MINIREVIEW

The ironclad truth: how in vivo transcriptomics and in vitro mechanistic studies shape our understanding of Neisseria gonorrhoeae gene regulation during mucosal infection

Matthew R. Moreau, Paola Massari and Caroline A. Genco*

Department of Integrative Physiology and Pathobiology, Tufts University School of Medicine, Boston, MA 02111, USA

*Corresponding author: Department of Integrative Physiology and Pathobiology, Tufts University School of Medicine, 136 Harrison Ave, M & V 701, Boston, MA 02111, USA. Tel: 617-636-6739. Fax: 617-636-0445; E-mail: caroline.genco@tufts.edu

One sentence summary: This article reviews how combining transcriptomics on pathogens from naturally infected hosts and other mechanistic studies can improve our understanding the genes involved in bacterial pathogenesis and how they are regulated.

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ABSTRACT

Neisseria gonorrhoeae is one of the most prevalent sexually transmitted infections worldwide. This obligate human pathogen has been extensively studied in vitro, where bacterial factors that are known to contribute to gonococcal disease and their regulation are relatively well defined. However, these in vitro experimental conditions only loosely replicate the host specific environment encountered by the bacteria in vivo. We recently reported on the complete gonococcal transcriptome expressed during natural human mucosal infection using RNA-seq analysis. Gene transcripts expressed in vivo (in vivo expressed factors) included genes encoding antibiotic resistance determinants, and a large number of hypothetical genes. A comparison of the gonococcal transcriptome expressed in vivo with the corresponding strain grown in vitro identified sets of genes regulated by infection, including those regulated by iron and the transcriptional regulatory protein Fur. We highlight here the role of Fur and gonococcal-specific regulatory processes important for infection and pathogenicity. We have determined that the genes controlled by Fur follow the same expression pattern in vivo as described previously in vitro, confirming Fur’s regulatory role during infection. Collectively, these studies provide new insights into how bacterial fitness and pathogenicity are modulated during human mucosal infection.

Keywords: Neisseria gonorrhoeae; gene regulation; iron; transcriptome; mucosal infection

INTRODUCTION

The Gram-negative obligate human pathogen Neisseria gonorrhoeae is the causative agent of the sexually transmitted infection gonorrhea, the second most common reportable disease in the USA. According to the Centers for Disease Control and Prevention (CDC) and the World Health Organization (WHO), there are approximately 800 000 new cases of gonorrhea in the USA and 106 million cases worldwide each year (World Health Organization 2012, 2016; Newman et al. 2015; CDC 2016). Neisseria gonorrhoeae infects the genitourinary tract of men and women; however, the disease sequelae differ between genders (Edwards and Apicella 2004). In men, gonorrhea is defined by a marked infiltration of polymorphonuclear neutrophils (PMNs), resulting
in symptomatic urethritis and epididymitis (World Health Organization 2012, 2016; Yu and Genco 2012a; Goire et al. 2014). In women, the disease is more insidious, remaining asymptomatic and often resulting in spread of the infection to the ascending genitourinary tract (Walker and Sweet 2011; Islam et al. 2015; Newman et al. 2015). If left untreated in women, gonococcal infection can result in pelvic inflammatory disease, endometritis, ectopic pregnancy and ultimately, infertility (Fichorova et al. 2001; World Health Organization 2012, 2016; Islam et al. 2015). Although the genitourinary tract is the primary site of infection in humans, N. gonorrhoeae can also infect extragenital sites such as the oropharynx and the rectum (Chan et al. 2016).

A rising trend in incidence of N. gonorrhoeae has recently been reported, and this is expected to continue to rise, due in part to increased antibiotic resistance of the organism and the lack of a preventative vaccine (Unemo et al. 2012; Unemo and Shafter 2014; Jerse, Bash and Russell 2015; Gottlieb et al. 2016). A better understanding of N. gonorrhoeae pathogenic mechanisms during this human mucosal disease would aid in development of novel therapeutics. There are a few in vivo models of gonococcal genital infection, but these present with several limitations. The human male urethral N. gonorrhoeae challenge model uses experimental infection of male volunteers as a model of urethritis (Cornelissen et al. 1998; Biswas et al. 1999). While studies in this model have expanded our understanding of acute infection, they do not address the consequences of long-term infection or infection specifically in women.

A mouse model of female gonococcal infection was developed in the late 1990s based on estradiol treatment of female mice, which allows for N. gonorrhoeae genitourinary tract infection in this otherwise resistant species (Jerse 1999; Jerse et al. 2011). Although successful, this model represents a surrogate of female infection in humans in an artificial hormone condition. A more recent model based on generation of ‘humanized’ mice expressing the human carcinoembryonic antigen cell adhesion molecule (CEACAM) 1 and 5, required for N. gonorrhoeae opacity (Opa) proteins interactions with host cells overcomes some of these limitations (Schmitter et al. 2007; Sadarangani, Pollard and Gray-Owen 2011). While this model has expanded the study of N. gonorrhoeae infection in both male and female mice without the use of estradiol (Gu et al. 2010; Sintsova et al. 2015), it does not mimic the unique environment of the human genital tract.

GONOCOCCAL GENE EXPRESSION DURING INFECTION

Like other human pathogens, Neisseria gonorrhoeae has evolved mechanisms to adapt to the specific environments encountered during infection. However, the majority of studies aimed at characterizing this response of the gonococcus to different environmental stimuli have examined N. gonorrhoeae cultured in vitro conditions that do not completely replicate the environment encountered in the human host (Biswas et al. 1999; Grifantini et al. 2003; Agarwal et al. 2008). In the female genital tract, N. gonorrhoeae is exposed to an environment characterized by low pH, varying oxygen and iron levels, and the presence of additional microbes and host cells (Fig. 1) (O’Hanlon, Moench and Cone 2013; McClure et al. 2015; Zozaya et al. 2016). Free iron is scarce in the female host and is complexed to host iron-binding proteins including lactoferrin or transferrin, making the female genital tract iron deplete (Agarwal et al. 2008). During menses, iron levels can rise and N. gonorrhoeae responds to these changing levels through regulation of gene expression (Anderson et al. 2001; Jerse et al. 2002; McClure et al. 2015). During infection, the gonococcus encounters several different host cells such as epithelial cells and PMNs, the latter of which function to engulf and degrade the organism (Fig. 1). However, N. gonorrhoeae has been demonstrated to survive and replicate within epithelial cells and PMNs and to evade the antibacterial actions of PMNs (Crisis and Seifert 2012). The gonococcus also encounters other microbes during genital tract infection in men and women (Weis and Nelson 2006; O’Hanlon, Moench and Cone 2013). Thus, the complexity of environmental signals during human mucosal infection is difficult to recapitulate by in vitro studies.

GONOCOCCAL GENE EXPRESSION DURING NATURAL MUCOSAL INFECTION IN HUMANS

We previously reported on the expression of a subset of iron-regulated genes during human mucosal infection in men and women. These studies revealed that the gene encoding the transcriptional regulatory protein Fur (fur) and the Fur-regulated tbpA/B and fbpA genes were expressed in mucosal samples obtained from men and women with uncomplicated gonorrhea (Agarwal et al. 2005). However, these studies were carried out using microarray and qRT-PCR analysis and were limited in their sensitivity and detection (Agarwal et al. 2005, 2008). To define gonococcal global gene responses during human mucosal infection, we recently utilized RNA-seq analysis to define the complete gonococcal transcriptome in cervico-vaginal lavage samples from naturally infected female subjects. These studies revealed that 65% of the gonococcal genome was expressed during natural mucosal infection of the lower genitourinary tract in women. We detected expression of 1700 gonococcal genes which represented 22 functional categories and included large groups of hypotheticals, rRNA, sRNA, phage-associated and translation-related genes all (Fig. 2). Within these categories, we observed high expression of genes encoding antimicrobial efflux pumps, iron transport, phage, pilin, outer membrane and hypothetical proteins (McClure et al. 2015).

The strains isolated from cervico-vaginal lavage specimens were also grown in vitro and resulting transcriptomes compared to those expressed during infection to define infection-specific expression profiles. This analysis established that a large portion of the gonococcal genome was regulated during mucosal infection relative to in vitro growth. Genes involved in DNA/RNA processing, genetic regulation, sugar uptake, amino acid processing and phage-associated proteins all displayed large differences in expression among these two datasets. Furthermore, expression levels of a group of gonococcal hypothetical genes were observed to vary between in vivo and in vitro conditions, leading to speculations that new metabolic or regulatory pathways may be discovered, as well as bacterial factors involved in virulence, evasion of host defense mechanisms (i.e. antibiotic resistance) and novel proteins that could represent new therapeutic targets (McClure et al. 2015). Our analysis also revealed increased expression of Fur and iron-regulated genes during infection in women as compared to growth in vitro, suggesting that during infection of the genital tract in women, the gonococcus is exposed to an iron-deplete environment.

TRANSCRIPTIONAL CONTROL BY FUR

The gonococcal Fur protein controls expression of iron homeostasis genes in response to intracellular iron levels (Fig. 3). This ensures a crucial balance between the requirement for iron,
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Figure 1. Mucosal environment during gonococcal infection. During infection, the gonococcus encounters several different host cells such as epithelial cells and PMNs, the latter of which function to engulf and degrade the organism. Gonococcal infection in men typically results in a robust immune infiltration by PMNs, whereas in women asymptomatic infection is common and N. gonorrhoeae exists as a biofilm. In both men and women, the genitourinary tract mucosa is an iron-deplete environment with most bioavailable iron being bound host proteins such as transferrin. The gonococcus also encounters other microbes during genital infection in men and women, although the microbiota in men is typically less robust and diverse than that of females. Gonococci have also been demonstrated to transverse the epithelial barrier.

as an essential element for growth, and the avoidance of iron toxicity, which occurs via production of hydroxyl or peroxide radicals (Cornelissen et al. 1998; Touati 2000; Seib et al. 2006; Bartnikas 2012; Troxell and Hassan 2013). Classically, Fur binds directly to DNA sequences to inhibit transcription of downstream genes (Mellin et al. 2007; Carpenter, Whitmire and Merrell 2009; Yu and Genco 2012a,b). One of the best-known examples involves transcriptional control of genes that scavenge iron from the host, including the transferrin binding proteins (tbpAB) and the ferric binding protein (fbp) (Gray-Owen and Schryvers 1996; Cornelissen et al. 1998; Agarwal et al. 2005; McClure et al. 2015). In the absence of iron, Fur exists as an inactive monomer that becomes active when intracellular levels of iron are high allowing Fur to bind to promoter regions bearing the Fur box (Fig. 3) (Bagg and Neilands 1987; Troxell and Hassan 2013). While this interaction typically acts to block subsequent binding by RNA polymerase, Fur can also function as a transcriptional activator (Yu and Genco 2012a). Fur can also regulate genes indirectly by repressing a series of trans elements that regulate downstream targets. For example, sRNAs can act as repressors as is the case for the Fur repressed sRNA NnrF, which controls transcription of the sdhC/A genes (Fig. 3). Fur-mediated repression of NnrF results in increased translation of sdhC/A transcripts; thus, expression of functional SdhC/A proteins (succinate dehydrogenases involved in the TCA cycle) is indirectly activated by Fur (Ducey et al. 2005; Agarwal et al. 2008; Jackson et al. 2010). Fur can also control additional regulatory proteins including ArsR, MpeR and OxyR (Fig. 3). OxyR is known to regulate resistance of the gonococcus to ROS (Seib et al. 2007). MpeR targets genes involved in antimicrobial resistance including MtrR (Lee et al. 2003; Warner et al. 2007; Jackson et al. 2010; Mercante et al. 2012; Yu et al. 2016). ArsR is a regulator of the norB gene involved in nitrous oxide reduction, as well as predicted to regulate NGO1411 and NGO1646 (encoding a hypothetical and phage-associated gene respectively) and has been shown to be important for intracellular survival in endocervical cells (Isabella et al. 2008; Yu et al. 2016).

Fur regulation of regulatory and stress proteins in vitro

Superoxide and other oxide-containing effectors are stresses that Neisseria gonorrhoeae encounters within the human genital tract. In response to infection, epithelial cells and resident macrophages produce nitric oxide (NO) via AKT kinase activation and iNOS in vitro (Householder et al. 2000; Seib et al. 2006, 2007; Isabella et al. 2008; Edwards 2010). To combat the effects of NO, N. gonorrhoeae expresses NorB, a NO reductase whose expression is repressed by the Fur-regulated ArsR regulatory protein (Isabella et al. 2008). In iron-replete conditions in vitro, Fur functions as a transcriptional activator of arsR, a gene that is essential for survival within endocervical cells in vitro (Yu et al. 2016).

Intrinsically, high levels of intracellular iron can react with the reduction and oxidation of NADH resulting in the formation of damaging reactive oxygen species (ROS) (Touati 2000; Seib et al., 2006, 2007). PMNs also express hydrogen peroxide (H2O2) and other oxidative species as antimicrobial compounds (Criss and Seifert 2012). The transcriptional regulator OxyR that is, in turn, regulated by active Fur regulates the ROS protection regulon; when Fur is active, it upregulates oxyR expression
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Of this operon is predicted to enhance... inferred to the development of resistance to antimicrobial and antibiotic stress. Fur plays an integral role in regulation of the antimicrobial resistance efflux pump system encoded by the mtr locus via upstream regulation of the MtrR transcriptional regulatory protein (Lee et al. 2003; Yu et al. 2016). During growth under iron-deplete conditions, such as those observed during human mucosal infection, Fur is inactive and as such expression of MpeR, the transcriptional regulator of the mtrR, is increased resulting in... expression (Fig. 3). The inhibition of mtrR expression results in expression of the mtrCDE operon that encodes the multidrug efflux pump proteins (Mercante et al. 2012). Interestingly, our analysis of gonococcal gene expression profiles in cervico-vaginal lavage specimens revealed that both mtrR and mtrCDE expression levels were similar to the levels expressed during in vitro culture in the presence of iron (McClure et al. 2015). These observations lead us to speculate that loss in regulation of these genes during growth in vivo could result from promoter allele changes altering the interaction between the repressor, MtrR, and the cis elements in the promoter. Indeed, allele changes in these operons have been reported to be involved in increases in antimicrobial resistance as well as intracellular survival in the gonococcus (Warner et al. 2007; Kirkcaldy, Kidd and Weinstock 2013; Unemo and Shafer 2014; Grad et al. 2016).

Fur control of gonococcal factors involved in host-pathogen interactions

During initial mucosal infection, the gonococcus interacts with epithelial cells via the Opa proteins and their cognate receptors, CEACAM 1, 3, 5 and 6 on the epithelial cell surface. This results in downstream signaling that favors subsequent gonococcal invasion of epithelial cells (Schmittet al. 2007; Sadarangani, Pollard and Gray-Owen 2011; Tchoupa, Schuhmacher and Hauck 2014; Sintsova et al. 2015). Fur has been demonstrated to bind to and repress the gonococcal opa promoter during growth of the gonococcus under iron-replete conditions in vitro (Sebastian et al. 2002). In concert with these observations, Opa-negative strains are predominantly recovered from infected female subjects during menses, when iron is abundant in the lower genital tract (Sebastian et al. 2002; Folster et al. 2009; Jackson et al. 2010; Sadarangani, Pollard and Gray-Owen 2011; Yu and Genco 2012b). Intracellular survival of N. gonorrhoeae within endocervical cells in vitro as well as colonization within the female mouse model is partially mediated by the expression of a Fur-controlled phage repressor npr (Neisseria phage repressor) (Daou et al. 2013). Despite the ability of Fur to bind the common promoter between npr and the four genes immediately downstream, we demonstrated that expression of npr is not regulated by iron or Fur in contradiction to previous studies (Ducey et al. 2005; Jackson et al. 2010; Daou et al. 2013).

Opa proteins also mediate interaction of the gonococcus with PMNs (Johnson et al. 2015). PMNs are the primary immune cells observed during N. gonorrhoeae infection in symptomatic males and females (albeit to a lesser extent in females compared to males) and the gonococcus has adapted mechanisms that enable the organism to survive within PMNs (Edwards and Apicella 2004). In women, N. gonorrhoeae infection is also associated with biofilm formation, protecting the organisms from immune cells...
Figure 3. Fur interaction with regulatory proteins and sRNAs. Fur has been shown or hypothesized to interact with other regulatory proteins and sRNAs. Interactions between Fur (tan circle) and other regulators (light blue circles) are shown. Downstream targets of these regulators are shown and defined as indirect targets of Fur. Rust colored circles indicate regulators identified in our studies. Expression under iron-replete conditions is depicted.

such PMNs (Fig. 1). Evidence suggests that iron is more replete within a biofilm based on expression of iron-responsive gene products that are upregulated during growth in iron-deplete conditions, such as FetA and TbpA/B (Phillips et al. 2012). Eventually, however, some bacteria escape the biofilm and enter into the iron-deplete luminal space where they make contact with PMNs (Steichen et al. 2011; Phillips et al. 2012). Survival after internalization of *N. gonorrhoeae* PMNs in vitro depends on Opa expression. Expression of Opa proteins results in efficient clearance mediated by serine protease activity (Ball and Criss 2013; Johnson and Criss 2013; Johnson et al. 2015).

OUTLOOK/CONCLUSIONS

*Neisseria gonorrhoeae* is one of the most common bacterial sexually transmitted infections worldwide (World Health Organization 2016). The incidence of infections has increased due in part to the continuing evolution of bacterial mechanisms of antimicrobial resistance and the asymptomatic nature of the disease which results in increased transmission. The lack of a good experimental model that can replicate the various microenvironmental conditions *N. gonorrhoeae* is exposed to within the human host has led to a significant gap in our understanding of its pathogenic strategies during natural mucosal infection. Transcriptome studies of gonococci during natural human mucosal infection reported by our group in combination with in vitro-based regulatory studies have shown that several regulatory circuits control *N. gonorrhoeae* genes important for colonization and survival. We demonstrated that the Fur transcriptional regulatory protein extends not only to iron-regulated genes, but also to genes involved in a number of other regulatory networks. All of these studies show that the general trend of activation or repression of genes by Fur observed in vitro is mimicked in vivo, further showing the importance of Fur as a central regulator during infection. A comparison of the gonococcal transcriptome expressed in *vivo* to the corresponding strain grown in *vitro* revealed increased expression of genes involved in iron transport including *tbpAB* and *fhpABC* in *vivo* (Agarwal et al. 2005, 2008; McClure et al. 2015). As TbpA and TbpB have been considered potential gonococcal vaccine candidates, defining the details of their regulation in *vivo* compared to *in vitro*, may be beneficial for future characterization of the therapeutic potential of these proteins. In addition, it is possible that other regulated factors, among both the known and hypothetical gene products revealed by our transcriptome studies, may represent novel targets for therapeutic and preventive strategies, i.e. new antimicrobial factors or vaccine candidates.

Current studies are focused on gonococcal transcriptional profiles expressed during infection in men. These studies have revealed a divergence in both the disease presentation in men and women, and gonococcal transcriptional programming. Initial results suggest that there is significant overlap in genes expressed by the gonococcus during mucosal infection in both men and women, including genes related to general metabolism and transport. Distinct gene sets expressed during infection in men were enriched for genes encoding host interaction and membrane-associated proteins. In contrast, distinct gene sets expressed during infection in women were enriched for genes encoding host interaction and membrane-associated proteins. In contrast, distinct gene sets expressed during infection in women were enriched for iron and DNA metabolism genes (Nudel et al., unpublished). Further analysis of these unique data sets obtained from infected male and female subjects will define gonococcal adaptions during natural mucosal infection, a long-term goal of our studies. Taken together, these studies offer powerful new insights into the pathobiology of *N. gonorrhoeae* and will not only lead to a better understanding of the mechanisms of gene regulation employed by *N. gonorrhoeae* during infection, but ultimately will allow for
the identification of novel virulence factors and consequently expand the potential for preventive and therapeutic strategies against N. gonorrhoeae infection.

Conflicts of interest. None declared.

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