from the 2.5 standard deviation cutoff. This data is not included in the tables, but was taken into consideration when determining genes for which data from multiple spots or experiments were not consistent.

RT-PCR. Total bacterial RNA was prepared as described above. cDNA was synthesized from 100 ng RNA with Superscript II RNase H^- reverse transcriptase (RT) (Invitrogen) using random nonamers (Sigma-Aldrich, St. Louis, MO). Gene specific primers for *rpoH, groES* and *16S rRNA* (internal control) were used in the subsequent PCR, which was carried out for 30 cycles. Negative controls for each experiment included a no template control and a no RT control where RNA was added as a template for the final PCR (to rule out genomic DNA contamination of RNA preparations). Products were analyzed by agarose gel electrophoresis and stained with EtBr for photographing and/or quantification using KODAK 1D Image Analysis Software (Eastman Kodak, Rochester, NY).

RESULTS

Analysis of global gene expression in gonococci adherent to cells in culture. Several cell lines have been used to characterize interactions between gonococci and host cells (19, 60, 164, 167, 182, 200, 246, 247, 274, 360, 419). Each cell line has slightly different features with respect to specifics of interactions with gonococci and handling in the laboratory. For the experiments described in this work, A431 cells (110), an epithelial cell line derived from the endocervix (275) were used. Gonococci have been shown to adhere to and invade A431 cells at a high frequency (19, 164, 209, 246, 371) and there have been several reports investigating the response of these cells to gonococcal adherence at the molecular level (20, 21, 40, 209).

Total RNA was isolated from A431 cells alone, bacteria alone (MS11), and A431 cells infected with MS11 for 3 h. A 3 h infection time was chosen since this has been reported as the minimum time where most gonococci that will adhere to cells have done so, yet few if any, have invaded (360). cDNA was synthesized from each RNA incorporating digoxigenin-11-dUTP (Roche Applied Science, Indianapolis, IN) and used to probe a Southern blot of *Cla*I-digested MS11 genomic DNA (19). The results of this experiment (data not shown) showed that cDNA from bacteria alone and cells infected with bacteria gave a strong signal following chemiluminescent detection, with intense bands at positions corresponding to the bacterial ribosomal RNAs, and a fainter banding pattern (upon extended exposure) that is presumably from mRNA. cDNA from uninfected A431 cells gave no signal at all, even after prolonged exposure. This indicated that any remaining eukaryotic RNA in the infected cell samples (minimal since the cells

are lysed before bacteria are harvested) would not create a background problem in DNA array hybridizations.

We next analyzed global gene expression in gonococci adherent to A431 cells. *N. gonorrhoeae* strain MS11 was used to infect A431 cells at a multiplicity of infection (MOI) of 100. An MOI of 100 was chosen to insure that sufficient quantities RNA would be isolated for hybridization experiments. At 3 h post-infection (p.i.), the cells were washed with PBS to remove non-adherent bacteria. Cells (with adherent bacteria) were lifted with 1% saponin in GC broth and samples diluted for plating to determine cell associated CFU. Duplicate plates containing media without cells were "infected" as the bacteria alone sample, and bacteria harvested without washing. Adhesion frequencies were determined by comparing the number of adherent CFU to the total number of CFU (CFU in the supernatant plus adherent CFU) at the end of the experiment. The total number of CFU in wells containing A431 cells was comparable to the number of CFU in the bacteria alone control, indicating that the growth of gonococci was not appreciably altered in the presence of epithelial cells. Adhesion frequencies varied from 40-66%, comparable to previous reports of gonococcal adherence under similar conditions at an MOI of 10 (19, 164, 246). Bacteria from both samples (adherent bacteria and bacteria alone) were concentrated by centrifugation and RNA isolated immediately using Trizol (Invitrogen). Labelled cDNA probes were generated from isolated RNA and hybridized to DNA arrays as described in Materials and Methods.

Global gene expression in adherent gonococci was compared with that of gonococci grown in cell culture media in the absence of A431 cells at 3 h p.i. Four independent experiments were performed and outliers were identified by iterative outlier

analysis in which three successive rounds of analysis are done to determine genes with ratios (log_2 transformed) greater than 2.5 standard deviations from the mean (47). Averages of ratios (and standard deviations) of genes identified as outliers in at least two experiments as differentially regulated by host cell contact are shown in Tables 4-1 (up-regulated) and 4-2 (down-regulated).

Of the 94 genes identified as differentially regulated, several genes (35/94) encode hypothetical proteins. This was not surprising, as nearly half of the genes in the annotated genome of *N. gonorrhoeae* encode putative proteins with no similarities to proteins of known function (43). The remaining 59 genes identified as differentially regulated encode proteins with a broad spectrum of functions in *N. gonorrhoeae* including amino acid metabolism, protein export, transcription, translation, protein modification, glycolysis, oxidation-reduction reactions, cell division and replication.

A control microarray experiment was done to determine whether saponin treatment (used to lift cells with adherent bacteria) affected gonococcal gene expression. The results of this experiment showed no genes significantly down-regulated, and six genes (*NG0387*, *NG0652*, *NG1055*, *NG1767*, *NG1779*, and *NG2065*) consistently up-regulated in bacteria treated with saponin for 10 min (data not shown). However, none of the genes up-regulated by saponin were identified as up-regulated by host cell contact (Table 4-1).

RpoH is induced upon contact with A431 cells. Among the genes identified as up-regulated following adherence to epithelial cells is *rpoH*, which encodes the putative heat shock sigma factor, σ^{32} (RpoH). In *E. coli*, RpoH has been shown to regulate the

expression of genes encoding a number of molecular chaperones and other proteins that are important for keeping proteins in the cell folded properly under conditions of stress such as increased temperature (131). Two major genes known to be transcribed from σ^{32}-dependent promoters in gram-negative bacteria are groEL and groES, which encode such molecular chaperones. Not surprisingly, our results showed the gonococcal *groEL* and *groES* homologs to be up-regulated in gonococci upon cell contact (Table 4-1). This is consistent with a previous report showing that transcription of the gonococcal *groESL* operon is increased following heat shock (391).

RT-PCR analysis of gonococcal genes induced upon host cell contact. In order to corroborate the DNA microarray results, quantitative RT-PCR was employed to measure differential expression of *rpoH* and *groES*. *16S rRNA* expression was used as an internal control, since its expression was not expected to be influenced by bacteria- host cell contact. *N. gonorrhoeae* strain MS11 was used to infect A431 cells at an MOI of 10. At 3 h p.i., RNA was isolated from adherent bacteria and from bacteria grown in cell culture medium in the absence of cells essentially as described for the DNA array experiments. RNA was quantified and subjected to RT-PCR using primers specific for *rpoH*, *groES*, and *16S rRNA*. Figure 4-1A shows the results of a representative RT-PCR experiment. These data show that expression of *rpoH* was greatly increased (4.32-fold) in wild-type gonococci (MS11) at 3 h p.i. Expression of *groES* was also significantly increased in MS11 (1.77-fold), while the expression of *16S rRNA* was unchanged. These data are consistent with the results of the DNA microarray experiments.

Table 4-1. Genes up-regulated in gonococci adherent to A431 cells

ORF ID[a]	gene	gene product	expression ratio (+cells/-cells)[b]	std dev
NG1684		conserved hypothetical protein	3.27	1.33
NG0288	*rpoH*	RpoH, σ^{32}	2.61	0.61
NG1363	*mtrE*	MtrE, multidrug efflux pump	2.48	0.88
NG2094	*groES*	GroES, heat shock chaperone	2.39	0.42
NG1989		Neisseria-specific protein, uncharacterized	2.38	0.67
NG0340	*cysK*	cysteine synthase	2.34	0.95
NG0238		conserved hypothetical protein	2.34	1.10
NG1210		conserved hypothetical protein	2.24	0.50
NG2095	*groEL*	GroEL, heat shock chaperone	2.10	0.24
NG0634		hypothetical protein	2.03	0.72
NG0372		ABC transporter	1.97	0.41
NG0791		conserved hypothetical protein	1.93	0.71
NG1927		Neisseria-specific protein, uncharacterized	1.91	0.43
NG0635		hypothetical protein	1.91	0.51
NG1083		hypothetical protein	1.89	0.50
NG0633	*nifU*	Fe-S scaffold protein	1.89	0.78
NG1988		conserved hypothetical protein	1.88	0.48
NG0234	*hemN*	porphyrin oxidoreductase	1.88	0.63
NG0376	*ppiB*	peptidyl-prolyl cis-trans isomerase B	1.87	0.86
NG0721		Neisseria-specific protein	1.85	0.38
NG1366	*mtrR*	MtrR, multidrug efflux regulator	1.84	0.65
NG1291		conserved hypothetical protein	1.83	0.25
NG0199	*rho*	transcription termination factor Rho	1.79	0.62
NG1515	*tyrC*	prephenate dehydrogenase TyrC	1.76	0.11
NG1493	*rpsT*	30S ribosomal protein S20	1.73	0.36
NG1439	*aqpZ*	ABC transporter, ATP-binding	1.72	0.34
NG0989		conserved hypothetical protein	1.69	0.44
NG1831	*rpmC*	50S ribosomal protein L29	1.68	0.61
NG1841	*rspJ*	30S ribosomal protein S10	1.68	0.24
NG1668	*pgi*	glucose-6-phosphate isomerase (Gpi)	1.67	0.38
NG0637		conserved hypothetical protein	1.67	0.18
NG1773	*sdaA*	L-serine dehydratase; L-serine deaminase	1.65	0.35
NG2180		conserved hypothetical protein	1.59	0.56
NG0962	*pncB*	nicotinate phosphoribosyltransferase	1.59	0.31
NG5041	*ydhB*	hypothetical protein, MS11 island	1.46	0.37
NG1587	*mafB2*	adhesin MafB2	1.41	0.39
NG0276	*comA*	competence protein (ComA)	1.32	0.30

[a]ORF IDs and gene designations are from the annotated *N. gonorrhoeae* genome sequence (43).

[b]Ratio of expression in adherent bacteria to expression in bacteria grown in the absence of epithelial cells.

Table 4-2. Genes down-regulated in gonococci adherent to A431 cells

ORF ID[a]	*gene*	gene product	expression ratio (-cells/+cells)[b]	std dev
NG1024		conserved hypothetical protein	5.44	1.26
NG0186	*ald*	Zn-alcohol dehydrogenase	2.55	1.24
NG0715	*g6pd*	glucose 6-phosphate 1-dehydrogenase	2.54	0.25
NG0186.1		transposase fragment of IS1016	2.54	1.57
NG0574	*cah*	carbonic anhydrase	2.52	0.72
NG0771	*recD*	RecD	2.35	0.26
NG1215		conserved hypothetical protein	2.24	0.70
NG1358	*gdhA*	glutamate dehydrogenase	2.04	0.50
NG1473	*mdaB*	drug activity modulator B	1.99	0.26
NG1882		hypothetical protein	1.96	0.79
NG1600	*glnA*	glutamine synthetase	1.92	0.44
NG1545		conserved hypothetical protein	1.89	0.56
NG1627		hypothetical protein	1.85	0.53
NG0656	*oxiT*	oxalate/formate antiporter	1.84	0.89
NG1630		lambda repressor-like protein *cI*	1.83	0.85
NG1698	*comE*	competence protein ComE	1.78	0.44
NG1139		Neisseria-specific protein	1.76	0.70
NG0479		phage-related repressor (*cI*)	1.76	0.26
NG0849	*proB*	ProB	1.73	0.57
NG1249		conserved hypothetical protein	1.71	0.45
NG1117		conserved hypothetical protein (phage-like)	1.71	0.68
NG0249	*accD*	acetyl-CoA carboxylase	1.70	0.20
NG1881	*pykA*	pyruvate kinase II	1.70	0.64
NG0177	*cpxR*	CpxR	1.70	0.62
NG0850	*proA*	ProA	1.69	0.33
NG1769	*yhjA*	cytochrome c peroxidase	1.68	0.37
NG0788		Neisseria-specific protein, uncharacterized	1.67	0.29
NG1258	*pgm*	phosphoglycerate mutase	1.67	0.20
NG0561		conserved hypothetical protein	1.67	0.33
NG1406	*gcsT*	aminomethyltransferase, glycine cleavage system	1.66	0.40
NG1815	*minD*	cell division protein, MinD	1.66	0.44
NG0904		conserved hypothetical protein	1.66	0.40
NG0062	*fthS*	formate-tetrahydrofolate ligase	1.65	0.46
NG0959		conserved hypothetical protein	1.65	0.35
NG0719	*g6pi*	glucose-6-phosphate isomerase	1.65	0.26
NG0794	*bfrA*	bacterioferritin A	1.64	0.58
NG0410	*cspA*	cold-shock protein	1.64	0.14
NG1122		hypothetical protein	1.64	0.37
NG0318	*recN*	RecN	1.64	0.50
NG0449		conserved hypothetical protein	1.63	0.50
NG0717	*hxkG*	glucokinase	1.63	0.40
NG1577	*omp3*	PIII	1.63	0.37
NG0732		Neisseria-specific protein, uncharacterized	1.62	0.59
NG0626	*mltB*	murein transglycosylase	1.62	0.16
NG0692		LysR family transcriptional regulator	1.62	0.34
NG0472		hypothetical protein	1.61	0.42
NG1300	*yfcB*	adenine specific methylase	1.61	0.29
NG0787		Neisseria-specific protein, uncharacterized	1.59	0.25
NG1374	*ccoN*	cytochrome c oxidase	1.59	0.31
NG1643		conserved hypothetical protein	1.58	0.14
NG1138		conserved hypothetical protein	1.57	0.19
NG0248	*trpA*	tryptophan synthase alpha chain	1.56	0.22
NG0203	*gph*	phosphoglycolate phosphatase	1.55	0.30
NG1110	*dnaB*	DnaB	1.55	0.42
NG0716	*6pgl*	6-phosphogluconolactonase	1.54	0.37
NG1381	*glr2*	glutaredoxin 2	1.51	0.41
NG1411		conserved hypothetical protein	1.40	0.66

[a]ORF IDs and gene designations are from the annotated *N. gonorrhoeae* genome sequence (43).
[b]Ratio of expression in bacteria grown in the absence of epithelial cells to expression in adherent bacteria.

An additional control was done to rule out effects of saponin (used to lift cells with adherent bacteria). Gonococci grown on plates were swabbed into GC broth and divided into two aliquots: the first into GC broth, the second into GC broth containing 1% saponin. After 10 min incubation, RNA was isolated from both samples and subjected to RT-PCR using *rpoH*-specific primers. Results of this experiment showed that *rpoH* expression was not affected by treatment of the bacteria with saponin (data not shown).

RpoE does <u>not</u> mediate the induction of *rpoH* in gonococci upon host cell contact. In *E. coli*, the expression of *rpoH* is under the control of multiple promoters, three that are transcribed under most growth conditions via the housekeeping sigma factor, σ^{70} (131), one of which is subject to catabolite repression (269). The fourth promoter is dependent on the extreme heat shock sigma factor, σ^{E} (encoded by *rpoE*) which responds to misfolded extra-cytoplasmic proteins (328). It is thought that RpoE is important for the maintenance of membrane and periplasm homeostasis, and has been shown to have a role in pathogenesis in several gram-negative bacteria (70, 197, 328).

Since *N. gonorrhoeae* has an annotated gene (*NG1944*) with high similarity to *rpoE* (43), we initially hypothesized that the observed response to host cell contact might be mediated by RpoE-dependent induction of *rpoH*, which in turn would lead to RpoH-dependent induction of *groEL/groES*. To test this hypothesis, an *rpoE* mutant of *N. gonorrhoeae* strain MS11 was constructed. The gonococcal *rpoE* gene was PCR amplified from *N. gonorrhoeae* genomic DNA and cloned into pDONR221, and the resulting plasmid (pNG1944) mutagenized by in vitro transposition using the transposon

TnErmUP as described (83). Two plasmids containing *rpoE* with transposon insertions approximately 320 bp (11F) and 500 bp (9G) from the *rpoE* start codon were isolated and used to transform *N. gonorrhoeae* strain MS11. Em^R transformants were obtained at a high frequency (~1 x 10^{-5} Em^R transformants/CFU), indicating that *rpoE* is not essential in *N. gonorrhoeae*. This is inconsistent with observations that *rpoE* is essential in several bacteria, including *E. coli* and *Y. enterocolitica* (75, 156). To rule out the possibility that the Em^R transformants were *rpoE* heterodiploids, having a copy of the intact *rpoE* gene in addition to the insertionally inactivated copy, PCR was done using primers specific for *rpoE*. Agarose gel analysis of the amplification products showed that the mutants yielded a band ~1.2 kb larger than the wild-type *rpoE*, consistent with the insertion of the TnErmUP transposon, (data not shown). None of the mutants yielded a band corresponding to the size of the wild-type *rpoE* gene. Expression of *rpoE* in the mutants was analyzed by RT-PCR, with no amplification products observed except in the wild-type (MS11) control. Hence, our results indicate that strains NG1944.11F and NG1944.9G are indeed *rpoE* null mutants and that *rpoE* is not essential to *N. gonorrhoeae*.

To examine the role of RpoE in the initial stages of a gonococcal infection, adhesion and invasion assays were done using A431 cells as described in Materials and Methods. Adhesion was scored as the number of CFU cell-associated/total CFU at the end of the experiment, while invasion was scored as the number of bacteria protected from gentamicin killing (Gm^R)/number of cell-associated CFU. The results of these assays are summarized in Figure 4-2. The *rpoE* mutant (NG1944.11F) adhered to and invaded A431 cells at essentially the same frequencies as the wild-type parent strain,

MS11 ($p > 0.05$, Student's t test). These results suggest that RpoE is not necessary for gonococcal adherence to or invasion of A431 cells.

To determine whether *rpoH* expression and induction upon adherence to epithelial cells was dependent on RpoE, RT-PCR was done using RNA isolated from NG1944.11F grown either alone in cell culture medium, or following adherence to A431 cells for 3 h. The results of these experiments (shown in Figure 4-1B) showed that *rpoH* is up-regulated in the *rpoE* mutant at 3 h p.i., similar to what was observed in the wild-type strain, MS11 (Figure 4-1A). Expression of *groES* followed a similar pattern. These data suggest that *rpoE* is not required for the up-regulation of *rpoH* or *groES* upon host cell contact.

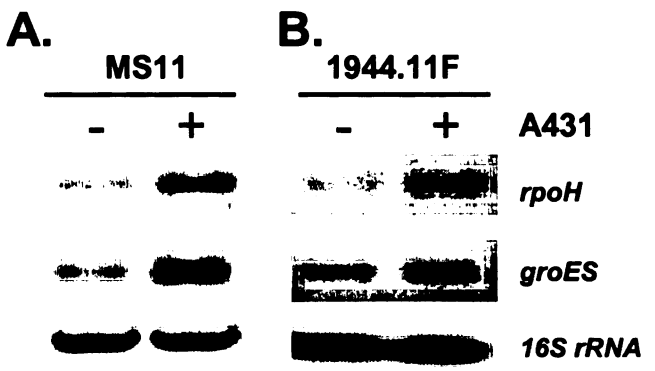


Figure 4-1. RT-PCR analysis of genes induced in gonococci adherent to human epithelial cells. RNA isolated from MS11 (wild-type) or NG1944.11F (*rpoE*::TnEmUP) adherent to A431 cells (+) or grown in the absence of A431 cells (-) was reverse transcribed using random primers. The subsequent PCR was performed with primers specific for *rpoH*, *groES* and *16S rRNA*, as indicated on the right. Images of EtBr stained gels were reversed using Adobe Photoshop for clarity and band intensities quantified using Kodak image analysis software, normalizing to *16S rRNA*. Results shown are representative of three independent determinations.

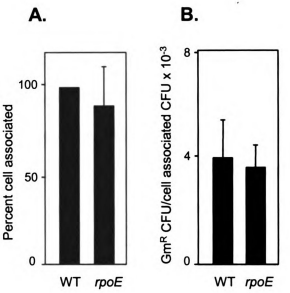


Figure 4-2. Adherence to and invasion of A431 cells is not affected in a gonococcal *rpoE* mutant. WT: MS11; *rpoE:* *rpoE*::TnEmUP. (A) Adherence to A431 cells. Adhesion data are presented as a percentage of the wild-type control (MS11) and are averages of five independent experiments performed in triplicate. (B) Invasion of A431 cells. Invasion data are presented as the ratio of Gentamicin-resistant (Gm^R) bacteria per number of cell associated bacteria and are averages of two independent experiments performed in triplicate. Error bars indicate standard deviation.

To analyze global gene expression in *rpoE* mutant gonococci, a DNA microarray experiment was performed. Labelled cDNA was synthesized from RNA isolated from NG1944.11F (*rpoE*::TnEmUP) following 3 h adherence to A431 cells as described above for wild-type gonococci. Analysis of the data showed a similar list of genes up-regulated by host cell contact as was observed for the wild-type parent, MS11, including *rpoH*, *groEL*, and *groES* (data not shown). Taken together, our results indicate that RpoE is not involved in regulating *rpoH* gene expression in gonococci, at least under the conditions tested.

RpoH is essential to *N. gonorrhoeae*. In order to examine the role of RpoH in gonococci and its interaction with host cells, we next attempted to construct an *rpoH* null mutant. The gonococcal *rpoH* gene was PCR amplified and cloned into pHSS6, and the resulting plasmid (pADC1) mutagenized with the transposon TnErmUP as described for *rpoE* above. Three different mutants with transposon insertions approximately 220 bp (1H), 435 bp (5D), and 700 bp (6G) from the *rpoH* start codon were used to transform *N. gonorrhoeae* strain MS11. Despite several attempts at transformation with increasing amounts of input DNA, Em^R mutants were never isolated. Positive transformation controls included plasmid DNA used to construct the *rpoE* mutants (described above), as well as genomic DNA from a previously characterized Em^R *N. gonorrhoeae* strain, MS11-306 (247). The inability to obtain Em^R *rpoH* null mutants strongly suggested that *rpoH* is essential in *N. gonorrhoeae*. An alternative explanation for our result could be a polar effect on expression of a gene downstream (3') of *rpoH*, however this is unlikely as

the gene immediately 3' of *rpoH* (NG0287) is transcribed in opposite direction (43). Hence, we concluded from these results that *rpoH* is essential to gonococci.

Construction of a conditional *rpoH* mutant. Since we were unable to construct an *rpoH* null mutant in *N. gonorrhoeae*, a strain was constructed in which *rpoH* expression was under the transcriptional control of the lactose repressor (LacI) such that *rpoH* expression could be controlled by the addition of the LacI inducer, IPTG, to the culture medium. We utilized a plasmid, pKH35 (142), which contains a segment of the *N. gonorrhoeae* chromosome (3.8 kb) in which large sequences can be inserted without being deleterious to the cell (H. S. Seifert, personal communication). Between the *aspC* and *lctP* genes on the plasmid is a selectable marker (Cm^R), a $lacI^Q$ gene (since *N. gonorrhoeae* has no *lac* operon of its own), two tandem *lac* promoter-operator sequences (351), and three *Neisseria* uptake sequences (NUS) which are necessary for gonococcal transformation (351). A gene of interest cloned downstream of the *lacOPOP* and transformed into *N. gonorrhoeae* results in recombination between the incoming DNA and the *aspC-lctP* region of the chromosome. Since pKH35 can replicate in *E. coli*, but not *N. gonorrhoeae*, the plasmid itself is lost following transformation. What remains on the chromosome is the selectable marker and the IPTG-inducible construct.

The *rpoH* gene was PCR amplified from MS11 genomic DNA and cloned immediately downstream of *lac* promoter on pKH35. This plasmid, pNGR18, was then used to transform MS11, selecting for Cm^R. A double crossover event following transformation results in the insertion of the inducible *rpoH* between *aspC* and *lctP*. The resulting strain, MP288, has two copies of *rpoH* on the genome, the native copy and the

inducible copy. To eliminate the native copy of *rpoH* (such that *rpoH* expression is solely under IPTG control) MP288 was transformed with the *rpoH*::TnErmUP plasmid, pNGR20.5D, and transformants selected for Em^R on plates containing IPTG, to insure some *rpoH* expression in the event the native copy of *rpoH* was inactivated, as intended. Since this incoming DNA could recombine with either the native copy of *rpoH* or the inducible *rpoH* locus, Em^R transformants were screened by PCR using primers that differentiated between the native *rpoH* locus and the inducible construct at the *lctP-aspC* locus. A mutant was identified in which only the wild-type copy of *rpoH* gene was insertionally inactivated, which was named MPD288 [*rpoH*::TnEmUP, *lacIOP-rpoH*Cm], and expresses *rpoH* only in the presence of IPTG. MPD288 was maintained on GC agar plates containing 20 μM IPTG, which allowed growth similar to the wild-type strain. Subsequent passage of this strain onto media lacking IPTG resulted in an eventual growth defect, but only after several passages. This may be due to a small amount of RpoH still existing in the cell, and/or leaky expression of *rpoH* from *lacOPOP* promoter.

The expression and inducibility of *rpoH* in strain MPD288 was examined by RT-PCR using RNA isolated from bacteria grown with and without IPTG. Figure 4-3 shows that *rpoH* is expressed at barely detectable levels in the absence of IPTG in MPD288, but at nearly wild-type levels in the presence of 20 μM IPTG. Increasing the IPTG resulted in corresponding increases in *rpoH* mRNA (data not shown).

Since *groES*, which is also induced in gonococci upon adherence to A431 cells (Table 4-1 and Figure 4-1), is expected to be dependent on RpoH, we also analyzed expression of this gene in the conditional *rpoH* strain. As predicted, expression of *groES*,

but not *16S rRNA*, was reduced in the conditional *rpoH* strain grown under *rpoH*-depletion conditions (no IPTG, Figure 4-3), confirming that *groES* expression in gonococci is dependent on RpoH.

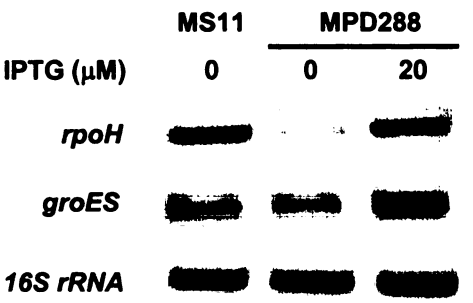


Figure 4-3. RT-PCR of *rpoH* and *groES* in an *N. gonorrhoeae* strain conditionally expressing *rpoH*. RNA isolated from MS11 (wild-type) or MPD288 (*rpoH*::TnEmUP, *lacIOP-rpoH*Cm) grown overnight on GC agar with or without 20 μmIPTG (as indicated) was reverse transcribed using random primers. The subsequent PCR was performed with primers specific for *rpoH*, *groES* and *16S rRNA*, as indicated on the left. Images of EtBr stained gels were reversed using Adobe Photoshop for clarity and band intensities quantified using Kodak image analysis software, normalizing to *16S rRNA*. Results shown are representative of three independent determinations.

Temperature sensitivity of a conditional *rpoH* strain. In gram-negative bacteria, the RpoH regulon is induced in response to a sudden increase in temperature, as well as other stresses. This response is necessary to maintain the proper folding of proteins in the cytoplasm under such conditions which could otherwise be lethal to the cell. An inability to respond to heat shock (as is mediated by RpoH) would likely make the bacterium more sensitive to heat shock. To test this in gonococci, we asked whether depletion of RpoH, using our conditional *rpoH* strain, MPD288, would affect the strains' ability to survive heat shock. *N. gonorrhoeae* strains MS11 (wild-type) and MPD288 (inducible *rpoH*) grown on GC plates with or without 20 μM IPTG were swabbed into GC broth and diluted to 10^7 CFU/ml. The bacterial suspensions were then subjected to heat shock at 42°C for various times over a 1 h period. At 10 min intervals, samples were collected and plated to determine viable CFU. The data obtained from four independent experiments was averaged and is plotted in Figure 4-4. Gonococci depleted for RpoH (MPD288 grown without IPTG) were more sensitive to heat shock than the wild-type strain (MS11) or the conditional *rpoH* strain grown in the presence of IPTG. This result indicates that *rpoH* expression contributes to gonococcal survival in response to heat shock.

Effect of RpoH on interactions between gonococci and A431 cells. DNA array analysis of gene expression in gonococci adherent to A431 cells showed that *rpoH* is up-regulated more than two-fold at 3 h p.i. (Table 4-1). Genes expected to be dependent on RpoH for expression, such as *groES* and *groEL* are also significantly up-regulated under these conditions. These results imply that RpoH and genes it regulates might be important

for subsequent steps in a gonococcal infection. Thus, we next asked whether *rpoH* expression is necessary for gonococci to adhere to and invade A431 cells in culture.

Wild-type *N. gonorrhoeae* strain MS11 and MPD288 were used to infect A431 cells at an MOI of 10. To deplete RpoH in MPD288, this strain was maintained on GC agar containing 20 μM IPTG, and then grown overnight on medium lacking IPTG prior to infection of A431 cells. For infection, bacteria were harvested from plates and a suspension prepared in cell culture medium with or without IPTG. At the end of the experiment, samples were plated on GC agar containing 20 μM IPTG and CFU enumerated following incubation at 37°C.

The results of these experiments are summarized in Figure 4-5. Adhesion frequencies, determined at 3 h p.i., showed no significant differences between MPD288 grown with or without IPTG, or the wild-type parent strain, MS11 ($p > 0.05$). However, the subsequent step in infection, invasion of epithelial cells, appeared to be reduced for the mutant MPD288 depleted for RpoH. The relative invasion frequency at 7 h p.i. for this strain was significantly less than that observed for the wild-type strain, MS11 ($p = 0.002$). MPD288 grown in conditions which express levels of *rpoH* comparable to the wild-type (Figure 4-3) invaded A431 cells at a higher frequency than this strain grown without IPTG, although the frequency is not as high as observed for the wild-type parent, and may not be significantly different ($p = 0.06$). The reduced ability of the *rpoH*-expressing MPD288 strain to invade A431 cells compared to MS11 may be due to an inability of this strain to induce *rpoH* expression upon adherence, since the native regulatory sequences are not present upstream of the expressed *rpoH* gene in this strain. Taken together, these data show that in wild-type gonococci, *rpoH* expression is turned

on in response to initial contact with epithelial cells, and this expression is necessary for the subsequent invasion of these cells. This demonstrates a mechanism for this bacterium to sense its environment and adjust gene expression in preparation for the next step of the infection.

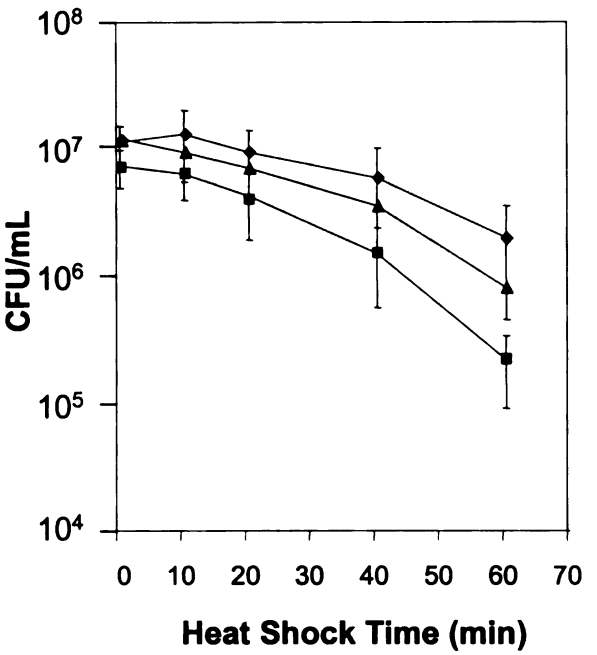


Figure 4-4. *N. gonorrhoeae* depleted for RpoH are hyper-sensitive to heat shock. Bacteria were grown overnight on GC agar plates with or without 20 μM IPTG (as indicated) and then swabbed into GC broth at 10^7 CFU/ml. Bacterial suspensions were subjected to a heat pulse at 42°C and aliquots removed at 10 min intervals. Serial dilutions of samples were plated on GC agar plates (+20 μM IPTG as necessary). Symbols: diamond (◆), MS11 (wild-type); triangle (▲), MDP288 (inducible *rpoH*) +20 μM IPTG; box (■), MDP288 (inducible *rpoH*) w/o IPTG. Error bars indicate standard deviation.

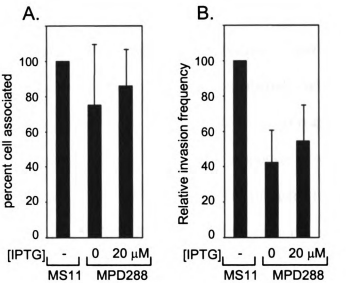


Figure 4-5. Depletion of RpoH in *N. gonorrhoeae* affects invasion of, but not adherence to A431 cells. MS11: wild-type; MDP288: *rpoH*::TnEmUP, *lacIOP-rpoH*Cm. (A) Adherence to A431 cells. Adhesion data are presented as a percentage of the wild-type control (MS11) and are averages of four independent experiments performed in triplicate. (B) Invasion of A431 cells. Invasion data are presented as relative invasion frequency, normalized to MS11. These data are averages of five independent experiments performed in triplicate. Error bars indicate standard deviation.

DISCUSSION

The interaction between a host and a pathogen is a dynamic process, involving responses from both the pathogen and the host. Several bacterial pathogens respond to changes in their environment by modulating gene expression, providing a selective advantage to organisms that are, at least initially, outnumbered, and must deal with differences in nutrient availability as well as with the (often hostile) response of the host. Often, these regulatory systems result in the integration of responses to several signals via complex networks to modulate the expression of a variety of genes that are important during an infection (79, 130, 225, 254, 339, 366). Some bacteria, such as *Yersinia pseudotuberculosis* (293) and uropathogenic *E. coli* (448) regulate gene expression specifically in response to contact with host cells. Since one of the first events to occur when the *N. gonorrhoeae* enters a new host is adherence to the mucosal epithelia, we hypothesized that this might serve as a signal for the bacterium to modulate the expression of genes that will allow it to colonize and proliferate in the new host. It has been shown that adherence of gonococci to epithelial cells in culture enhances their invasiveness (60), and it is likely that this increased invasiveness results from changes in gene expression that occur in response to adherence to cells. Thus, in this work, we sought to identify the genes that are differentially expressed in gonococci in response to attachment to epithelial cells and to identify the regulatory networks involved.

A PCR-amplicon based genome microarray was used to examine changes in gene expression in *N. gonorrhoeae* upon adherence to human epithelial cells. The results of these experiments showed 37 genes up-regulated (Table 4-1) and 57 genes down-regulated (Table 4-2) in adherent gonococci relative to bacteria grown in the absence of

host cells. These genes fall into several categories including transcriptional regulation, bacterial metabolism, molecular transport and (as expected) several hypothetical genes of unknown function.

Of the genes down-regulated upon adherence, several of them (22/57) encode proteins involved in central housekeeping functions such as glycolysis, amino acid metabolism, nitrogen assimilation, respiration, and cell division. This suggests that there might be a general shift in cellular metabolism in response to host-cell contact, indicating a mechanism for the bacterium to begin to adapt to different nutrient availability in the new host and/or the different environment inside of the host cell. Other down-regulated genes encode proteins involved in DNA replication, recombination, and repair (RecD, RecN, DnaB, YfcB), transformation competence (ComE), and four potential transcriptional regulators. Interestingly, one of the down-regulated genes, *NG*1473, encodes MdaB, a protein thought to be a drug activity modulator (43). However, MdaB has recently been shown to be an NADPH quinone reductase that plays an important role in managing oxidative stress in bacteria, suggestive of a role in pathogenesis (421).

In addition to the hypothetical genes up-regulated upon adherence (16/37) were several genes encoding proteins involved in cellular metabolism and general gene expression, again suggesting a general shift in bacterial metabolism in response to host-cell contact. *NG*1587, which was slightly up-regulated on adherence, is annotated as *mafB2*, one of several genes of the multiple adhesin family (*maf*) which encode glycolipid adhesins (276). This up-regulation of *mafB2* could indicate a need for MafB2 at subsequent steps of an infection, consistent with reports that MafB2 is involved in Opa-independent invasion (276).

Of particular interest was the observation that several putative transcriptional regulators were differentially regulated upon host cell contact, two up-regulated (Table 4-1) and four down-regulated (Table 4-2). The identification of multiple putative transcriptional regulators as repressed as well as induced upon adherence to epithelial cells suggests that there are indeed complex regulatory networks involved in the modulation of gene expression in gonococci in response to host cell contact. Interestingly, *NG0177*, which is annotated as *cpxR* (43) is down-regulated upon host cell contact. In *E. coli,* CpxR is a regulator that responds to cell envelope stress, in particular to mis-folded proteins at the inner membrane, (314)]. However, *NG0177* is also annotated as *ompR* in *N. gonorrhoeae*, *phoQ* in *N. meningitidis* serogroup B (173), and *misR* in *N. meningitidis* serogroup A (406). Thus, the function of the protein encoded by NG0177 remains to be determined.

One of the highest up-regulated genes identified in our experiments was *rpoH*, which encodes a heat shock-specific sigma factor (RpoH, σ^{32}). In gram-negative bacteria, σ^{32} up-regulates the expression of genes encoding a number of proteins (particularly chaperones and proteases) that are necessary for keeping proteins in the cell folded properly under conditions of stress such as increased temperature (131, 443). *groEL* and *groES*, which encode such molecular chaperones and are known to be transcribed from σ^{32}-dependent promoters in many gram-negative bacteria, were also identified as up-regulated in gonococci upon adherence to host cells (Table 4-1, Figure 4-1). Our initial hypothesis was that the up-regulation of gonococcal *rpoH* and the RpoH regulon would be dependent on the extra-cytoplasmic sigma factor σ^{E} (encoded by *rpoE*). σ^{E} is known to control *rpoH* expression in *E. coli* and *S. typhimurium* in response to misfolded extra-

cytoplasmic proteins (256, 328). Our rationale was that an interaction between host cells and the outer surface of the gonococcus might be a signal to which RpoE would respond and subsequently up-regulate *rpoH* expression. Construction and analysis of a gonococcal strain with a transposon insertion in *rpoE*, however, showed that RpoE was not necessary for the induction of *rpoH* upon host-cell contact (Figure 4-1). This may be due in part to the fact that there are no apparent gonococcal homologs to the anti-sigma factor, RseA, and its accessory protein, RseB, which serve to control RpoE activity in response to extra-cytoplasmic signals (75).

While it is clear that RpoE does not control *rpoH* gene expression in gonococci in response to host cell contact, the regulatory mechanism used by gonococci to up-regulate *rpoH* gene expression in response to host cell contact is still to be determined. It is possible that transcriptional control of *rpoH* expression in gonococci is mediated by other regulators in response to host cell contact, as yet to be identified. Clearly, expression of *rpoH* is important in gonococci, since we (and others (205)), have been unable to construct an *rpoH* null mutant, suggesting it is an essential gene.

Control of RpoH activity in gram-negative bacteria is complex, and occurs only partially at the level of *rpoH* transcription (131, 443). Transcription of *rpoH* is usually high under steady state conditions, but the message is not efficiently translated until a shift in growth temperature, which results in an increase in translation of the pre-existing *rpoH* mRNA by destabilization of a secondary structure in the mRNA which overlaps the *rpoH* start codon (260). In addition, RpoH activity is controlled by two RpoH-dependent chaperone systems, DnaK/J and GroEL/S which can sequester σ^{32}, preventing its

association with RNA polymerase holoenzyme (134, 215). Thus, the heat shock response is modulated by the protein folding status of the cell.

A recent report suggests that regulation of RpoH in *N. gonorrhoeae* may differ from that observed in other bacteria (205). While an increase in *rpoH* expression was observed upon heat shock, the time following heat shock that the increase was observed was less than the time observed for induction of the RpoH-dependent genes, *dnaJ*, *dnaK*, and *grpE*. The level of RpoH protein, as shown by immunoblotting with antisera raised against *E. coli* RpoH, correlated with *rpoH* expression, which the authors concluded ruled out translational regulation, as has been demonstrated for *E. coli rpoH* (260). The conclusion of this work was that regulation of RpoH activity is the primary level of regulation in response to heat shock in gonococci.

Our observation that *rpoH* is induced upon host cell contact in gonococci is a determination of the relative levels of *rpoH* transcript at a single point in time (3 h p.i.). The increase in *rpoH* transcript levels in adherent bacteria relative to that in non-adherent bacteria could be due to increased transcription or to an increase in *rpoH* mRNA stability, which cannot be distinguished from one another in these experiments. It is clear from our results, however, that the interaction between gonococci and epithelial cells serves to transmit a signal to the bacterium, by an as yet undefined mechanism, that results in the induction of *rpoH* and at least some genes of the RpoH regulon. Regulation of RpoH activity was not directly assessed, and could, in part, play a role in the induction of *groEL*, *groES*, and other genes upon host cell contact. Further experiments are required to examine this.

The observation that *rpoH*, as well as two genes of the RpoH regulon, *groES* and *groEL*, but not *dnaJ*, *dnaK*, and *grpE*, which are known to be RpoH-dependent in gonococci (205) were up-regulated in our studies could indicate the involvement of multiple regulatory systems, as yet unidentified. RpoH may respond to more than heat shock in the gonococcus. As a strict human pathogen with no environmental niche outside its host, *N. gonorrhoeae* is likely to encounter only slight temperature fluctuations, but numerous other environmental stresses in an infection or upon transmission. In other bacteria, RpoH responds to stresses such as increased temperature, as well as other factors such as increased hydrostatic pressure and hyperosmotic shock (8, 32) enabling the bacterium to detect and correct misfolded proteins in the cytoplasm either by chaperone-assisted folding, or degradation by proteases (131).

Bacterial pathogens often up-regulate the production of virulence factors upon infection, many of which are directly involved in host interactions, and are on the surface of the bacterium. These proteins are often large, complex, multi-subunit proteins that require the assistance of chaperones and other proteins in the bacterial cell to assemble correctly and/or to be exported to their final destination. If the assembly and export functions are not adequate, accumulation of misfolded proteins in the cytoplasm would need to be degraded by proteases. An increase in the production of chaperones to aid in the proper folding and assembly of virulence factors concommitant with an increase in their expression would be critical in the adaptation process.

Our results demonstrating a role for the heat shock response in gonococcal pathogenesis suggests that bacterial heat shock regulons might have a significant role in the regulation of virulence. Another example is *Vibrio cholerae*, in which the expression of several

virulence genes including the cholera toxin gene (*ctx*) are modulated by growth temperature as well as other environmental factors (198, 290)].

A final interesting note is that while many bacteria use several sigma factors, even regulatory cascades of sigma factors, to control gene expression in response to a variety of environmental conditions, *N. gonorrhoeae* only appears to encode four sigma factors: σ^E (RpoE), σ^{32} (RpoH), σ^{70} (RpoD), and σ^{54} (RpoN). σ^{70} (RpoD) is the primary "house-keeping" sigma factor, highly conserved among gram-negative bacteria (222). In *N. gonorrhoeae*, σ^{54} (RpoN) has been shown to be non-functional (132). As we have shown in this work, σ^{32} (RpoH), but not σ^E (RpoE) is essential for viability of gonococci, and also plays a role in interactions between gonococci and its host. However, σ^E (RpoE) does not appear to play a role in controlling expression of *rpoH*, at least under the conditions tested, and preliminary experiments in this laboratory have yet to identify a role for RpoE in gonococcal gene expression. In addition, gonococci do not appear to have a homolog of the general stress sigma factor, σ^s, that in many gram-negative bacteria regulates gene expression in resonse to stresses such as starvation, hyperosmolarity, pH downshift, or nonoptimal temperature (153).

In summary, we have examined global gene expression in gonococci in response to adherence to epithelial cells in culture. The results of our array experiments indicate that the interaction between gonococci and its host results in changes in transcription of several genes which can lead to changes in bacterial cell metabolism and other basic functions, enabling the bacteria to quickly adapt to its new environment.

Chapter 5

RpoH mediates the expression of some, but not all genes induced in *Neisseria gonorrhoeae* adherent to epithelial cells

This manuscript has been published in Infection and Immunity.

Du,Y and C.G. Arvidson. 2006 RpoH mediates the expression of some, but not all genes induced in *Neisseria gonorrhoeae* adherent to epithelial cells. *Infect. Immun.* **74:** 2767-2776

ABSTRACT

Neisseria gonorrhoeae (gonococcus) is highly adapted to the human host, the only known reservoir for gonococcal infection. However, since it is sexually-transmitted, infection of a new host likely requires a regulatory response on the part of the gonococcus to respond to this significant change in environment. We previously showed that adherence of gonococci to epithelial cells results in changes of gene expression in the bacteria that presumably prepares them for subsequent steps in the infection process. Expression of the heat shock sigma factor gene, *rpoH*, was shown to be important for the invasion step, as gonococci depleted for *rpoH* were reduced in their ability to invade epithelial cells. Here, we show that of the genes induced in adherent gonococci, two are part of the gonococcal RpoH regulon. When RpoH is depleted, expression of these genes is no longer induced by host cell contact, indicating that RpoH is mediating the host cell induction response of these genes. One RpoH-dependent gene, *NG0376,* is shown to be important for invasion of epithelial cells, consistent with earlier observations that RpoH is necessary for this step of an infection. Two genes, *NG1684* and *NG0340,* while greatly induced by host cell contact, were found to be RpoH-independent, indicating that more than one regulator is involved in the response to host cell contact. Furthermore, *NG0340*, but not *NG1684*, was shown to be important for both adherence and invasion of epithelial cells, suggesting a complex regulatory network in the response of gonococci to contact with host cells.

INTRODUCTION

Neisseria gonorrhoeae (GC, gonococcus) is a Gram-negative diplococcus which causes the sexually-transmitted disease, gonorrhea. It is an obligate human pathogen which typically infects the urethra in men and endocervix in women, although it occasionally infects other tissues as well. Gonorrhea is a continuing threat to public health worldwide, especially in developing countries and some lower socioeconomic groups in developed countries. According to the World Health Organization (WHO; www.who.org), about 30 million incidents of gonococcal disease occur each year, a figure that is likely underestimated due to the large reservoir of asymptomatic carriers (404). Another serious problem, in light of the global AIDS epidemic, is the finding that gonorrhea can significantly enhance the transmission of HIV (202, 447). Unfortunately, no effective vaccine for gonococcal infection is available, due in part to the high variability of its surface antigens (46, 352, 370, 445) and treatment has heavily depended on antibiotics. This too is a problem in that gonococci show an incredibly rapid ability to develop resistance to antibiotics (23, 100, 361, 425).

The initial step of a gonococcal infection is colonization of mucosal tissues, a complex process involving multiple components of both the bacteria and host cells. This process is critical for the production of an infection. If gonococci are unable to adhere to mucosal epithelial cells, they will be washed away with the normal flow of vaginal fluid (in women) or urine (in men). The primary adhesins on the gonococcal cell surface are the type IV pili, without which gonococci do not cause infection in human volunteers (380, 381). Binding of gonococcal pili to host cells results in the induction of signal

transduction pathways in the eukaryotic cell that affects multiple cellular processes (20, 165, 180, 209, 276, 278, 304) and adherence of piliated gonococci results in a variety of rearrangements in the components of the cellular cytoskeleton (reviewed in (249)). Little is known about the response of *N. gonorrhoeae* to this interaction, although our previous studies have begun to address this issue (84). In this work, we further these studies of the examination of gonococcal gene expression in response to contact with epithelial cells to test the hypothesis that attachment to epithelial cells signals the bacterium to modulate the expression of genes necessary for proliferation in the host.

Our previous studies showed that adherence to epithelial cells in culture results in changes of gene expression in gonococci, and that this response is in part mediated by RpoH, a heat shock sigma factor (84). RpoH is a general transcriptional regulator in several bacteria, regulating a broad range of genes responding to different stimuli inside or outside the bacterium (8, 32, 269, 439, 443). Our previous work showed that RpoH played a role in virulence in *N. gonorrhoeae* in that depletion of RpoH reduced invasion of, but not adherence to epithelial cells in culture. Here we show that some of the genes whose expression induced in adherent gonococci require RpoH for this induction, while others are RpoH-independent for induction. In addition, analysis of insertion mutants show that one of the RpoH-dependent genes is important for invasion of but not adherence to epithelial cells, consistent with the previous conclusion that RpoH is important for the induction of one or more genes upon adherence that is important for subsequent steps in gonococcal infection. In addition, we show that one of the RpoH-independent genes is important for both adherence to and invasion of epithelial cells,

suggesting the existence of multiple regulators in *N. gonorrhoeae* to mediate the response to host cell contact.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* strain DH5α was used for all recombinant DNA manipulations and was grown in Luria broth supplemented as necessary with chloramphenicol (Cm) at 30 mg/liter, kanamycin (Kn) at 50 mg/liter, or erythromycin (Em) at 300 mg/liter. *Neisseria gonorrhoeae* strain MS11 (P^+, Tr (349)) was grown in GC medium (Difco Laboratories, Sparks, MD) with Kellogg's supplements (187) in a humidified 5% CO_2 atmosphere. Em (3 mg/liter) and/or Cm (7 mg/liter) were added when necessary.

DNA manipulations. Polymerase chain reaction (PCR) was performed in a GeneAmp 9700 thermocycler (Applied Biosystems, Foster City, CA) using Taq DNA polymerase as described (84, 91). PCR products were purified using the QIAEX II Gel Extraction kit (Qiagen, Valencia, CA) when necessary. Oligonucleotide primers were synthesized at the Michigan State University Research Technology Support Facility (MSU RTSF). DNA sequencing was done by the MSU RTSF. Restriction enzymes and T4 DNA ligase were from New England Biolabs (Beverly, MA) and used as recommended. Plasmid pKH35 (142) was used for molecular complementation constructions. *N. gonorrhoeae* transformation and genomic DNA isolation was performed as described previously (349, 355).

Construction of mutants. Plasmids containing genes of interest were from the *N. gonorrhoeae* clone set (44) and used as targets for transposon mutagenesis. In vitro transposition was carried out using EZ::TN™ transposase (Epicentre Technologies,

Madison, WI) and the modified transposon, TnErmUP (83). Determination of the position of transposon insertions was determined by PCR using primers homologous to the ends of transposon (SqFP and SqRP; Epicentre) and to the ends of the gene in question and corroborated by restriction analysis.

Infection Assays. Human epithelial cell line A431 (ATCC CRL 1555) was grown at 37°C with 5% CO_2 in Dulbecco's modified Eagle medium (DMEM; Gibco/Invitrogen, Carlsbad, CA) supplemented with 5% fetal calf serum (FCS; Gibco). Adhesion and invasion assays were performed as described previously (84). Assays were performed three or more times using fresh bacterial isolates, with each sample assayed in triplicate in each experiment. Adhesion frequencies were scored as the number of cell-associated colony forming units (CFU) divided by the number of total CFU per well in that experiment. Invasion frequencies were calculated as the number of gentamicin-resistant (Gm^R) CFU per cell-associated CFU at the end of the experiment. Statistical analysis of data was done using the Student's *t* test, with *p* values indicated where appropriate.

RNA isolation and cDNA synthesis. RNA was isolated using TRIzol™ reagent (Invitrogen). An additional DNase treatment was performed using Turbo DNA-free kit (Ambion, Austin, TX) to remove any remaining contaminating DNA. Purified RNA was eluted in RNAse-free H_2O and stored at -80°C until further use. RNA was quantified spectrophotometrically, and quality assessed by agarose gel electrophoresis. cDNA was synthesized by reverse transcription using Superscript II RNase H^- reverse transcriptase (RT; Invitrogen) using random hexamers (Invitrogen). Briefly, RNA was mixed with

random hexamers and denatured at 75°C for 5 min. Mixtures were cooled to room temperature, and reaction buffer, dNTPs and reverse transcriptase were then added to a total volume of 20 µl. First strand synthesis was performed at 42°C for 50 min and reaction terminated by heating to 75°C for 15 min. Controls without RT were included for all samples.

Microarray hybridization and data analysis. Global gene expression was measured using *N. gonorrhoeae* DNA microarrays composed of PCR amplicons of 2035 of the 2250 predicted open reading frames (ORFs) of the *N. gonorrhoeae* genome spotted onto glass slides and have been described previously (44). Ten micrograms of total bacterial RNA was reverse transcribed and labeled using the CyScribe post labeling kit (Amersham Biosciences, Piscataway, NJ). Cy3 and Cy5 labeled cDNAs were purified on QIAquick Cleanup columns separately (Qiagen), and combined and concentrated to 15 µl using Microcon YM-30 microcentrifugation units (Millipore, Billerica, MA). Labelled probes were mixed with 1 µl of 1 mg/ml sheared, sonicated herring sperm DNA and denatured by boiling for 5 min, followed by chilling on ice. DNA array slides were pre-hybridized, hybridized with labeled probes at 42°C overnight and washed as described (84). Slides were scanned using a GenePix 4000B scanner (Axon Instruments, Union City, CA) and images were processed and analyzed using GenePix version 4.1 software. Four independent experiments (including a dye swap) were performed and data analysis was performed as described previously (47, 84).

Quantitative RT-PCR. Oligonucleotides for genes of interest (*rpoH*, *groES*, *NG0372*, *NG0376*, and *NG1684*) were designed using Primer Express software (Applied Biosystems) and sequences will be provided on request. Oligonucleotide primers for *16S rRNA* were described previously (93, 322). Real-time PCR was performed using the SYBR Green detection method (Applied Biosystems). Amplification reactions were in a total volume of 15 µl and contained 1.5 µl 10X SYBR Green PCR buffer, 1.5 µl 25 mM $MgCl_2$, 1.2 µl 10 mM dNTP, 20 nM of each oligonucleotide, 0.15 µl AmpErase® UNG (1U/µl), 0.075 µl AmpliTaq Gold® DNA polymerase (5U/µl) and appropriate diluted template. The reactions were held at 50°C for 2 min, then heated to 95°C for 10 min and then cycled 40 times using parameters of 95°C for 15 s and 60°C for 30 s. Reactions were run on an ABI Prism model 7900HT Sequence Detection System (Applied Biosystems) at the MSU RTSF.

Three independent experiments were performed using freshly extracted RNA for each experiment (biological replicates). In each single experiment, dilutions of each reverse transcription product were loaded in triplicate for each pair of oligonucleotides. Data generated from real-time PCR experiments was processed and analyzed using Microsoft Excel. Differential gene expression was calculated using the relative expression method as recommended (Applied Biosystems). The relative amount of target was normalized to the internal control *16S rRNA* (322), a house-keeping gene not regulated by RpoH or host cell contact (84). Data were subjected to statistical analysis using the Student's *t* test, and *p* values are indicated where appropriate.

RESULTS

Transcriptional analysis of gonococcal genes in an *rpoH* conditional strain. In our previous work we identified several genes, including *rpoH* and two genes (*groEL* and *groES*) known to be regulated by RpoH in *N. gonorrhoeae* (391) as induced upon contact with epithelial cells (84). Based on these observations, we hypothesized that RpoH would mediate the induction of expression of some of these genes upon adherence to epithelial cells. To identify genes in addition to *groEL* and *groES* that are regulated by RpoH, global gene expression was examined in a gonococcal strain conditionally expressing *rpoH* (MPD288 [*rpoH*::TnEmUP, *lacIOP-rpoH*Cm] (84). In this strain, the wild-type copy of *rpoH* on the chromosome was disrupted by insertion of the transposon TnEmUP (83), and an additional copy of *rpoH* under control of a *lac* promoter was placed between *aspC* and *lctP* on the chromosome (142). MPD288 was grown in GC broth either with or without 20 μM IPTG for 6 h and RNA isolated from each culture. RNA was then labeled and used to hybridize to *N. gonorrhoeae* DNA microarrays as described in Materials and Methods. Four independent experiments (including one dye swap) were performed and data was analyzed as described previously (84).

Table 5-1 lists the average ratios of expression of genes identified previously as induced upon host-cell contact (84). As expected, the expression of *rpoH* was highly induced in the presence of IPTG (5.33-fold), consistent with the fact that *rpoH* is under control of a *lac* promoter in this strain. Also as expected, expression of *groES* and *groEL* were significantly increased (2.58-fold and 2.06-fold respectively) in IPTG-treated samples. In addition, expression of *NG0376*, predicted to encode a homolog of peptidyl-prolyl cis-trans isomerase B (148), was induced 2.71-fold in the presence of IPTG,

indicating that this gene is regulated by RpoH. The expression ratios for the remainder of the genes induced upon host cell contact (84) were not significantly larger than 2-fold in the presence of IPTG, suggesting that they are not regulated by RpoH, at least not under the conditions tested.

Quantitative PCR analysis of selected host cell contact-induced genes in a conditional *rpoH* strain. To corroborate the microarray results and confirm that expression of *NG0376*, but not *NG0372* or *NG1684*, are induced by RpoH, real-time RT-PCR was performed. *N. gonorrhoeae* strain MPD288, was grown with and without the inducer IPTG as for the microarray experiment described above. RNA was isolated and cDNA synthesized using random primers as described in Materials and Methods. Gene specific primers for *NG0372, NG0376, NG1684,* as well as *groES* and *rpoH* (as controls) were designed and used in real-time PCR to determine the relative levels of transcription of these genes in IPTG-treated and untreated samples. The relative amounts of products were normalized to an internal *16S rRNA* control, as it is a housekeeping gene whose expression is not influenced by RpoH (84).

As expected, the expression of *rpoH* was highly induced bacteria grown in the presence of IPTG (7.5-fold), consistent with the fact that *rpoH* is under control of a *lac* promoter in this strain. *NG1684*, annotated as encoding a conserved hypothetical protein (43), is the gene whose expression was most highly induced in gonococci adherent to A431 cells (3.27-fold, Table 5-1) (84). Microarray analysis of the conditional *rpoH* strain MPD288 showed that this gene was not increased in expression in inducing conditions, suggesting that it is not regulated by RpoH (ratio 0.80 ± 0.13; Table 5-1). Consistent with

this observation, real-time PCR analyses show that *NG1684* is not induced in MPD288 grown with IPTG, and may actually be slightlyrepressed under these conditions (ratio 0.43 ± 0.12, $p < 0.0001$; Figure 5-1). *NG0372*, annotated as encoding a periplasmic amino acid binding component of an ABC-transporter (43), was also shown to be significantly up-regulated in adherent gonococci (1.97-fold, Table 5-1) (84). Like *NG1684, NG0372* was not significantly induced by IPTG in the DNA microarray analysis of the conditional *rpoH* strain, MPD288 (ratio 1.06 ± 0.48, Table 5-1) nor was it significantly differentially expressed in the real-time PCR analyses (ratio 0.99 ± 0.40, $p = 0.958$; Figure 5-1). In contrast, *groES*, known to be RpoH-regulated in several bacterial species, including *N. gonorrhoeae* (84), (391) was induced more than 3-fold in IPTG-treated samples as determined by real-time PCR (ratio 3.39 ± 0.92, $p = 0.0002$; Figure 5-1). DNA microarray analyses showed expression of *NG0376* to be induced nearly 3-fold in IPTG-induced samples, and real-time PCR results are consistent with this observation in that an expression ratio of 3.12 ± 1.09 was determined ($p = 0.0004$; Figure 5-1). These results indicate that while *NG0376* and *groES* are regulated by RpoH, *NG1684* and *NG0372* are not.

Table 5-1. Expression ratios of host cell contact-induced genes in the presence or absence of RpoH

ORF ID[a]	*gene*	gene product	expression ratio (+cells/-cells)[a]	expression ratio (+IPTG/-IPTG)[b]	(SD)[c]
NG0288	*rpoH*	RpoH, σ^{32}	2.61	5.33	(2.56)
NG2094	*groES*	GroES, heat shock chaperone	2.39	2.58	(0.47)
NG2095	*groEL*	GroEL, heat shock chaperone	2.10	2.06	(0.17)
NG0376	*ppiB*	peptidyl-prolyl cis-trans isomerase B	1.87	2.71	(0.60)
NG1684		conserved hypothetical protein	3.27	0.80	(0.13)
NG1363	*mtrE*	MtrE, multidrug efflux pump	2.48	0.95	(0.06)
NG1989		*Neisseria*-specific protein, uncharacterized	2.38	0.71	(0.11)
NG0340	*cysK*	cysteine synthase	2.34	1.06	(0.36)
NG0238		conserved hypothetical protein	2.34	1.01	(0.33)
NG1210		conserved hypothetical protein	2.24	N.A.[d]	-
NG0634		hypothetical protein	2.03	1.16	(0.51)
NG0372		ABC transporter	1.97	1.06	(0.48)
NG0791		conserved hypothetical protein	1.93	N.A.	-
NG1927		*Neisseria*-specific protein, uncharacterized	1.91	1.04	(0.51)
NG0635		hypothetical protein	1.91	1.19	(0.35)
NG1083		hypothetical protein	1.89	1.04	(0.44)
NG0633	*nifU*	Fe-S scaffold protein	1.89	N.A.	-
NG1988		conserved hypothetical protein	1.88	0.68	(0.21)
NG0234	*hemN*	porphyrin oxidoreductase	1.88	1.03	(0.09)
NG0721		Neisseria-specific protein	1.85	1.49	(0.76)
NG1366	*mtrR*	MtrR, multidrug efflux regulator	1.84	0.99	(0.16)
NG1291		conserved hypothetical protein	1.83	1.01	(0.21)
NG0199	*rho*	transcription termination factor Rho	1.79	0.70	(0.17)
NG1515	*tyrC*	prephenate dehydrogenase TyrC	1.76	N.A.	-
NG1493	*rpsT*	30S ribosomal protein S20	1.73	0.96	(0.43)
NG1439	*aqpZ*	ABC transporter, ATP-binding	1.72	0.87	(0.14)
NG0989		conserved hypothetical protein	1.69	N.A.	-
NG1831	*rpmC*	50S ribosomal protein L29	1.68	N.A.	-
NG1841	*rspJ*	30S ribosomal protein S10	1.68	1.08	(0.15)
NG1668	*pgi*	glucose-6-phosphate isomerase (Gpi)	1.67	1.01	(0.56)
NG0637		conserved hypothetical protein	1.67	0.98	(0.41)
NG1773	*sdaA*	L-serine dehydratase; L-serine deaminase	1.65	1.20	(0.50)
NG2180		conserved hypothetical protein	1.59	0.82	(0.26)
NG0962	*pncB*	nicotinate phosphoribosyltransferase	1.59	1.10	(0.15)
NG5041	*ydhB*	hypothetical protein, MS11 island	1.46	1.28	(0.53)
NG1587	*mafB2*	adhesin MafB2	1.41	1.01	(0.23)

NG0276	*comA*	competence protein (ComA)	1.32	0.96	(0.26)

[a] Gene list and ratios are from (84), and ratios are gene expression in gonococci adherent to A431 cells relative to expression in gonococci grown in the absence of cells.
[b] +IPTG indicates growth in GCB + 20 μM IPTG, - IPTG indicates growth in GCB alone.
[c] SD: standard deviation
[d] N.A.: not available
(Table 5-1 cont'd)

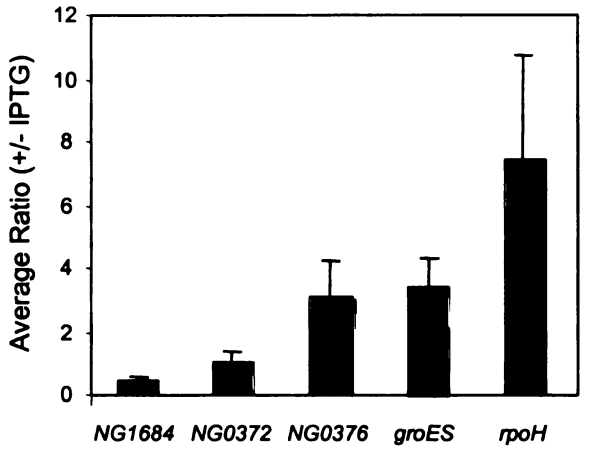


Figure 5-1. Real-time quantitative PCR analysis of expression of *NG1684, NG0372, NG0376, gorES* and *rpoH* in the conditional *rpoH* strain MPD288 grown with and without inducer (IPTG). The expression of each gene was normalized to that of the house-keeping gene *16S rRNA*. Ratios were calculated as the expression level of a gene in MPD288 grown with 20 μM IPTG divided by that from MPD288 grown without IPTG. Data presented is the average of three independent experiments and error bars represent standard deviation.

Induction of *NG0376* and *groESL* upon host cell contact is mediated by RpoH. Previous results showed that *NG0376* was induced in gonococci adherent to epithelial cells (84). Results obtained by both microarray (Table 5-1) and real-time PCR (Figure 5-1) showed that *NG0376* was regulated by RpoH in standard laboratory medium (GCB). Thus, we next asked whether the induction of *NG0376* expression in adherent gonococci was dependent on RpoH. *N. gonorrhoeae* strain MPD288, which conditonally expresses *rpoH*, was grown in the absence of the inducer, IPTG, to deplete RpoH in the cell, and then used to infect A431 cells. For comparison, RpoH-depleted bacteria were grown in cell culture media without cells. At 3 h post-infection (p.i.), cells were washed to remove non-adherent bacteria, and bacteria adherent to cells lifted by lysing the epithelial cells with saponin. Adherent bacteria and grown in the absence of cells were harvested by centrifugation and RNA was isolated as described in Materials and Methods. cDNA was made from the RNA using random primers and levels of expression from *NG0376*, *groES*, and *NG1684* in the samples were quantified by real-time PCR. *NG1684* and *groES* were used as negative and positive controls respectively. *NG1684*, while induced in gonococci adherent to epithelial cells (84), did not appear to be regulated by RpoH in either microarray (Table 5-1) or quantitative PCR experiments (Figure 5-1). *groES* expression has been shown to be regulated by RpoH in *N. gonorrhoeae* (Table 5-1, Figure 5-1) (84, 391); as well as induced upon host-cell contact (84).

As expected, the expression of *groES* in adherent gonococci depleted of RpoH was similar to the levels observed in gonococci grown in cell culture medium alone, indicating that *groES* was no longer induced by contact with host cells in the absence of RpoH (ratio 1.22 ± 0.17, p = 0.040; Figure 5-2). A similar expression pattern was

observed for *NG0376*, which was also no longer induced in adherent gonococci in the absence of RpoH (ratio 1.39 ± 1.20, $p = 0.357$; Figure5-2). Interestingly, expression of *NG1684* was still induced in adherent gonococci depleted of RpoH (ratio 4.84 ± 3.47, $p = 0.042$; Figure 5-2), suggesting that another regulatory mechanism, independent of RpoH, is involved in modulating the expression of this gene in response to contact with epithelial cells. Taken together, these results show that host-cell contact induction of *NG0376* and *groES*, but not *NG1684*, is mediated by RpoH.

Identification of a putative RpoH-like promoter upstream of *NG0376*. Several genes have been identified in *E. coli* as being transcribed from σ^{32} (RpoH)-containing RNA polymerase, leading to the determination of a consensus RpoH-regulated promoter (195, 212, 442). Tauschek and coworkers identified a putative RpoH-binding site upstream of *groES* in *N. gonorrhoeae,* which is near a transcriptional start site that was more active following a 10 min pulse at 42°C (391). This promoter sequence shares high similarity (13/20 nt match) to the consensus RpoH promoter identified in *E. coli* (442). Analysis of the promoter region of *NG0376* revealed a sequence with high similarity to both the gonococcal *groES* promoter and the consensus *E. coli* RpoH promoter (13/20 nt match). The identification of a putative RpoH-binding site upstream of *NG0376* is consistent with the observations that this gene is regulated by RpoH and suggests that the regulation is direct, with *NG0376* transcribed from a σ^{32}(RpoH)-containing RNA polymerase in *N. gonorrhoeae*.

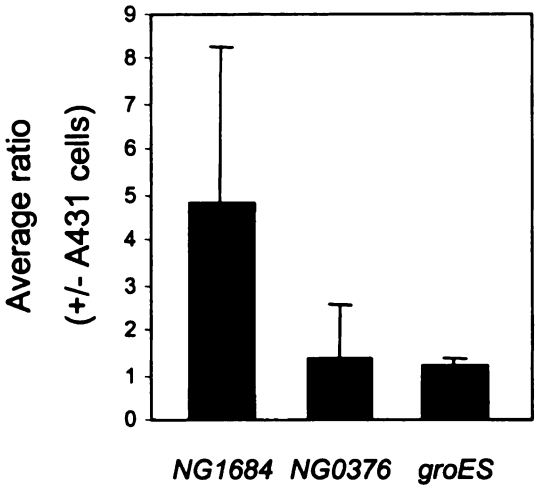


Figure 5-2. Real-time quantitative PCR analysis of expression of *NG1684, NG0376* and *groES* in MPD288 depleted for RpoH and adherent to A431 cells. RNA isolated from MPD288 depleted for RpoH adherent to A431 cells (+) or grown in the absence of A431 cells (-) was reverse transcribed using random primers. Real-time PCR was performed with primers specific for *NG1684, NG0376, groES* and *16S rRNA* as described in the text. Ratios were calculated as the expression level of a gene in adherent MPD288 divided by that of MPD288 grown in cell-free culture. Data presented is the average of three independent experiments and error bars represent standard deviation.

Construction of gonococcal strains with mutations in host cell contact-induced genes. Since our previous results indicated that several genes were induced in gonococci adherent to epithelial cells (84), we hypothesized that one or more of those genes would be important for subsequent steps of a gonococcal infection. To test this, several host cell contact-induced genes were selected for further investigation of their roles in gonococcal infection. The rationale for the selection of genes to mutate was as follows: *NG0340, NG0634, NG1363, NG1684, NG1989*, and *NG2094* were selected based on the observation that they showed a ≥ 2-fold increase in expression in adherent gonococci (Table 5-1) (84). While *NG0238* and *NG1210* were also induced ≥ 2-fold upon adherence, neither was present in the clone set used as targets for mutagenesis (44), and therefore we were not able to mutagenize them. *NG2095 (groEL)* is the second gene of the *groES-groEL* operon (391), and only *NG2094 (groES)* was selected for mutational analysis. *NG0372* was selected as it was induced nearly 2-fold upon adherence and was also found to be differentially expressed in other experiments (Lenz, J. and C. G. Arvidson, unpublished results), and was therefore of interest. *NG0376* was selected since it was found to be RpoH-dependent for induction in adherent gonococci (Table 5-1, Figure 5-1 and 5-2), and *NG1366 (mtrR)* was selected as it encodes a previously characterized transcriptional regulator (139).

Plasmids containing genes of interest were insertionally inactivated by in vitro transposition using the transposon TnErmUP as described previously (83, 84). Plasmids containing mutated genes were then used to transform *N. gonorrhoeae* strain MS11 to Em^R. Allelic exchange between the wild-type copy on the chromosome and transforming

DNA containing the mutant copy containing the Em^R determinant should result in null mutations of each of these genes. Not surprisingly, despite several transformation attempts, we were unable to construct a gonococcal *groES* null mutant in that no Em^R transformants were obtained. Since *groES* is essential in other bacterial species (94), it is likely that it is essential in gonococci as well, although additional experiments will be required to prove this. We were also unable to contruct null mutations in three additional genes, *NG0372, NG0634,* and *NG1363.* The reasons for this were not clear, but for this work these genes were not studied further. Once mutants were successfully constructed in *N. gonorrhoeae* strain MS11, each mutant was backcrossed with the parent strain by transformation with genomic DNA. This was to insure that the mutation was caused by the insertion of the transposon in the gene of interest. A list of the mutant strains constructed and the gene mutated in each is in Table 5-2.

Analysis of mutants for interactions with epithelial cells in culture. Once gonococcal strains were constructed with insertion mutations in individual genes identified as induced upon contact with epithelial cells, we next asked whether any of the mutations affected the ability of gonococci to adhere to and/or invade A431 cells in culture. Wild-type MS11 and mutant strains were grown overnight on GC agar and then used to infect A431 cells at an MOI of 10 as described in Materials and Methods.

Figure 5-3A summarizes the results of three independent experiments in which adherence was measured at 3 h p.i, and adhesion frequencies expressed as the percent cell-associated CFU as a function of the total CFU in the well at the end of the experiment. These results show that insertion mutations in *NG0376, NG1366, NG1684,*

and *NG1989* had no appreciable affect on gonococcal adherence to A431 cells compared to the wild-type parent, MS11. However, mutant M0340 was significantly reduced in adherence, with an adhesion frequency more than 3-fold lower than that determined for MS11 (32.9 ± 9.3%, $p < 0.0001$). A possible explanation for this phenotype could be that the mutant expressed a variant *pilE* gene, which encodes pilin, the major subunit of the gonococcal pilus (374). Type IV pili are the primary adhesins for gonococci (380) and undergo antigenic variation at a high frequency (353), thus a variant pilin could alter the ability of the strain to adhere to cells in culture. To test this possibility, the *pilE* genes from M0340 and from the wild-type parent strain, MS11, were PCR amplified and DNA sequences were determined. The sequencing results showed that the two *pilE* sequences were identical at nucleotide level, indicating that the reduced adhesion phenotype of M0340 is not due to the production of a variant pilin in the M0340 mutant strain.

Figure 5-3B summarizes the invasion assay results obtained from three independent assays performed in triplicate. Assays were performed as described (84) and invasion was scored as the number of Gm^R CFU per cell-associated CFU at 7 h p.i. The results of these experiments show that two mutants, M0340 and M0376, were significantly reduced in their ability to invade A431 cells at 7 h p.i.. Mutant M0340, which was reduced in adherence to A431 cells (Figure 5-3A), also invaded cells ~ 70% (70.1 ± 10.4%, $p < 0.0001$) as well as the wild-type parent strain, indicating that this mutant is generally deficient in its ability to interact with epithelial cells. A second mutant, M0376, was also reduced in its ability to invade A431 cells, invading ~40% (40.9 ± 8.5%, $p < 0.0001$) as well as the wild-type MS11, although it appeared to adhere to

cells as well as MS11 (Figure 5-3A). The other three mutants assayed, M1366, M1684, and M1989, invaded with essentially the same frequencies as the wild-type strain.

Molecular complementation of the *NG0340* and *NG0376* mutations. In order to demonstrate that the reduced adhesion and invasion phenotypes observed for strains M0340 and M0376 were indeed due to insertion mutations in *NG0340* and *NG0376*, molecular complementation experiments were performed in which each of the genes was cloned and expressed *in trans* in the corresponding insertion mutant strains. *NG0340* and *NG0376*, along with 400-500 bp of 5' and 3' flanking sequences were PCR amplified and cloned into a derivative of the plasmid pKH35 in which the P_{lac}-*lacI*^{Q} fragment had been removed, to generate pC340 and pC376. Adjacent to the cloned genes on these plasmids is a marker for Cm^R, and flanking the cloned gene-marker construct are two genes, *aspC* and *lctP* of the *N. gonorrhoeae* chromosome between which is has been reported that large sequences can be inserted without being deleterious to the cell (84), (142). Transformation of *N. gonorrhoeae* with linearized pC340 and pC376 and selection for Cm^R resulted in recombination between the incoming DNA and the *aspC-lctP* region of the chromosome. The resulting heterodiploids are such that the chromosomal copy of *NG0340* or *NG0376* had the TnErmUP insertion, and an additional wild-type copy of the corresponding gene is at the *aspC-lctP* locus. Transformants were confirmed as having both a wild-type and Tn-mutated copy of *NG0340* or *NG0376* by PCR using combinations of gene-specific and transposon-specific primers.

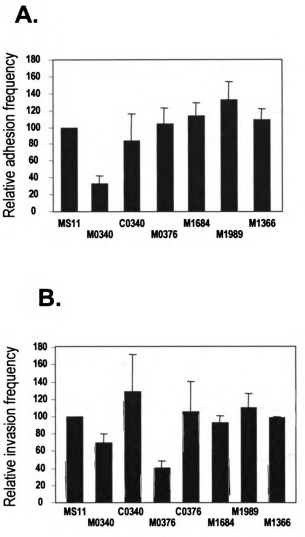


Figure 5-3. Interaction of *N. gonorrhoeae* mutants with A431 cells. (A) Adherence to A431 cells. Adhesion data are presented as relative adhesion frequency, normalized to the wild-type control (MS11), which was arbitrarily set at 100%. (B) Invasion of A431 cells. Invasion data are presented as relative invasion frequency, normalized to MS11, which was arbitrarily set at 100%. These data are averages of three independent experiments performed in triplicate, and error bars indicate standard deviation.

Table 5-2. Mutants constructed in host cell contact induced genes.

gene	ORF ID	Mutant	RpoH-dependent[a]
cysK	NG0340	M0340	no
	NG0372	N.D.[b]	no
ppiB	NG0376	M0376	yes
	NG0634	N.D.	no
mtrE	NG1363	N.D.	no
mtrR	NG1366	M1366	no
	NG1684	M1684	no
	NG1989	M1989	no
groES	NG2094	N.D.	yes

[a] Regulation is mediated by RpoH as determined by DNA microarray (Table 5-1) and Q-PCR (Figure 5-1).

[b] N.D.: no mutant obtained

The resulting strains, C340 and C376, were then used to infect A431 cells to measure adherence and invasion as described in Materials and Methods, and the data is shown graphically in Figure 5-3. The complemented mutant, C340, adhered to and invaded A431 cells as efficiently as the original wild-type parent strain, MS11 ($p = 0.436$ and 0.296, respectively). The complemented mutant, C376, also invaded A431 cells as efficiently as the original wild-type parent strain ($p = 0.798$). These results indicate that providing the wild-type *NG0340* and *NG0376 in trans* indeed complemented the mutations, restoring the adherence and invasion capabilities of the two mutant strains.

DISCUSSION

Adherence to mucosal epithelial cells is a criticial first step in a gonococcal infection. As gonorrhea is a sexually-transmitted disease, transmission to a new host often results in the bacterium entering a very different environment from which it came, and the initial interaction with epithelial cells has the potential to serve as a signal to the bacterium that it is in a new host. Previous studies from our laboratory have demonstrated that attachment of gonococci to epithelial cells results in the modulation of expression of several genes (84). Genes differentially expressed in adherent gonococci included those involved in a variety of functions such as glycolysis, amino acid metabolism, protein synthesis, protein transport, as well as regulation of gene expression. In particular, we found that *rpoH*, which encodes the heat shock sigma factor, σ^{32} (RpoH), was induced in adherent gonococci and that this induction was necessary for optimal invasion of epithelial cells.

RpoH is a critical regulator of the bacterial heat shock response, and is highly conserved in bacteria (271, 452). Studies in other bacteria have shown that RpoH is a general transcription factor not only involved in the heat shock response, but also involved in the response to osmotic pressure, misfolded proteins, nutrient limitation and other stimuli (8, 18, 32, 444). Our observation that RpoH is involved in modulating gene expression in gonococci upon contact with host cells suggests that this interaction is yet another stress response mediated by RpoH in a bacterium.

The goals of this work were two-fold. First, we sought to determine which genes induced upon adherence to epithelial cells require RpoH for this induction. Second, we

examined several host cell contact-induced genes to determine which, if any, play a role in adherence to and invasion of epithelial cells.

NG0376, encoding a putative rotamase, was found to be dependent on RpoH for induction upon contact with epithelial cells. Three pieces of evidence support this conclusion. First, in a standard laboratory medium (GCB), expression of this gene was significantly induced in the conditional *rpoH* strain grown in the presence of inducer as determined by DNA microarray analysis (Table 5-1) and quantitative RT-PCR (Figure 5-1) indicating regulation by RpoH. Second, when the conditional *rpoH* strain, MPD288, was grown in RpoH-depletion conditions (no IPTG), induction of *NG0376* expression in adherent gonococci was abated (Figure 5-2), indicating that RpoH was required for the cell contact-mediated induction. Third, a putative RpoH-binding site with significant sequence similarity to RpoH-dependent promoters was identified upstream of *NG0376* (data not shown). Similar observations were made for *groES*, a well-characterized RpoH-dependent gene in several bacteria including *N. gonorrhoeae* (84, 105, 199, 391, 440). Taken together, these results demonstrate that like *groEL* and *groES*, *NG0376* is part of the gonococcal RpoH regulon and requires RpoH for induction upon contact with epithelial cells.

In contrast to our observations that *groEL* and *groES* and *NG0376* are induced in adherent gonococci in an RpoH-dependent manner, *NG1684*, whose expression was the most highly induced in gonococci adherent to A431 cells (84) appeared to be RpoH-independent. Expression of *NG1684* in the conditional *rpoH* strain in the presence of inducer was essentially the same as in its absence (Table 5-1). However, when the conditional *rpoH* strain, MPD288, was grown in RpoH-depletion conditions and used to

infect A431 cells, expression of *NG1684* in adherent gonococci was still induced (Figure 5-2). These results clearly indicate that the induction of *NG1684* in adherent gonococci is independent of RpoH and is likely mediated by another, as yet unidentified regulator.

We previously showed that several genes were induced in gonococci adherent to epithelial cells (84). This led to the hypothesis that one or more of those genes would be important for adherence to and/or invasion of epithelial cells. To test this, mutations in several host cell contact-induced genes were constructed and analyzed for their effects on adherence to and invasion of A431 cells in culture.

We were successful in isolating null mutants of *NG0340, NG0376, NG1366, NG1684,* and *NG1989* (listed in Table 5-2) but were unable to isolate transformants with insertion mutations in *groES, NG0634, NG0372,* and *NG1363*, in spite of several attempts. It is possible that these genes are essential in *N. gonorrhoeae*, and is very likely for *groES*, which is known to be essential in other bacterial species (218). However, there have been reports of *mtrE* (*NG1363*) mutants isolated in other *N. gonorrhoeae* strains (76), thus there may be other explanations for our inability to construct a *mtrE* mutant in MS11. *NG0372* encodes a putative ABC-transporter component (43), and is induced in adherent gonococci in an RpoH-independent manner. Whether or not the putative ABC-transport system is essential will require further experimentation. Another explanation for the inability to obtain some of these mutants is that there may be polar effects on genes downstream of those we attempted to mutate. Additional experiments, such as using plasmid constructs with more flanking sequences on either side of the transposon insertion to facilitate homologous crossover and/or the development and use of a non-

polar transposon might result in viable mutants in these genes. To prove that either of these is essential will require the construction of conditional mutants.

Assays for adherence to A431 epithelial cells using the six transposon insertion mutants isolated (listed in Table 5-2) showed that one mutant, M0340, was significantly reduced in its ability to adhere to cells (Figure 5-3A). This mutant was also reduced in its ability to invade A431 cells (Figure 5-3B). Since invasion is scored as a function of adherence (Gm^R per cell associated CFU), the observed reduction in invasion frequency indicates that each step (adherence and invasion) is defective in this mutant, and that the protein encoded by this gene, thought to be involved in cysteine biosynthesis, is important for gonococcal-host cell interactions. While expression of *NG0340* is induced in adherent gonococci, this induction is independent of RpoH (Table 5-1, Figure 5-1), therefore, our previous observation that depletion of RpoH in gonococci reduces its ability to invade A431 cells (84) is not due to a lack of induction of *NG0340* expression. However, this does provide evidence for the existence of additional regulators that are involved in the host cell contact response.

In the gonococcal genome database (43), *NG0340* is annotated as encoding an *O*-acetylserine (thiol)-lyase-A, which catalyzes the last step in the synthesis of L-cysteine (EC 2.5.1.47). In *E. coli* and the closely related *Salmonella typhimurium*, there are two genes encoding *O*-acetylserine (thiol)-lyase-A, *cysK* and *cysM* (53), however only one (annotated as *cysK*) has been identified in the gonococcal genome. Cysteine has been shown to be very important for growth of gonococci in that cysteine is required for colony formation on chemically defined agar medium (56) and cystine (formed from cysteine in oxidizing conditions) is a limiting factor in continuous culture of gonococci

(185). Cysteine is an essential amino acid, not synthesized by the human host, thus it is likely that cysteine will be limiting in the natural environment of the gonococcus. Cysteine, as one of two sulfur-containing amino acids serves as a source of organic sulfur in the bacterial cell, and is also critical for the folding of many proteins via the formation of disulfide bonds. It is this feature that may account for our observations that *cysK* is necessary for gonococcal host cell interactions. The interaction between gonococci and host cells involves several proteinaceous structures on the surface of the bacterium, including pili (380), opacity proteins (232, 419, 427), and porins (176, 194). Cysteine residues are highly conserved in the otherwise highly antigenicially variable pilin, the major protein component of the gonococcal pilus (357), and are critical in the formation of disulfide bonds during assembly of the pilus fiber (288). As pili are critical for gonococcal attachment to epithelial cells this could explain why *cysK* mutants do not adhere well to A431 cells (Figure 5-3A), although since pili do not promote invasion, and may even impede the process (232, 360), this does not explain our observation that M0340 invades A431 cells less efficiently than the wild-type. The reason for this observation is less clear and will require additional experimentation.

A second mutant, M0376, adhered to A431 cells essentially the same as the wild-type (Figure 5-3A). However, this mutant did not invade A431 cells well at all, ~40% as efficiently as the wild-type (Figure 5-3B). Furthermore, the induction of expression of *NG0376* in adherent gonococci is dependent on RpoH (Table 5-1, Figure 5-1 and 5-2), consistent with our prior observation that RpoH is necessary for optimal invasion of A431 cells (84). In the gonococcal genome database (43), *NG0376* is annotated as encoding a peptidyl-prolyl cis-trans isomerase B (also referred to as rotamase B, PPIase,

PpiB). Rotamases facilitate the folding of proteins by catalyzing the cis-trans isomerization of proline imidic peptide bonds of proline-containing peptides and were first identified as cyclophilins in higher organisms, targets of the immunosuppressive drug, cyclosporin (96, 388). Analysis of the many sequenced genomes shows that rotamases are present in nearly all organisms prokaryotic and eukaryotic, many having several rotamases with slightly differing functions and characteristics (107). Rotamases can be divided into three families: 1) the cyclophilin family, which are homologous to cyclosporin binding proteins; 2) the FKBP (FK506 binding protein) family, which bind another immunosuppressive drug, FK506, and are not similar in sequence to cyclophilins (143, 362); and 3) a recently identified family called parvulins, so named for their small size (10.1 kDa) (313). Bacterial rotamases are found in the cytoplasm, periplasm, and can also be surface exposed (87, 148, 155).

Rotamases play a role in the proper folding of many bacterial proteins, including virulence factors in bacterial pathogens. A *Legionella pneumophila* rotamase mutant was shown to invade the amoeba, *Acanthamoeba castellanii* 10-fold less well than wild-type (343). Hermans and co-workers recently identified a surface exposed rotamase, SlrA, in *Streptococcus pneumoniae* that was important for colonization, but not invasive disease in a mouse model of infection (155). In addition, *slrA* mutant pneumococci were impaired in their ability to adhere to multiple cell lines in culture, clearly demonstrating a role for SlrA in pneumococcal-host cell interactions. Of the nine rotamases identified in *E. coli*, five are cytoplasmic and four are periplasmic (87). A quadruple mutant in which each periplasmic rotamase gene was inactivated was viable, but reduced in its ability to assemble pili (both type I and P) (178). As pili are adhesins in uropathogenic *E. coli*

(UPEC) that mediate their attachment to cells in the urogenital tract, a defect in pilus assembly would likely affect the ability of UPEC to interact with host cells in an infection. Our observation that a null mutation in a gene encoding a putative rotamase in *N. gonorrhoeae* results in a defect in invasion is consistent with these observations that rotamases are important in pathogen-host interactions.

There are five putative rotamases encoded in the gonococcal genome (43): one of the parvulin family (*NG0766*, *ppiD*), two of the cyclophilin family (*NG0376*, *ppiB*; *NG0544*, *ppiA*), and two of the FKBP family (*NG0981*, *slyD*; *NG1225*, *mip*). Leuzzi and co-workers recently reported that *NG1225*, which encodes a FKBP-type rotamase, is involved in persistance of gonococci in macrophages (213). Indeed, *NG1225* is annotated as encoding a macrophage infectivity potentiator (MIP), a family of proteins found on the surface of several intracellular pathogens that plays a role in the interactions between the pathogen and phagocytic cells. Null mutations in *NG1225* are viable, and the mutants appear not to be defective in production of pili or Opa proteins, and are bound by and taken up by macrophages as well as the wild-type parent strain (213). However, *NG1225* mutants appeared to be more sensitive to killing by the macrophages, as fewer intracellular bacteria were recovered from macrophages infected with the mutant compared to the wild-type. This phenotype was specific for macrophages, as the mutant behaved similarly to the wild-type in adhesion, invasion, and intracellular survival assays using ME-180 (human cervical epithelial) cells. Little is known of the roles played by the other gonococcal rotamases.

NG0376 is the only rotamase gene identified as regulated upon contact with epithelial cells (84). DNA microarray experiments showed that expression of *NG0544*,

NG0766, NG0981, and *NG1225* were essentially the same in gonococci grown either in the absence of or adherent to epithelial cells. Regulation of *NG0376* upon contact with epithelial cells is dependent on RpoH (Table 5-1, Figure 5-1 and 5-2), similar to regulation of expression of rotamase genes in *E. coli*, which varies for the many rotamases identified in this bacterium. Not surprisingly, most are controlled by stress regulators such as CpxR and the heat-shock sigma factors, σ^{32} (RpoH) and σ^{E} (RpoE) (69, 71, 300).

In this work, we showed that a *NG0376/ppiB* mutation affects invasion of, but not adherence to epithelial cells (Figure 5-3). Our previous studies showed that *ppiB* expression is increased when gonococci are adherent to cells (84). These observations suggest that gonococci may be responding to an increase in the synthesis of proline-containing proteins that are important for the invasion process and need to increase the production of PpiB to facilitate proper folding of these proteins. That PpiB is cytoplasmic suggests that its target proteins are at least folded in the cytoplasm, if not remaining there. The identity of these targets remains a mystery.

In summary, we have shown that adherence of gonococci to epithelial cells, a critical early step in an infection, results in the modulation of expression of several genes that are important for subsequent steps. In particular, two of these genes, *NG0340* and *NG0376*, encoding cysteine synthetase and rotamase, respectively, are likely important in the proper synthesis and assembly of proteins that are needed following the attachment step. That the heat shock sigma factor, RpoH, in part mediates this regulation is consistent with observations in other bacteria that this regulatory system responds to environmental stresses, and indicating that signalling is occurring across the bacterial

membrane. Additionally, the identification of genes that are induced by host cell contact independent of RpoH indicates that there are multiple regulators involved in this response. Further characterization of this regulatory network will be key to a more in depth understanding of the communication between gonococci and the human host.

Chapter 6

Summary and Future work

Neisseria gonorrhoeae infects more than 60 million people each year worldwide (www.who.org), posing a significant public health threat. Treatment has heavily depended on antibiotics; however, *N. gonorrhoeae*'s susceptibility to these antibiotics has been declining. Thus, developing new strategies to prevent and treat gonococcal infections is necessary in this modern society. Although much effort has been made to understand the pathogenesis of this pathogen, our knowledge about this pathogen, particularly the interaction between gonococcus and host cells is still limited.

In order to fully understand the pathogenesis of *N. gonorrhoeae*, this dissertation investigated the interaction between this pathogen and epithelial cells using a tissue culture model for infection. While *N. gonorrhoeae* typically establishes infection in the subepithelial space of the urogenital mucosa, adherence to epithelial cells is the critical first step in a gonococcal infection. The underlying hypothesis of this dissertation is that this initial attachment to host cells transmits signals into the bacterium, resulting in the modulation of gene expression to facilitate its subsequent survival and amplification in the host.

The important tool used for the experiments in this dissertation is the *N. gonorrhoeae* DNA microarray, which was designed and constructed in our lab in collaboration with several research groups around the country (44, Appendix A). This technique now allows us to measure gene expression at the genome-wide level.

Using the gonococcal DNA microarray and a cell culture model of infection, this dissertation reports the changes of gene expression within *N. gonorrhoeae* in response to host cell interaction. The genes differentially regulated upon host cell contact encode products with a broad range of functions, including transcriptional regulation, bacterial

metabolism, protein transport, and cell division, *etc.* (Table 3-1 & 3-2). The shift in expression of many metabolic genes suggests a general shift in the cellular metabolism in response to host cell contact, likely to allow the pathogen to adapt to the different environment inside the host cell. These observations are consistent with our hypothesis and show that host cell contact does indeed serve as an important signal to induce changes of gene expression inside the bacterium. Characterizing each single gene differentially expressed would be intriguing, as it will provide more information on the pathogenesis of *N. gonorrhoeae.*

In this work, RpoH was identified as an important regulator in gonococcal infection, as depletion of RpoH from the bacteria reduces gonococcal ability to invade epithelial cells. This phenotype is partially due to the failure of induction of an RpoH-regulated gene, *NG0376*, as the *NG0376* mutant was not able to invade epithelial cells as efficiently as the wild-type. In addition to the identification of *rpoH* and *NG0376*, two genes, *NG1684* and *NG0340*, which were greatly induced upon host cell contact, were not dependent on RpoH for this regulation. Futhermore, *NG0340* was identified as important in both adhesion and invasion steps. These results indicate that RpoH is not the only regulator involved in gonococci-host cell interactions. It is likely that a complex regulatory network is engaged in coordinating gonococcal responses to host cell contact (Chapter 5).

This dissertation has added some new information to our understanding of the complex regulatory network of *N. gonorrhoeae* in gonococcal infections. However, the more we learn about this pathogen, the more questions have emerged.

First, *NG0340* and RpoH-regulated *NG0376* were identified as important in infection, as the *NG0376* mutant was reduced in its ability to invade epithelial cells and *NG0340* mutant was compromised in both adhesion and invasion steps (Chapter 5). According to the annotated gonococcal genome (43), *NG0376* encodes a putative peptidyl-prolyl *cis-trans* isomerase B (rotamase B or PpiB) and *NG0340* encodes a putative *O*-acetylserine(thiol)-lyase A (Cysteine synthase). However, these predictions are solely based on sequence homologies to characterized genes in *E. coli* and other bacteria. Their functions in *N. gonohorroeae* are still unclear. If *NG0376* does encode a peptidyl-prolyl cis-trans isomerase B (PpiB) in *N. gonorrhoeae*, what are the targets? The same question also applies to *NG0340*. Is the gene product of *NG0340* a cysteine synthase? It is likely that some proteins important in a gonococcal infection require cysteine to maintain their conformation. If so, what are these targets? As NG0376 and NG0340 are likely involved in protein modification, techniques such as 2D SDS-PAGE and mass spectroscopy may be used to identify their target proteins and to analyze the locations of these proteins. Overall, characterizing the roles of these two genes in *N. gonohorroeae* would be necessary to shed light on why they are required in gonococcal pathogenesis.

Second, of the genes that were differentially regulated upon host cell contact, this dissertation only characterized a few of genes that were induced. In fact, our results have identified 37 genes as induced (Table 4-1) and 57 genes as respressed (Table 4-2) in adherent gonococci in response to host cell contact (Chapter 5). The products of these genes are predicted to be involved in a variety of cellular functions, transcriptional regulation, bacterial metabolism, molecular transport and so on. Particularly, of the genes

down-regulated upon adherence, more than half of them encode proteins involved in central housekeeping functions, such as glycolysis, amino acid metabolism, nitrogen assimilation, respiration and cell division (Chapter 5), which again indicates a general shift in cellular metabolism within gonococci in response to host cell contact. In addition, some of them encode putative drug activity modulator and proteins important in DNA replication, recombination or transformation competence. However, our knowledge about roles of these gene products in gonococcal infection is still incomplete. What are their roles in gonococcal infection? Are they required in the subsequent steps and why are they required? It will be necessary and intriguing to further individually characterize the genes up- and down regulated upon adherence, in particular, their roles in the subsequent infection steps. Transposon mutagenesis, DNA arrays and cell culture models may be used to characterize these genes.

Another aspect worthy of mention is that nearly half of genes differentially regulated upon host contact encode hypothetical proteins, and some are *Neisseria* specific. Those genes are intriguing to study, as they may contribute to the unique characteristics of this pathogen and are potential drug targets or vaccine candidates.

In addition to characterizing each single gene, another critical question is how these genes are co-regulated upon adherence to the host cell, that is, what are the regulators involved in modulating gene expression? In this work, RpoH was identified as a critical transcription factor regulating gene expression in response to adherence (Chapter 5). However, many genes induced in adherent gonococci were not dependent on RpoH for this regulation, such as *NG1684*, the gene induced most upon adherence. Furthermore, an RpoH-independent gene (*NG0340*) was found to be important in both

adhesion and invasion steps. These results clearly indicate that regulators other than RpoH contribute to the changes of gene expression upon host cell contact. Thus, identifying and characterizing these regulators will be of great significance, as it will help us to dissect the signaling pathways and piece together the complicated network involved in a gonococcal infection. In the long run, it will help us to design and develop new strategies to treat this disease.

However, the current techniques may not be sensitive enough to identify these regulators, as typically in response to stimuli the regulators themselves may not necessarily undergo dramatic changes to regulate subsequent cascades and thus may pose challenges for detection and identification. In the gonococcal genome sequence database, there are about 50 genes encoding putative transcription factors in *N. gonorrhoeae* (Chapter 1, Table 1-1), some of which are likely involved in the regulation of gene expression upon contact with host cells. Thus, transposon mutagenesis, cell culture models of infection and microarray analysis can be used to define the regulon of each individual regulator. Then, its regulon can be compared to the list of genes differentially regulated upon host cell contact. Thus, genes present in both lists will suggest that these genes are regulated by this specific regulator. In Chapter 5 we have used this method to identify RpoH as the transcription factor required for the induction of *NG0376* upon adherence.

In this work, the analysis of gonococcal gene expression is limited to gonococci adhered to A431 cells (110), which only represents a single tissue that the gonococcus can colonize. Clinical studies show that gonococci can infect the mucous membrane of the cervix, throat, rectum and conjunctiva. Occasionally, this bacterium can also infect

the joints and cause arthritis. It is likely that *N. gonorrhoeae* can evolve slightly distinct responses upon attaching to different tissues. Thus, it is necessary to use other cell lines to investigate the adhesion step in the future studies.

Finally, in this dissertation we only examine gene expression profiles of *N. gonorrhoeae* at a 3-h post infection (p.i.). Gonococcal infection is known as a dynamic process (249). Upon initial adhesion, gonococci undergo consecutive changes, forming and dispersing microcolonies and the subsequent loss of pili from the surface. Additionally, gonococci can trigger host cell responses rapidly after attachment (240). For example the transportation of the transcription factor nuclear factor kappa B (NF-κB) into the nucleus happens very quickly and NF-κB can be detected in the nucleus within minutes of attachment of gonococci (240). Therefore, different genes are likely required at different times of infection and gene expression is likely coordinated within the bacterium. Thus, it will be important to examine gene expression in this bacterium during the course of the adhesion process. Hence a time course study is necessary to perform towards this aim. The finding will be crucial in disclosing why and how gonococcus responses to host cell contact over time.

Appendix A

Verification of gonococcal DNA microarray

ABSTRACT

A gonococcal DNA microarray was developed in our lab in collaboration with several research groups around the country. This DNA array represents 90% (2035/2250) of the predicted open reading frames of *N. gonorrhoeae*, including 1977 ORFs from the sequenced *N. gonorrhoeae* FA1090 genome and 58 of the gonococcal genetic island from the *N. gonorrhoeae* strain MS11. In this work, validation experiments were performed to compare gene expression in otherwise isogenic piliated (P+) and non-piliated (P-) strains of *N. gonorrhoeae* strain MS11. Consistent with previous research results, our results showed that *pilE*, encoding the major pilus subunit was the most highly differentially expressed gene and demonstrated that this array is suitable for genome wide transcriptional analysis in gonococci.

INTRODUCTION

Neisseria gonorrhoeae (GC, gonococcus) is an obligate human pathogen, with no known animal host, thus creating challenges for *in vivo* gonococcal studies. Research characterizing gonococcal infections shows that the interaction between gonococcus and its host involves many signaling pathways in both the pathogen and host cells (146, 249). To thoroughly understand this interaction, it will be necessary to study gene expression profiles of *N. gonorrhoeae* during infection. DNA microarray technology allows one to examine changes of gene expression at a genome wide level in a single experiment. This technique has been successfully used for several pathogens to demonstrate that the modulation of gene expression is important and required for a pathogen to establish an infection in its host (9, 88).

Taking advantage of the complete sequence of the *N. gonorrhoeae* FA1090 genome (43), we developed a PCR amplicon-based gonococcal DNA microarray spotted onto glass slides (44). This DNA microarray represents more than 90% (1977/2035) of the predicted ORFs from the sequenced genome of *N. gonorrhoeae* strain FA1090 (www.stdgen.lanl.gov) and 58 of the 61 ORFs of a gonococcal genetic island (GGI) of *N. gonorrhoeae* strain MS11 (78). To verify that this DNA microarray was suitable for studying transcriptional profiles in *N. gonorrhoeae*, experiments were performed to compare gene expression in otherwise isogenic piliated (P+) and non-piliated (P-) strains of *N. gonorrhoeae*.

MATERIALS AND METHODS

RNA isolation and labeling. *N. gonorrhoeae* strains MS11 and MS11-307 (247) were grown in GC broth with supplements (187) and 0.042% sodium bicarbonate in a humidified 5% CO_2 environment . RNA was isolated from midlog phase cultures using Trizol reagent (Invitrogen, Carlsbad, CA). RNA was analyzed quantitatively by spectrophotometry, and for quality by agarose gel electrophoresis. RNA was labeled with either Cy3 or Cy5 dyes using the CyScribe first-strand labeling kit (Amersham Biosciences, Piscataway, NJ) and the cDNA probes were purified using the Qiagen PCR cleanup kit (Qiagen, Valencia, CA). Probes were combined and concentrated to 15 µl using Microcon® YM-30 centrifugal filtration units (Millipore, Billerica, Massachusetts).

Hybridizations and data analysis. DNA array slides were UV-crosslinked at 60 mJ energy, and then washed twice in 0.1% SDS for 3 min at room temperature to remove the unbound DNA. The slide was then washed in H_2O for 2 min at room temperature and boiled in H_2O for 3 min to denature the double-stranded DNA on the slide. The slide was plunged briefly into room temperature H_2O to cool and dried by centrifugation for 1 min at 800 xg in a 50 ml conical tube. Prehybridization was performed by incubating the slide with 60 µl pre-warmed prehybridization buffer (5X SSC, 0.1% SDS, 1% BSA) in a Corning hybridization chamber (Corning Life Sciences, Acton, MA) at 42°C for 45 minutes. The labelled probes were denatured by boiling for 5 min, along with 1 µl 1 mg/ml sheared, sonicated herring sperm DNA and chilled on ice. DNA was then mixed with 15 µl 4X hybridization buffer (Amersham) and 30 µl formamide, pipetted onto the prehybridized array slide, assembled in the hybridization chamber, and hybridized at

42°C overnight. Following hybridization, the coverslip was removed and the slide was washed as follows: once in 1X SSC; 0.2% SDS at 42°C, twice in 0.1X SSC; 0.2% SDS at room temperature, once in 0.1X SSC. After the last wash, the slide was dried by centrifugation and scanned using a GenePix 4000B scanner (Axon Instruments, Union City, CA). Images were processed and analyzed using GenePix 4.0 software. Data normalization and analysis were done as described (Chapter 3 and 4).

RESULTS AND DISCUSSION

Gene expression in P+ /- MS11 strains

Gonococcal pili play very important roles in gonococcal pathogenesis and are the major surface structure mediating the adhesion between gonococci and host cells (169, 380, 383). Without pili, gonococci are non-adherent and avirulent (311, 380). Gonococcal pili are of the type IV class (287, 377) and the major subunit, pilin, is encoded by the *pilE* expression loci on the genome.

In this work, we used a gonococcal DNA microarray to compare gene expression between wild-type gonococcal strain MS11 and a mutant, MS11-307, which cannot produce type IV pili on its surface, due to deletions in the pilin expression loci, *pilE* (247). Strains were grown in GC broth, total RNA isolated and labeled with Cy3 or Cy5 by reverse transcription. The labeled cDNAs were then hybridized to the gonococcal DNA microarray slide, as described in Materials and Methods. A total of three independent experiments (including one dye swap) were done and data normalization and analysis was performed as described (47). Outliers were identified as those with expression ratios ($\log_2$ transformed) greater than 2.5 standard deviations from the mean and are listed in Tables A-1 and -2.

As expected, *pilE* was the most highly expressed gene in the wild-type and up-regulated about 70-fold. Several *pilS* loci also had high expression ratios (5-15 fold) due to homologous sequences they share with *pilE* (Table A-1). Additionally, *bfrA* and *bfrB* were also significantly increased in expression in the wildtype strain, with ratios of 3.8 and 4.0 respectively. These two gene products comprise bacterioferritin, an important iron storage protein for bacteria. However, whether pili have some role in iron-

uptake/storage in *N. gonorrhoeae* remains to be determined. The up-regulation of several other genes in wild-type gonococci is also worth mentioning here. One such gene is *ppiB*, which encodes a putative rotamase, an enzyme involved in protein folding (96, 388). This gene was induced 2.82-fold in wild-type relative to non-piliated gonococci. As piliated gonococci express abundant pilin proteins, the induction of this gene in the piliated gonococcal strain may suggest the requirement of this gene product in the folding of pilin. Additionally, *himA* and *recN* are slightly induced in wild-type gonococci. *HimA* encodes a subunit of IHF (Integration Host Factor), a site-specific DNA-binding protein that with roles in DNA recombination, transcription and DNA replication (453). It has been shown that IHF is required for *pilE* transcription (453), which is consistent with our observation of the induction of *himA* in wild-type gonococci. *RecN* encodes a DNA repair protein RecN, required for both DNA repair and DNA transformation in *N. gonorrhoeae* (454). Research has shown that DNA transformation is important in pilus antigenic variation in *N. gonorrhoeae* by providing pilin sequences for recombination (354). Thus, the induction of *recN* in this study may further indicate that RecN plays an active role in the DNA transformation-related pilus antigenic variation.

Compared to the number of genes up-regulated in P^+ gonococci, fewer genes were identified as significantly up-regulated in P^- bacteria (MS11-307, Table A-2). Moreover, several genes were consistently induced ~1.5 fold in P^- strains and most of them were involved in transcription and translation. This may be due to the observation that unlike P^+ gonococci, in which pilus biosynthesis and assembly takes lots of energy, P^- gonococci don't require such energy and usually grow faster. The increased growth is consistent with the up-regulation of transcription and translation systems.

In this work we used the newly developed gonococcal DNA arrays to compare gene expression in a wild-type strain and a *pilE* mutant. The deletion of the complete *pilE* locus in this mutant completely abolishes *pilE* expression. As expected, our results are highly consistent with this prediction, showing that *pilE* had the highest expression ratio and was essentially expressed only in the wild-type. These results demonstrate that this DNA array is suitable for analyzing gonococcal gene expression at a genome-wide level.

In summary, this first version of a gonococcal DNA array provides a feasible means to analyze global gene expression in *N. gonorrhoeae*. With the advent of this DNA array, it is now possible to examine gene expression profiles during the course of gonococcal infection, which will substantially increase our knowledge about this pathogen and the disease it causes and hence provide more opportunities to design new strategies to prevent and treat it.

Table A-1. Genes expressed higher in P^+ (MS11A) than P^- (MS11-307)

gene	comments	ORF ID	P+/P- ave	Stdev
pilE	pilin expression locus	NG2061	67.13	53.66
pilS	silent pilin locus	NG1512.1	15.09	8.67
pilS	silent pilin locus	NG2062	12.04	6.91
island		NG3002	6.58	3.55
bfrB	bacterioferritin	NG0795	4.04	0.77
bfrA	bacterioferritin	NG0794	3.79	0.82
island		NG5001	3.44	0.30
clpB	endopeptidase Clp ATP-binding chain B	NG1046	2.86	0.71
ppiB	peptidyl-prolyl cis-trans isomerase B (rotamase B)	NG0376	2.82	0.39
grpE	HSP-70 cofactor; nucleotide exchange factor	NG1422	2.77	0.50
island		NG5041	2.36	0.79
pgi	glucose-6-phosphate isomerase	NG1668	2.32	0.80
	conserved hypothetical protein	NG0904	2.26	0.20
recN	DNA repair protein RecN	NG0318	2.18	0.36
	int gene product	NG0733	2.12	0.35
	probable iron-sulphur protein	NG0906	2.11	0.25
	conserved hypothetical protein	NG0905	2.11	0.38
	hypothetical protein	NG0634	2.04	0.64
pglC	pilin glycosylation protein	NG0084	2.02	0.14
yhjA	cytochrome c peroxidase	NG1769	2.02	0.50
htpX	heat shock protein (probable membrane-bound zinc metallopeptidase)	NG0399	1.97	0.71
pgm	phosphoglucomutase	NG0375	1.95	0.42
	hypothetical protein	NG0635	1.92	0.55
	hypothetical protein	NG1633	1.90	0.33
himA	IHF alpha subunit	NG0305	1.89	0.57
groEL	heat shock protein, 60 Kd subunit	NG2095	1.85	0.42
dnaJ	HSP-40/chaperone DnaJ	NG1901	1.80	0.21
	IS1016 transposase fragment	NG0186.1	1.78	0.12

Table A-2. Genes expressed higher in P^- (MS11-307) than P^+ (MS11A)

gene	comments	ORF ID	P+/P- ave	Stdev
mod	type III restriction/modification system modification methylase	NG0641	0.36	0.09
lrp	leucine-responsive regulatory protein	NG1294	0.42	0.11
mdaB	drug activity modulator B	NG1473	0.51	0.16
rplU	50S ribosomal protein L21 (RplU)	NG1676	0.56	0.06
rplD	50S ribosomal protein L4	NG1837	0.57	0.05
rpoA	DNA-directed RNA polymerase alpha chain (RpoA)	NG1818	0.57	0.22
secY	preprotein translocase SecY	NG1822	0.59	0.19
rplW	50S ribosomal protein L23	NG1836	0.60	0.04
rplT	50S ribosomal protein L20	NG0298	0.60	0.21
rplA	50S ribosomal protein L1	NG1854	0.60	0.23
rplF	50S ribosomal protein L6	NG1825	0.61	0.19
rplB	50S ribosomal protein L2	NG1835	0.61	0.18
rpsM	30S ribosomal protein S13	NG1821	0.62	0.26
rplQ	50S ribosomal protein L17	NG1817	0.62	0.19
rplO	50S ribosomal protein L15	NG1823	0.63	0.23
rpsH	30S ribosomal protein S8	NG1826	0.64	0.20
bolA	BolA/YrbA family protein	NG1657	0.64	0.21
RS14	30S ribosomal protein S14	NG1826.1	0.64	0.27
fusA	translation elongation factor EF-G	NG1843	0.65	0.12
rhlE	ATP-dependent RNA helicase	NG0650	0.65	0.14
rplK	50S ribosomal protein L11	NG1855	0.65	0.15
rplL	50S ribosomal protein L7/L12	NG1852	0.66	0.16
rpmA	50S ribosomal protein L27	NG1677	0.68	0.19
rplN	50S ribosomal protein L14	NG1829	0.68	0.21
rplE	50S ribosomal protein L5	NG1827	0.69	0.23
fbpA	periplasmic iron-binding protein (major iron-regulated protein), ABC solute binding protein	NG0217	0.77	0.52

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