Resistance of *Neisseria gonorrhoeae* to nutritional immunity protein-mediated zinc limitation during epithelial infection

Jocelyn Carroll Ray

Baltimore, Maryland

B.S., College of William and Mary, 2013

M.S., University of Virginia School of Medicine, 2019

A Dissertation presented to the Graduate Faculty

of the University of Virginia in Candidacy for the Degree of

Doctor of Philosophy

Department of Microbiology, Immunology, and Cancer Biology

University of Virginia

May, 2022

Abstract

Neisseria gonorrhoeae (Gc) is an obligate human pathogen that infects mucosal epithelia to cause the common sexually-transmitted infection (STI) gonorrhea, and antimicrobial-resistant Gc is an urgent public health threat. Gonorrhea has the potential to cause serious sequelae when untreated, especially in women, who may be asymptomatic and unaware that they are infected. The initial site of infection of the female reproductive tract is usually the cervix. In order to establish infection, Gc must attach to the epithelium and acquire essential nutrients such as iron and zinc. The host innate immune system can withhold these nutrients through a process called nutritional immunity, where trace metals are bound by host metal binding proteins. Zinc-binding proteins, including the S100-family proteins calprotectin (S100A8/S100A9) and psoriasin (S100A7), are produced by epithelial cells in response to inflammatory cytokines. These proteins sequester free zinc and make it unavailable to pathogens, and can kill multiple species of bacteria and fungi through zinc starvation. Studies of suspension-grown Gc revealed that expression of the outer membrane transporters TdfH and TdfJ which bind calprotectin and psoriasin, respectively, can pirate zinc from the host.

In contrast to Gc in suspension, the role of zinc acquisition in Gc that are adherent to mucosal epithelial cells was unknown. In studying adherent Gc I found that, under zinc sequestration conditions that reduced viability of suspension-grown Gc, adherent Gc were able to survive and replicate. In

suspension-grown bacteria, TdfH and TdfJ were necessary to survive zinc sequestration by human calprotectin and psoriasin. TdfH and TdfJ deletion mutant (*ΔtdfH ΔtdfJ)* Gc adherent to Ect1 immortalized ectocervical cells survived in spite of this zinc sequestration. I examined this further by using murine calprotectin or Site 1 KO calprotectin (neither of which WT Gc can use as a zinc source), as well as the membrane- permeable zinc-chelator TPEN. In each of these conditions, the negative effect of zinc sequestration on viable CFU was abrogated in the adherent compared to suspension Gc. This protection from zinc sequestration occurred along with a significant change in gene expression between adherent and suspension Gc during zinc sequestration, as assessed by RNAseq. The contribution of the periplasmic zinc shuttle protein ZnuA was assessed after *znuA* was found to be more greatly expressed in adherent vs. suspension Gc when zinc was sequestered. ZnuA was necessary but not sufficient for enhanced survival of adherent Gc under zinc sequestration. Suspension Gc that overexpressed ZnuA did not exhibit increased survival when zinc was sequestered compared to those with wildtype ZnuA expression.

In addition to ZnuA, numerous other candidate genes emerged as potential actors in the defense of adherent Gc from zinc sequestration. Additionally, new questions can be raised about the effect of adherence on Gc biology, signaling mechanisms, control of gene expression, zinc stores in Gc, and the role of thus far uncharacterized proteins in zinc uptake. These findings may open new avenues in understanding Gc biology, as well as lend insight to

the development of new drugs and vaccines to combat the global public health threat of Gc.

Table of Contents

List of Figures

List of Tables

List of Abbreviations in alphabetical order

A431: immortal vaginal squamous cell carcinoma cell line

Adh: Adherent (to acid-washed glass coverslips)

ANOVA: analysis of variance (statistical analysis)

ASGP-R: asialoglycoprotein receptor

Asp: aspartic acid (amino acid)

Asn: asparagine

ATP: adenosine triphosphate

B. subtilis: *Bacillus subtilis*

BSA: bovine serum albumin

CDM: chemically defined medium

cGAS: cyclic GMP-AMP synthase

CEACAM: carcinoembryonic antigen-related cellular adhesion molecule

CFA: cystic fibrosis antigen: archaic name for calprotectin

CFU: colony forming units

Cp: calprotectin ($S100A8_2S100A9_2$) also called MRP8/14, calgranulin A+B

CR3: complement receptor 3 (also called $\alpha_M\beta_2$ or CD11b/CD18)

Cu: copper

DGI: disseminated gonococcal infection

dKO Cp: double knockout calprotectin: site 1 and 2 mutated

DNA: deoxyribonucleic acid

E. coli: *Escherichia coli*

Fe: iron

Fpn1: Ferroportin (mammalian iron efflux pump)

Gc: Neisseria gonorrhoeae

GF: growth factors (human epidermal growth factor and bovine pituitary extract)

H: histidine (amino acid)

His: histidine (amino acid)

HPV: human papillomavirus

HSPG: heparin sulfate proteoglycan

ICP-MS: inductively coupled plasma mass spectrometry

ICP-OES: inductively coupled optical emission spectrometry

IL-: interleukin

IPTG: Isopropyl β-D-1-thiogalactopytanoside: *lac* operon inducer

KSFM: keratinocyte serum-free medium

L1: leukocyte-associated protein 1: archaic name for calprotectin

L2FC: Log_2 fold-change. +1= 2-fold increased; -1= 2-fold decreased

LOS: lipooligosaccharide

mCp: murine (mouse) calprotectin

Mn: manganese

MRP: myeloid related protein (MRP8 is S100A8; MRP14 is S100A9)

MT: metallothionein

NET: Neutrophil extracellular trap

ORF: open reading frame

PBS: phosphate buffered saline

PC: principal component

PCA: principal component analysis

PCR: polymerase chain reaction

PID: pelvic inflammatory disease

Ps: psoriasin ($S100A7_2$)

qPCR: quantitative PCR

RNA: ribonucleic acid

RNAseq: ribonucleic acid sequencing

ROS: reactive oxygen species

S1KO Cp: site 1 (6his) knockout

S2KO Cp: site 2 (3his1asp) knockout

STI: sexually transmitted infection

Sus: suspension-grown

TBST: tris-buffered saline with tween-20

TGF-β: Transforming growth factor beta

Th: T-helper lymphocyte

TLR4: Toll-like receptor 4

TNF-α: Tumor necrosis factor alpha

TdT: TonB-dependent transporter

TPEN: N,N,N’,N’-tetrakis(1-pyridinylmethyl)-1,2-ethanediamine, a zinc chelator

TZ: Transition/transformation zone (of the cervix)

WT: wildtype

Zn: zinc

ZIP: zinc import protein

Zur: zinc uptake repressor

Acknowledgements

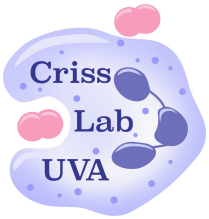
I would like to take this opportunity to thank some of the people who have helped me along the way. My mentor, Dr. Alison Criss, PhD, whose support, guidance, and knowledge made all of this possible.

My lab members, who made me excited to come in to work every day: Aimee, Allie, Amaris, Ian, Lacie, Evan, Mary, Keena, Chris, Amy, and Asya.

My thesis committee: Hervé Agaisse, Melissa Kendall, Barbara Mann, and Sarah Ewald. Thank you for your advice and insightful suggestions. The UVA MSTP, especially Dean Kedes, Ashley Woodard, and my fantastic colleagues.

I would especially like to thank my previous mentors: Dr. Deborah Persaud, MD, Dr. Patty Zwollo, PhD, Dr. Jim Arthos, PhD, Dr. Claudia Cicala, PhD, and Dr. Anthony Fauci, MD for giving me positions in their laboratories and contributing to my scientific training.

My friends: both new and old, near and far. My parents, Stuart and Sharon Ray, who continue to support me and root for me. My husband, C.J. Trachta, for always believing in me and taking care of me every day for the last 12 years.



CHAPTER 1 INTRODUCTION

1.1 Introduction to Neisseria gonorrhoeae

Neisseria gonorrhoeae (Gc) is a Gram-negative diplococcus which causes the common sexually transmitted infection, gonorrhea. Gc has adapted for millennia to the point where humans are its obligate host (Seifert 2019).

1.1.1 Public health threat

Gc is a pathogen of global concern because it is widely prevalent, with approximately 87 million cases per year (Rowley, Vander Hoorn et al. 2019), and because it is increasingly antibiotic resistant (Workowski, Bachmann et al. 2021). Gc readily acquires antibiotic resistance, likely because it is constitutively competent (Goire, Lahra et al. 2014), (Snyder and Saunders 2006), (Unemo, Seifert et al. 2019). As a result, Gc has developed resistance against every effective antibiotic introduced to combat infection, including sulfonamides, penicillins, tetracyclines, macrolides, fluoroquinolones, and cephalosporins (Unemo, Seifert et al. 2019), (Quillin and Seifert 2018). The result is the current urgent public health threat: strains of Gc exist which are resistant to all approved antibiotics (Unemo, Seifert et al. 2019), (Workowski, Bachmann et al. 2021). The only current empiric treatment for uncomplicated gonorrhea in the United States is the third-generation extended-spectrum cephalosporin ceftriaxone (Workowski, Bachmann et al. 2021), despite the existence of circulating strains which are resistant to this antibiotic (Quillin and Seifert 2018), (Unemo, Seifert et al. 2019), (Centers for Disease and Prevention 2013). The recommended antibiotic

treatment for gonorrhea is continually evolving as new antibiotic resistance is detected. Between the beginning of my dissertation work in 2017 and now (2022), the previous recommendation for dual therapy with ceftriaxone and azithromycin has been revoked due to increasing antibiotic minimum inhibitory concentrations of azithromycin against Gc (St Cyr, Barbee et al. 2020). Despite significant efforts, there is no vaccine protective against infection with Gc. Infection with Gc elicits little protective immune memory, and previously infected individuals are susceptible to repeat infections (Workowski, Bachmann et al. 2021). Many of the most immunodominant epitopes on Gc are phase- or antigenically-variable, enabling immune escape and complicating vaccine efforts (Stern, Brown et al. 1986). An ideal antigenic target would be a protein that is stably surface-expressed and well conserved between strains, as well as essential for colonization. One proposed strategy aims to both “starve and kill” the pathogen by simultaneously preventing acquisition of essential nutrients by eliciting antibodies against outer membrane nutrient transporters and killing the pathogen through neutralizing immune memory.

1.1.2 Typical clinical presentation

Gc is transmitted through sexual contact with an infected person. Sites of infection include the urethra (in men), uterine cervix (in women), pharynx and rectum (both sexes) and conjunctiva (especially in neonates born to mothers with untreated cervical infection) (Workowski, Bachmann et al. 2021).

Male urethral infection typically presents with urethritis, painful urination, and purulent urethral discharge, although men may also be asymptomatic (Grimley, Annang et al. 2006). Invasion of urethral epithelium occurs only beyond the fossa navicularis, where the stratified squamous epithelium of the external meatus transitions to simple columnar epithelium (Harkness 1948). Urethral gonorrhea can often be detected in women (Bhattacharyya, Jephcott et al. 1973), but the relatively short female urethra is predominantly squamous epithelium, which likely allows for colonization but not invasion and therefore is without symptoms (Harkness 1948). Cervical infection in women may be asymptomatic, or may present with mucopurulent vaginal discharge and/or vaginal itching (Detels, Green et al. 2011, Tuddenham, Hamill et al. 2022). The uterine cervix presents a physical barrier between the lower female genital tract (vulva, vagina, and ectocervix), which possesses a distinct microbiome, and the relatively sterile upper reproductive tract (endocervix, uterus, fallopian tubes, ovaries) (Chen, Song et al. 2017). The lower genital tract is characterized by stratified squamous epithelium with variable degrees of keratinization, while the upper reproductive tract is composed primarily of simple columnar epithelium (Blaskewicz, Pudney et al. 2011). The transition between these realms occurs within the cervix, stratified squamous epithelium of the ectocervix thins and transitions to simple columnar epithelium of the endocervix at the transition or transformation zone (TZ) (Reich, Regauer et al. 2017). These distinct tissue types are relevant for Gc infection because colonization and biofilm formation are frequently observed on

the ectocervix, where Gc bind complement receptor 3 (CR3) and prevent exfoliation of the squamous layers (Edwards, Shao et al. 2000, Falsetta, Bair et al. 2009). Invasive Gc have not been reported beneath the squamous epithelium of the ectocervix (Yu, Wang et al. 2019), (Harkness 1948). By contrast, invaded, submucosal Gc at the transition zone and endocervix are thought to contribute to the development of cervicitis and ascending infection (Yu, Wang et al. 2019).

Pharyngeal infections are often asymptomatic, although one study reported that 1% of 192 adults presenting to general medicine practices with sore throat were positive for pharyngeal gonorrhea (Komaroff, Aronson et al. 1980). Rectal infection with Gc is most common in men who have sex with men (MSM) who engage in (and report) receptive anal intercourse, but has also been found in women and men who do not report anal intercourse (Klein, Fisher et al. 1977), (Trebach, Chaulk et al. 2015). Rectal gonorrhea is also often asymptomatic but can present as proctitis which cannot be clinically differentiated from other causes (Klein, Fisher et al. 1977, Trebach, Chaulk et al. 2015).

1.1.3 Sequelae

Gc can cause serious sequelae if left untreated. Rarely (in approximately 0.5-3% of infections), Gc can escape the mucosal origin of infection and spread through the body, causing disseminated gonococcal infection (DGI) (O'Brien, Goldenberg et al. 1983, Rice 2005). DGI can involve septic arthritis, fever, painful skin lesions, and sometimes meningitis or endocarditis. More locally, ascending infection from the male urethra into the testes can cause epididymitis and

possible sterility (McConaghy and Panchal 2016). In women, lower genital tract infections are often undiagnosed and left untreated as they may be asymptomatic or display symptoms that are nonspecific (Workowski, Bachmann et al. 2021). Ascending Gc infection in women can cause pelvic inflammatory disease, a painful condition of upper reproductive tract inflammation that can involve fallopian tube scarring and, with repeated episodes, potentially infertility (Brunham, Gottlieb et al. 2015). Therefore, a need exists to find new ways to prevent infection by this prevalent pathogen, particularly in women whose infections may not be detected until irreversible damage has been done.

1.1.4 Infection models for Gc

Because Gc's only natural host is humans, there are limited *in vivo* models in which to study infection (Edwards and Butler 2011). Experimental challenge studies of male participants have been done to study urethral infection, but the risk of serious sequelae in women means that experimental infection to study cervical infection is not ethically tenable (Cornelissen, Kelley et al. 1998), (Edwards and Butler 2011). Female mice treated with estradiol can be transiently infected with Gc during the proestrus phase of their ovulatory cycle, but infection is cleared much more rapidly than it is in infected humans (Jerse, Wu et al. 2011). Recently, advances have been made in developing humanized mouse models (Li and Dewey 2011), (Sintsova, Sarantis et al. 2014), but these models are engineered to replace mouse proteins with human ones and require foreknowledge of which proteins must be replaced. In the case of nutritional

immunity, where we know that Gc can take advantage of, for example, human but not mouse calprotectin (Kammerman, Bera et al. 2020), we would likely miss a large part of the picture by selectively “humanizing” only the aspects that we already know to be important. Tissue explants, such as the fallopian tube organ culture model used by Joe Dillard (Lenz and Dillard 2018) and cervical explant culture used by Wenxia Song, have been important advances, but are also more fragile, require access to a regular source of hysterectomy samples, and display additional complexity (Yu, Wang et al. 2019).

Human-derived immortalized cell lines are easy to acquire, are standardized, and can mimic human tissue without introducing additional complexity of multiple cell types and donor variability. Unfortunately, the other face of this simplicity is also a shortcoming of cell lines. Immortalized cell lines cannot reproduce nuanced aspects of human tissues such as heterogeneous cell types, tissue architecture, systemic immune responses, and variation between individuals. Immortalized cell lines often do not perfectly replicate the receptor expression, cellular architecture, and polarization of their parent tissue (DiPaolo, Popescu et al. 1993). Additionally, most immortalized cell lines have been transformed using oncogenes or transforming viral proteins such as HPV E6/E7, which leads to hyperploid cells and the accumulation of abnormal features (DiPaolo, Popescu et al. 1993). For these reasons, the utility of cell lines depends on the specificity of the phenotype being studied, and discoveries should be confirmed in more complex systems.

1.2 Attachment and Gc pathogenesis

1.2.1 Pili

Regardless of the anatomical site of infection, attachment to epithelial surfaces is extremely important to Gc pathogenesis. Gc express multiple adhesion factors, but early attachment to primary urethral cells, primary endocervical and ectocervical cells, and cervical explants has been demonstrated to be greatly enhanced by Type IV pili (pili) (Edwards and Apicella 2005), (Edwards 2002), (Yu, Wang et al. 2019). While studies on ME180 cervical carcinoma cells indicated that initial pili-mediated adherence depended on CD46 (Kallstrom, Liszewski et al. 1997), Edwards *et al* showed that CD46 was not required for adherence to primary cervical epithelial cells, which only express CD46 on their basolateral surface (Edwards 2002), (Edwards and Apicella 2005). Edwards *et al* demonstrated that the ligand for pili on primary cervical cells is the I-domain of complement receptor 3 (CR3) (Edwards 2002). CR3 is made up of the integrin subunits α_M (CD11b) and β_2 (CD18), and is present on cervical and rectal epithelial cells but was not detected on the surface of primary urethral epithelial cells (Edwards, Brown et al. 2001), (Hussain, Kelly et al. 1995). In addition to α_M, α_1 and α_2 also contain an I-domain which is highly conserved with that of α_M (Edwards and Apicella 2005). $\alpha_1\beta_1$ and $\alpha_2\beta_1$ are found on primary urethral epithelial cells (Edwards and Apicella 2005). Edwards and Apicella infected primary urethral epithelial cells with MS11 Gc and demonstrated that

complexes pulled down using antibodies against integrins α_1 and α_2 co-immunoprecipitated with pili (Edwards and Apicella 2005). Taken together, these data suggest an important role for pili in attachment of Gc to human epithelial cells through interaction with I-domain containing integrin receptors.

Beyond attachment, pili are required for twitching motility, the only known form of motility in Gc (Anderson, Dewenter et al. 2014). Cyclical pilus extension, binding to a substrate (such as glass, agar, or cell surfaces) and retraction enable movement (Merz, So et al. 2000). This has been studied through use of *ΔpilT* mutants, which lack the ability to retract pili (Wolfgang, Lauer et al. 1998). These bacteria retain normal levels of piliation and the ability to bind to host cells, but are nonmotile (Park, Wolfgang et al. 2002). Retraction of pili is also necessary for the formation of microcolonies, which are aggregations of Gc that form on surfaces such as epithelial cells or glass coverslips and dynamically merge and migrate as a unit (Higashi, Lee et al. 2007),(Merz, So et al. 2000).

Antigenic variation of the type-IV pilus occurs through genetic substitution of epitopes with silent versions of the major pilin subunit (Wainwright, Frangipane et al. 1997). Antigenic variation in PilE was shown to affect the degree of adherence of Gc to endometrial, cervical, and prostatic cell lines (Rudel, van Putten et al. 1992). In addition to antigenic variation, phase variation of the pilus also occurs, so that the pilus is turned on or off in an average of 10^{-4} CFU per generation (Seifert 1996).

1.2.2 Opa proteins

Opacity-associated proteins (Opa) are another major Gc adhesion factor, and exhibit significant variability. Within a single Gc isolate, ~ 11 distinct *opa* genes may be encoded, which vary in their extracellular loops and in their affinity for members of the human carcinoembryonic antigen cellular adhesion molecules (CEACAM) family or heparin-sulfate proteoglycan (HSPG) antigens (Wang, Gray-Owen et al. 1998, Virji, Evans et al. 1999). These *opa* genes are phase-variable and also vary significantly from one isolate to another (Wang, Gray-Owen et al. 1998). The beginning of each *opa* gene contains pentameric CTCTT repeats which can be duplicated or skipped when the chromosome is copied between generations (Murphy, Connell et al. 1989). Changes in the number of repeats can lead to the gene being shifted in- or out- of frame. This happens in a stochastic manner, such that these genes turn on or off randomly at a rate of 10^{-3} to 10^{-4} replications *in vitro* (Mayer 1982). This enables dynamic changes in Opa expression within a population from one point in time or space to another.

CEACAM and HSPG expression varies depending upon the cell type investigated, even within the same tissue (Yu, Wang et al. 2019). Therefore, the relative importance of adhesion factors also varies depending on cellular context. When assessing cervical explant cultures, Yu et al. reported that CEACAM-binding Opa (Opa_{CEA}) expression significantly improved colonization of ectocervical cells and to a lesser extent endocervical cells through activating integrin β_1 and inhibiting cellular exfoliation (Yu, Wang et al. 2019). However,

Opa_{CEA} expression was completely dispensable for colonization of transition zone epithelium, which expresses much lower levels of CEACAM compared to ectocervix or endocervix (Yu, Wang et al. 2019). Intriguingly, Opa_{CEA} expression may actually antagonize invasion of CEACAM-expressing cells, because invasion of endocervix was reduced in Opa_{CEA}-expressing Gc while invasion of transition zone was unaffected (Yu, Wang et al. 2019). The ability of Gc to invade cells depends highly on the cell type, as non-Opa_{CEA}-expressing Gc were still not internalized into the stratified squamous cells of the ectocervix (Yu, Wang et al. 2019).

1.2.3 Other adhesion factors

Other surface components of Gc have also been described as being important for cell attachment, including PorB (Edwards 2002) NhbA (NGO_1958) (Semchenko, Everest-Dass et al. 2019) and the autotransporter NGO_2105 (Huang, Zhang et al. 2020). Gc endocytosis into urethral epithelial cells has been shown to be dependent on lipooligosaccharide (LOS) binding to human asialoglycoprotein receptors (ASGP-R) on the urethral cells (Harvey, Jennings et al. 2001). Finally, recent work has demonstrated that a subset of Gc proteins have the capacity to bind mannosylated proteins on epithelial cell surfaces, including TbpA, TdfJ, TdfH, PorB, and Opa proteins (Semchenko, Everest-Dass et al. 2019). Thus, even in nonpiliated and Opa- bacteria, there are multiple mechanisms for attachment and colony formation on epithelial cells.

1.2.4 Development of the Gc parent strain used in these studies

Different PilE and Opa gene variants affect the dynamics of attachment and interaction with host cells (Ball and Criss 2013), (Quillin and Seifert 2018), (Gray-Owen, Lorenzen et al. 1997), (Merz, So et al. 2000). These variants are heritable and as a result the Opa- and PilE- proteins expressed by a Gc population can shift with repeated passaging.

Therefore, the bacterial parent strain used in this work is FA1090 that has been modified so that antigenic variation in PilE and phase variation of Opa proteins are no longer possible (Ball and Criss 2013). The only *pilE* sequence expressed is 1-81-S2 (Seifert, Wright et al. 1994) through a point mutation which disrupts the *pilE* G-quadroplex structure and prevents antigenic variation (Cahoon and Seifert 2009). All *opa* genes were deleted, generating Δ*opa*. OpaD is an Opa protein that binds CEACAM-1, CEACAM-3, and CEACAM-5, which are expressed on neutrophils (CEACAM-1 and -3) and epithelial cells (CEACAM-1 and -5)(de Jonge, Hamstra et al. 2003, Werner, Palmer et al. 2020). The sequence for the *opaD* gene was engineered to replace the CTCTT pentameric repeat-rich section of the DNA with sequences encoding the same amino acids but unable to phase vary (Ball and Criss 2013). This *opaD* gene was then cloned back into its native locus to create OpaD+$_{NV}$ which was used as WT in most of these studies.

Use of this controlled genetic background increases the likelihood that mutants that are engineered are truly isogenic, and do not have an altered Opa

and pilin landscape relative to the parent strain. This is especially important given the ultimate focus of my project on adherent Gc, because a shift in adhesion factor expression between parent and mutant strains would have been a major confounding variable.

1.2.5 Immune response to Gc infection

In addition to facilitating colonization, surface-expressed components of Gc contribute to immune detection by the host. Neutrophils are extremely abundant in symptomatic Gc infection, making up the majority of the cellular content of characteristic gonorrheal purulent exudate (Evans 1977, Apicella, Ketterer et al. 1996). Remarkably, viable Gc can be recovered from within the neutrophils in these exudates (Criss and Seifert 2012). Work from the Criss lab and others has demonstrated that variability of surface components such as *opa* genes contribute to immune detection of Gc and affect the manner in which host immune cells respond to the presence of bacteria (Sarantis and Gray-Owen 2012, Ball and Criss 2013, Palmer and Criss 2018). For example, Opa_{CEA} proteins, which contribute to epithelial colonization through binding to CEACAMs (**section 1.2.2**) also contribute to immune detection of Gc through their affinity for CEACAMs expressed on neutrophils (Gray-Owen, Dehio et al. 1997). Gc that do not express any Opa genes are less likely to associate with neutrophils and more likely to survive over time in the presence of neutrophils (Johnson, Ball et al. 2015)(Allison Alcott *et al* in press 2022).

Gc infection induces a predominantly T-helper 17 (Th17) type immune response (Stevens and Criss 2018), and in a mouse model of female Gc infection, neutrophil influx to the site of Gc infection depended in large part on interleukin-17 (IL-17) signaling (Feinen, Jerse et al. 2010). A murine model also showed that expression of Opa proteins by Gc stimulated transforming growth factor beta (TGFβ) production and therefore suppressed Th1 and Th2 responses, and this could be inhibited using an anti-murine CEACAM-1 antibody, even though Opa is not thought to bind to murine CEACAMs (Liu, Islam et al. 2012). TGFβ stimulation can induce $CD4^+$ T cells to differentiate into regulatory T (Treg) cells, so the cytokine interleukin-6 (IL-6) is required suppress Treg differentiation and shift $CD4^+$ T cells toward Th17 (Bettelli, Carrier et al. 2006, Tanaka, Narazaki et al. 2014). Gc lipooligosaccharide (LOS) has been shown to stimulate IL-6 production in murine bone marrow-derived macrophages through Toll-like receptor 4 (TLR4) signaling (Andrade, Agarwal et al. 2016).

LOS signaling locally and IL-6 release into the bloodstream are also key players in the acute-phase response, leading to systemic and local sequestration of the essential metals iron and zinc (Gaetke, McClain et al. 1997). As will be discussed in **Section 1.4.2**, IL-6 can act locally to induce upregulation of zinc import and storage proteins in mammalian cells (Gammoh and Rink 2017). The cytokine-stimulated reduction of extracellular free zinc levels is part of an immune paradigm called “nutritional immunity”, where the host accumulates metals within

cells or produces proteins to chelate free metal ions in body fluids. This is described in more detail in the following section.

1.3 Nutritional Immunity

Nutritional immunity refers to a stress exerted on microbes by the host through limitation of essential nutrients during infection. This was first characterized in iron acquisition, but has been expanded to include other metals, including zinc, manganese, and copper (Cassat and Skaar 2013), (Corbin, Seeley et al. 2008), (Kehl-Fie and Skaar 2010). Iron is an important cofactor in many enzymes due to its redox potential and ability to act both as an electron acceptor and electron donor, depending on its oxidation state (Fe^{2+} or Fe^{3+})(Cassat and Skaar 2013). In Gc, it is essential for SodB, and is expected to play a role in many other proteins (~3.5% of the *Neisseria meningitidis* proteome)(Andreini, Banci et al. 2007). Similarly, copper can cycle between Cu^{2+} and Cu^{+} redox states and act as a catalytic cofactor, although proteins containing copper are less abundant than iron-containing proteins (~0.65% of the *Neisseria meningitidis* proteome) (Andreini, Banci et al. 2008). Manganese is used to overcome oxidative stress by some bacteria (Anjem, Varghese et al. 2009). Zinc is notable for its abundance (4.5% of the *Neisseria meningitidis* proteome) in both structural and catalytic roles in proteins (Auld 2001, Andreini 2006), and as the focus of this dissertation its role is described in detail in section 1.5.1.

Iron and zinc are the most abundant trace metals in the human body (Gammoh and Rink 2017) and are therefore the best-studied during

inflammation. At steady-state, most zinc in the bloodstream is nonspecifically bound to albumin, and iron is bound to transferrin (Parrow, Fleming et al. 2013), (Gammoh and Rink 2017). Nutrient sequestration occurs both on a systemic and a local level as a result of inflammation. During the acute phase immune response, cytokines such as IL-6 are released. IL-6 stimulates hepatocytes in the liver to upregulate the zinc importer ZIP-14 and metallothioneines (MTs) to increase zinc uptake and storage capacity, respectively (Gammoh and Rink 2017). IL-6 also stimulates hepatocytes to release hepcidin, a hormone which stimulates multiple cell types to remove iron from serum by expressing the cytoplasmic iron-binding protein ferritin and degrading the iron exporter ferroportin (Fpn1). The ultimate result is that zinc accumulates in the liver and iron accumulates in macrophages, while metal ion levels in the blood drop (Aydemir, Chang et al. 2012) (Gaetke, McClain et al. 1997) (Parrow, Fleming et al. 2013). Locally, cytokine production stimulates increased metal protein expression by epithelial cells and the release of metal binding proteins into the milieu (Gammoh and Rink 2017), (Hegyi, Zwicker et al. 2012, Parrow, Fleming et al. 2013), (Glaser, Meyer-Hoffert et al. 2009), (Liang, Tan et al. 2006). These metal binding proteins include lactoferrin, which binds iron; psoriasin, which binds zinc; and calprotectin, which binds zinc and manganese (Parrow, Fleming et al. 2013), (Kehl-Fie and Skaar 2010).

Nutritional immunity is nonspecific and affects pathogens and commensal species alike, for instance *Salmonella typhimurium* is better able to outcompete

commensal gut microbes when inflammation causes an increase in calprotectin, which *Salmonella* is better able to tolerate relative to commensal microbes (Liu, Jellbauer et al. 2012, Diaz-Ochoa, Lam et al. 2016). Nutrient sequestration is also not without cost to the host, as illustrated by the prevalence of “anemia of chronic disease,” which is the most common cause of anemia in hospitalized patients (Madu and Ughasoro 2017). This anemia is not fully understood and likely occurs through multiple mechanisms depending upon the underlying illness, which can include chronic infection, cancer, autoimmune disease, transplant rejection, or chronic kidney disease (Madu and Ughasoro 2017). Normally hepatocytes are the gatekeepers of iron homeostasis and when serum iron levels rise, they produce the hormone hepcidin. Hepcidin acts at multiple sites to induce degradation of the iron exporter ferroportin (Fpn1) and increase expression of ferritin, the intracellular iron storage molecule (Lopez, Cacoub et al. 2016). This controls dietary zinc uptake in the duodenum as well as release from splenic macrophages and liver cells (Ganz 2006). During anemia of chronic disease, IL-6 acts on hepatocytes to stimulate hepcidin production. Ultimately, reticuloendothelial macrophages in the bone marrow are signaled to withhold iron from erythrocyte progenitors, which results in blood cells which do not possess enough iron to efficiently oxygenate the body (Madu and Ughasoro 2017).

1.3.1 Pathogen defenses against nutritional immunity

Various pathogens have different methods to overcome nutritional immunity, which have been extensively reviewed elsewhere, and which I will

describe in brief (Neumann, Hadley et al. 2017), (Stork, Grijpstra et al. 2013), (Diaz-Ochoa, Jellbauer et al. 2014), (Lonergan and Skaar 2019), (Kehl-Fie and Skaar 2010), (Capdevila, Wang et al. 2016).

One of the most basic methods of overcoming metal restriction is expression of high-affinity transporters to scavenge any remaining free metal in the environment. This strategy is illustrated in the case of *Salmonella typhimurium*, which expresses the high affinity zinc transporter ZnuABC, allowing it to outcompete commensal bacteria in the inflamed gut (Liu, Jellbauer et al. 2012). This method is often used in parallel with other metal acquisition methods, as will be described later for Gc. Another method of scavenging scarce metals is the expression of siderophores, which are small iron chelating molecules produced by bacteria to outcompete host metal-binding proteins. Once iron is bound to the siderophore, it is imported into the bacterium by active so that the iron can be used (Behnsen and Raffatellu 2016). Examples include enterobactin, yersiniabactin, and salmochelin, which vary in their ability to evade host responses and their ability to bind other metals. For example, yersiniabactin has been shown to bind copper and zinc, and is required for the establishment of septicemic plague by *Yersinia pestis* in mice (Bobrov, Kirillina et al. 2014), (Bobrov, Kirillina et al. 2017), (Chaturvedi, Hung et al. 2012). Some siderophores, such as enterobactin, are conserved across bacterial species. Some pathogens are able to intercept the siderophores produced by other bacterial species; for instance, Gc express FetA to acquire iron from

enterobactin, which they do not produce (Carson, Klebba et al. 1999). In order to outcompete siderophore-mediated metal acquisition, mammals express siderocalin (also called lipocalin-2) to intercept enterobactin and prevent its reuptake by bacteria (Flo, Smith et al. 2004), (Becker and Skaar 2014). Illustrating the constant push-pull dynamic of nutritional immunity, bacteria have further evolved to prevent interception of their siderophores by expressing so-called “stealth siderophores,” such as petrobactin in *Bacillus anthracis* (Abergel, Wilson et al. 2006) and salmochelin in *Salmonella enterica* (Crouch, Castor et al. 2008), (Raffatellu, George et al. 2009). These alternative siderophores scavenge iron and are taken up by the bacteria that produce them, but evade binding by mammalian siderocalin (Becker and Skaar 2014).

Finally, some bacteria do not always try to out-compete the binding affinity of the host metal-binding proteins. Instead, they engage in “metal piracy” and use TonB-dependent transporters (TdTs) to acquire metals directly from the exact proteins that were produced by the host to sequester metal from pathogens (Cornelissen 2018), (Yadav, Noinaj et al. 2019).

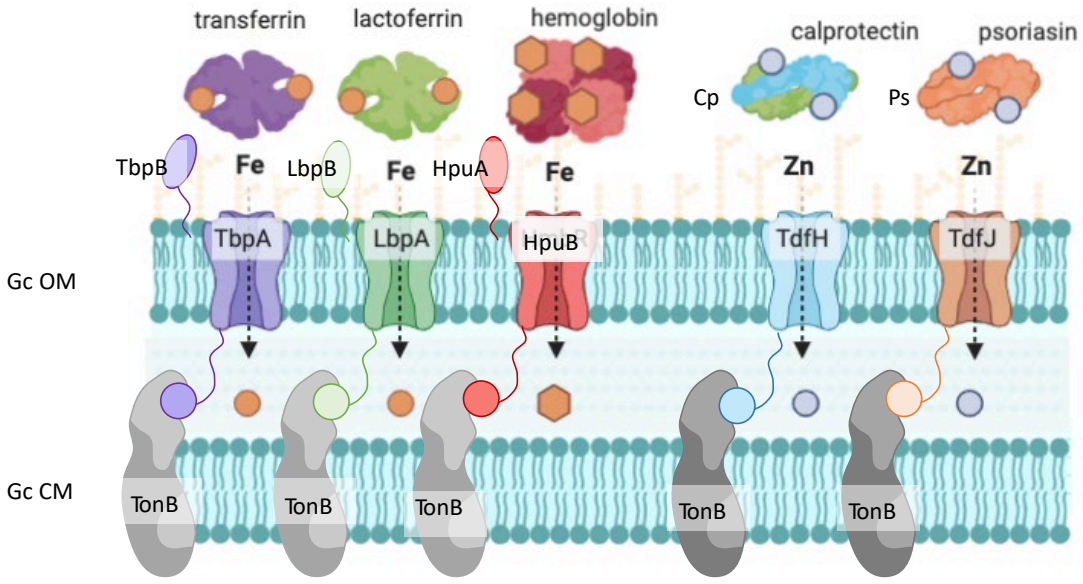
1.3.2 TonB-dependent Transporters:

TdTs are outer membrane proteins consisting of a beta-barrel pore and a plug domain (Yadav, Noinaj et al. 2019). The plug domain interacts with the TonB complex, an inner membrane protein complex made up of TonB, ExbB, and ExbD (Noinaj, Guillier et al. 2010), (Postle 2007). The TonB complex harnesses the proton gradient across the inner membrane and couples it to

movement of the nutrient across the outer membrane (Calmettes, Ing et al. 2015). When the TdT binds its ligand, a conformational change occurs and TonB pulls the plug domain so that the substrate is transported into the periplasm (Calmettes, Ing et al. 2015). In other gram-negative bacteria, TdTs help import essential nutrients such as vitamin B12, or iron in the context of siderophores (Noinaj, Guillier et al. 2010), (Postle 2007).

The pathogenic *Neisseria* species, *Neisseria meningitidis* and Gc, have evolved TonB-dependent transporters (TdTs) that are specific to the human metal-binding proteins transferrin, lactoferrin, hemoglobin, calprotectin, and psoriasin (Cornelissen and Hollander 2011), (Jean, Juneau et al. 2016), (Maurakis, Keller et al. 2019) (**Figure 1.3.2**). They bind host proteins and strip the metal from them before releasing the proteins back into the milieu to bind metal anew (Yadav, Noinaj et al. 2019). This host protein specificity of *Neisserial* TdTs was first described for the acquisition of iron from human transferrin via TbpA (Cornelissen, Biswas et al. 1992). TbpA and its lipoprotein cofactor TbpB bind to human transferrin, strip the iron from the protein, and internalize it through the transmembrane pore in TbpA (Cornelissen, Biswas et al. 1992).

Figure 1.3.2 Gc overcome nutritional immunity by acquiring iron and zinc from human metal-binding proteins.
Five Gc TonB-dependent transporters (TdTs) are known to bind to human proteins and acquire metal. “TonB” in this figure represents the TonB-ExbB-ExbD protein complex on the cytoplasmic membrane which binds the plug domain of a TdT to energize cargo transport across the outer membrane by harnessing the proton gradient at the cytoplasmic membrane. TbpA and its lipoprotein cofactor TbpB acquire iron (Fe) from human transferrin. TbpB enhances efficiency of this process by having higher affinity for holo-transferrin and releasing transferrin once iron has been removed by TbpA. LbpA (TdT)and LbpB (lipoprotein) likely function similarly to acquire iron from human lactoferrin. HpuB (TdT) and HpuA (lipoprotein) acquire heme-complexed-iron from hemoglobin. TdfH (CbpA in *Neisseria meningitidis*) acquires zinc from human calprotectin ($S100A8_2S100A9_2$). TdfJ (ZnuD in *Neisseria meningitidis*) acquires zinc from human psoriasin ($S100A7_2$). Periplasmic and cytoplasmic membrane transporters which are required to internalize metal cargo fully into the cytoplasm from the periplasm are not illustrated here.
Gc OM: outer membrane, GC CM: cytoplasmic membrane.



Cornelissen *et al* demonstrated the importance of iron uptake from transferrin with an experimental urethral challenge experiment which was conducted on male volunteers. A strain of *ΔtbpB-ΔtbpA* Gc was created without antibiotic resistance markers by replacing the antibiotic resistance cassette with a transcriptional and translational stop signal to prevent expression of either TbpA or TbpB. Without TbpAB, the bacteria were unable to establish infection, while the men infected with wildtype quickly exhibited symptoms (Cornelissen, Kelley et al. 1998).

Human challenge studies have not been conducted with mutants in each known human-specific TdT, and challenge studies have not been conducted in women at all due to the potential for undetected asymptomatic infection and serious sequelae (Edwards and Butler 2011). The results on TbpA nevertheless provide a demonstration of the importance of metal uptake from human proteins during *in vivo* infection.

1.3.3 TdTs as vaccine antigens:

Unlike the many variable surface-expressed proteins in Gc, some of the TdTs are stably expressed and well-conserved across strains (Baarda, Zielke et al. 2021). This, along with their necessity during infection, recommends them as good vaccine antigens to test. Interestingly, while wildtype TbpB is not very immunogenic, TbpB that has been mutated to no longer bind transferrin is a far superior immunogen. Whether this is due to epitope masking by transferrin binding or activation of self-reactive Treg cells when transferrin epitopes are

presented along with TbpB, has yet to be determined (Frandoloso, Martinez-Martinez et al. 2015). TbpA and TdfJ have both been specifically named as good vaccine candidates (Baarda, Zielke et al. 2021), and ZnuD, TdfJ's homolog in *Neisseria meningitidis*, has also been proposed as an immunogen (Stork, Bos et al. 2010), (Hubert, Devos et al. 2013), (Calmettes, Ing et al. 2015). In addition to vaccine targets, the external location and low degree of variability makes TdTs tempting targets for small molecule inhibitors of metal uptake. Each TdT may vary in its essentiality depending on the site of infection. For instance, the heme-uptake receptor *hpuB* is phase-variable and Gc with *hpuB* phase-on are more likely to be isolated from women during the bleeding phase of their menstrual cycle (Anderson, Leone et al. 2001). In order to determine which TdTs will make the best antigens or drug targets, it is important to understand the roles they play during infection, and when and where they are expressed.

1.4 Zinc Nutritional Immunity

1.4.1 Necessity of zinc in biological systems

Zinc is an essential element for all domains of life (Andreini 2006). It serves important structural roles in many proteins, stabilizing tertiary structures and facilitating dimerization (Auld 2001). Across all domains of life, zinc is an essential cofactor in proteases, phosphatases, and many other hydrolytic enzymes (Reyes-Caballero, Campanello et al. 2011). Zinc's electron configuration has four valence electrons, which constitute an incomplete octet. It readily accepts the valence electrons of oxygen, and therefore acts as a strong

Lewis acid to coordinate and “activate” water and enable hydrolysis of an enzyme’s substrate (Auld 2001), (Schroeder and Cousins 1990). (Andreini, Bertini et al. 2008). Zinc is required for approximately 10% of all human proteins (Andreini 2006), (Andreini, Banci et al. 2006) and 4-6% of all bacterial proteins (~4.5% of the *Neisseria meningitidis* proteome) (Andreini 2006), (Andreini, Bertini et al. 2008), (Capdevila, Wang et al. 2016). In humans, a large number of these include zinc-finger transcription factors, in which zinc plays a structural role. In bacteria, essential roles include tRNA synthesis, folate biosynthesis, metalloproteases, transcription (as a component of RNA polymerase), and transcriptional regulation (zinc is required for proper folding of zinc-finger transcription factors such as DksA in E. coli) (Capdevila, Wang et al. 2016), (Cheng-Guang and Gualerzi 2020), (Blaby-Haas, Furman et al. 2011).

Excess zinc is toxic, both to bacteria and to humans. Some pathogens express zinc efflux pumps, which are necessary to overcome zinc toxicity (as demonstrated in Group A *Streptococcus* for the zinc efflux pump CzcD) (Ong, Gillen et al. 2014). In humans, zinc uptake from the gut is an active process, so zinc toxicity is uncommon (Alker and Haase 2018). There have, however, been cases of extreme coin ingestion, leading to multi-organ failure and death due to zinc toxicity (Bennett, Baird et al. 1997). The mechanism of zinc toxicity may be through competition with other metals at their active sites, due to the greater stability of zinc-complexes compared to other metals (Becker and Skaar 2014). Zinc, along with the much rarer trace metal copper, is at the top of the Irving-

Williams series, a model predicting the relative stabilities of complexes formed with first-row transition metals (Waldron, Rutherford et al. 2009).

1.4.2 Host control of zinc levels:

Zinc levels in the human host are kept controlled at multiple levels, including regulation of zinc uptake from the gut and sequestration of zinc within cells of the liver during systemic inflammation (Alker and Haase 2018). Levels of free zinc within cells of all types are estimated to be in the low picomolar range, despite total concentrations of zinc in cells being in the hundreds of micromolar. Zinc within the mammalian cell exists incorporated into proteins or bound to chelators called metallothioneins (MTs) (Lonergan and Skaar 2019), (Colvin, Holmes et al. 2010). The human genome encodes ten functional MT genes, and while MT1/2 isoforms are present in most cells, MT3 is restricted to the brain and MT4 is restricted to epithelial cells (Gammoh and Rink 2017). Further, MT1/2 isoforms are inducible by cytokines such as IL-6 or excess zinc (Liuzzi, Lichten et al. 2005, Vasak and Meloni 2011, Gammoh and Rink 2017).

Import of zinc into cells is enabled by Zrt/Irt-like protein (ZIP) family members (Gammoh and Rink 2017). Some ZIP proteins are expressed ubiquitously on all cells, but ZIP-14 is predominantly expressed in liver cells and induced by cytokines, including IL-6 (Lonergan and Skaar 2019), (Aydemir, Chang et al. 2012), (Schroeder and Cousins 1990), (Gammoh and Rink 2017). Because all blood is filtered through the liver, this organ is uniquely poised to

quickly alter levels of serum zinc during systemic inflammation (Gaetke, McClain et al. 1997).

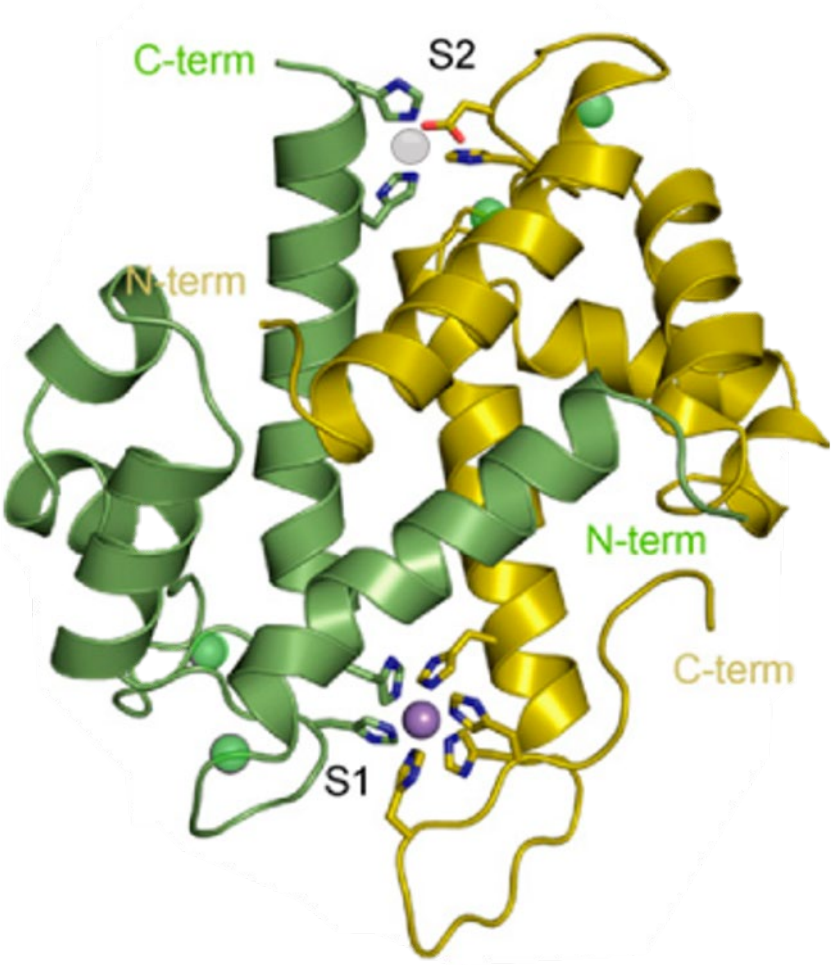
On a more local level, immune cells and epithelial cells at the site of infection can be stimulated by IL-6 to increase production of MTs and ZIPs to remove zinc from the extracellular milieu and sequester it within cells (Liuzzi, Lichten et al. 2005, Gammoh and Rink 2017). MT1, Zip6 and Zip14 have all been shown to be upregulated in mice treated with LPS (Liuzzi, Lichten et al. 2005). Beyond removing zinc from the environment, squamous mucosal epithelial cells increase expression of zinc-binding proteins such as calprotectin and psoriasin in response to the inflammatory cytokines IL-17, IL-22, TNF-α, and IL-1α (Liang, Tan et al. 2006),(Hegyi, Zwicker et al. 2012),(Glaser, Meyer-Hoffert et al. 2009),(Abtin, Eckhart et al. 2008). Zinc-binding S100-proteins such as psoriasin and calprotectin are released without metals bound, and bind zinc with high affinity extracellularly, as will be described in the next section.

1.4.3 Calprotectin

Calprotectin is an S100-family protein composed of S100A8 and S100A9. Calprotectin has been given a confusing number of names, having been "discovered" as significant in multiple fields (Johne, Fagerhol et al. 1997). Names include: Leukocyte protein 1 (L1), cystic fibrosis antigen (CFA), myeloid related protein 8/14 (MRP8/14), and calgranulin A/B. Calprotectin is produced in abundance by neutrophils, and has been estimated to constitute about 45% of the protein content of neutrophil cytoplasm (Edgeworth, Gorman et al. 1991).

Neutrophils can be stimulated to release webs of DNA decorated with antimicrobial peptides and proteins including abundant calprotectin (Jean, Juneau et al. 2016). Calprotectin has also been detected in neutrophil secondary granules, and released by activated neutrophils when these granules are mobilized (Stroncek, Shankar et al. 2005). Additionally, calprotectin is ubiquitously produced by many normal squamous mucosal epithelia, including the ectocervix, vaginal epithelium, gingiva, tongue, and esophagus (Wilkinson, Busuttil et al. 1988, Coleman and Stanley 1994, Ross and Herzberg 2001, Uhlen, Fagerberg et al. 2015),(Bastian, Roux et al. 2021). It has also been detected in cervicovaginal lavage fluid and cervical mucus by unbiased proteomics studies (Zegels, Van Raemdonck et al. 2009, Panicker, Ye et al. 2010, Birse, Burgener et al. 2013).

Figure 1.4.3 Calprotectin crystal structure.
Calprotectin possesses two distinct binding sites.
Site 1 can bind either zinc or manganese (shown complexed with manganese), and is composed of 6 histidine residues: (clockwise from top left) S100A8 H17 and H27, S100A9 H103, H95, H105, and H91. Site 2 binds zinc but not manganese and is composed of S100A8 H87 and H83, S100A9 H20 and D30
Green ribbons: S100A8; Yellow ribbons: S100A9
Purple circle: manganese; green circles: calcium, gray circle: zinc
Modified from a crystal structure of calprotectin bound to manganese by (Damo, Kehl-Fie et al. 2013) refined to 1.6 Å resolution. Depiction of zinc at site 2 has been added for the purposes of illustration.
PDB DOI: 10.2210/pdb4GGF/pdb



Calprotectin possesses two transition metal binding sites (Damo, Kehl-Fie et al. 2013)(**Figure 1.4.3**). Site 1 is made up of six histidines and is capable of binding zinc, manganese, iron, or copper (Brophy, Hayden et al. 2012),(Brophy, Nakashige et al. 2013, Hayden, Brophy et al. 2013, Zygiel and Nolan 2019). While zinc coordination only requires four histidines, the crystal structure of manganese-bound calprotectin showed involvement of all 6 histidines at site 1 (Damo, Kehl-Fie et al. 2013). Site 2 is made up of three histidines and one aspartate, and is only known to bind zinc or copper with high affinity (Damo, Kehl-Fie et al. 2013). Each subunit (S100A8 and S100A9) contains two EF-hand domains which bind calcium (Leukert, Vogl et al. 2006). Calcium binding is required for the formation of calprotectin tetramers, which are made up of two S100A8/S100A9 heterodimers (Leukert, Vogl et al. 2006). Once calcium is bound and calprotectin oligomerizes, its affinity for zinc and/or manganese increases significantly (Brophy, Hayden et al. 2012). Calprotectin has been shown to restrict the growth of some pathogenic bacteria and fungi through zinc and/or manganese sequestration (Urban, Ermert et al. 2009), (Brophy, Hayden et al. 2012), (Nakashige, Stephan et al. 2016). Calcium levels within human cells are low (100nM free Ca intracellularly)(Clapham 2007), so it is likely that calprotectin only efficiently chelates zinc once it has been released extracellularly (Brophy, Nakashige et al. 2013). In the presence of excess calcium, calprotectin has a dissociation constant of ~200nM for manganese at site 1, ~250pM for zinc at site

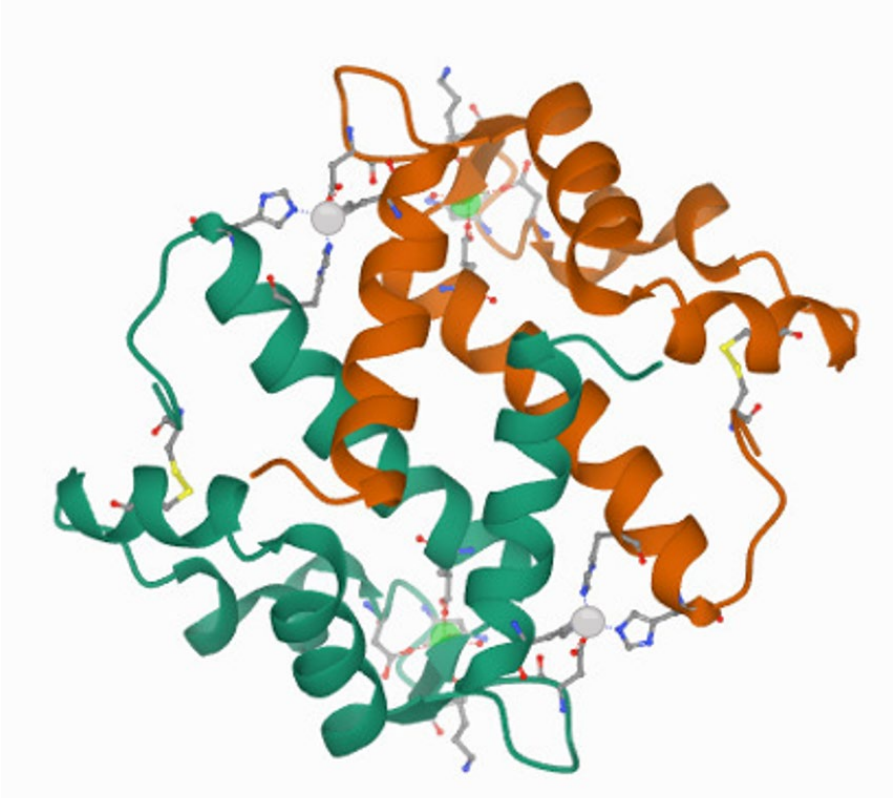
1, and ~10pM for zinc at site 2 (Brophy, Hayden et al. 2012), (Hayden, Brophy et al. 2013). *Note: the Nolan group uses a different notation and reverses the site 1 and site 2 nomenclature relative to the Chazin group. This dissertation uses the Chazin group notation.*

1.4.4 Psoriasin

Calprotectin is the most extensively studied zinc-binding S100-family protein, but other S100 proteins have been reported to bind zinc, including S100s A1, A2, A3, A5, A6, A7, A12, A16, and S100B (Gilston, Skaar et al. 2016). The role of most of these proteins in bacterial infection and nutritional immunity is not well established, except for in the case of S100A7 and, to a lesser extent, S100A12. S100A7 is also called psoriasin, originally named because it was discovered in the inflamed epidermis of psoriatic lesions, and has also been called RIS-1 for retinoid-inducible skin-specific gene. It was later shown not to be specific to psoriasis, but to inflammatory skin diseases including acne and atopic dermatitis (Zouboulis, Beutler et al. 2017). While psoriasin expression in the skin is generally indicative of pathology, it is constitutively expressed in many mucosal squamous epithelia including the vagina, ectocervix, tongue, nasopharynx, corneal and conjunctival cells (Mildner, Stichenwirth et al. 2010, Zouboulis, Beutler et al. 2017),(Meyer, Harder et al. 2008), (Bryborn, Adner et al. 2005), (Garreis, Gottschalt et al. 2011), existing as a homodimer of S100A7 (**Figure 1.4.4**). S100A7's two transition metal binding sites are identical, and both sites are only known to bind zinc with high affinity (Gilston, Skaar et al. 2016),

(Cunden, Brophy et al. 2017). Psoriasin has also been demonstrated as an effective zinc sequestering agent against a selection of bacteria and fungi (Cunden, Brophy et al. 2017), (Hein, Takahashi et al. 2015), (Mildner, Stichenwirth et al. 2010).

Figure 1.4.4 Psoriasin crystal structure
Psoriasin is a homodimer, and the two identical binding sites bind zinc.
Green and orange ribbons represent two monomers of S100A7
Green circles: calcium, gray circles: zinc
Yellow sticks: disulfide bridges between S100A7 monomers
Modified from a crystal structure of psoriasin bound to zinc and calcium by (Brodersen, Nyborg et al. 1999) refined to 2.05 Å resolution. PDB DOI: 10.2210/pdb2PSR/pdb



1.4.5 Zinc in the Female Genital Tract

Calprotectin and psoriasin are both among the most abundantly detected proteins in cervical mucus and cervicovaginal lavage fluid and increase in concentration in cervicovaginal lavage fluid during inflammation (Birse, Burgener et al. 2013), (Zegels, Van Raemdonck et al. 2009), (Borgdorff, Gautam et al. 2016). The mean concentration of calprotectin in the cervical mucus of 100 healthy women was reported to be approximately 1 μM (Kunimi, Maegawa et al. 2006). The mean psoriasin concentration from a 2 mL cervicovaginal lavage wash was reported as ~62 nM (Mildner, Stichenwirth et al. 2010). While high-quality data are not currently available on calprotectin and psoriasin levels in women specifically infected with Gc, it is logical to predict that the highly inflammatory response to Gc infection would cause an increase in these proteins (discussed in **section 1.2.5**). IL-17 and IL-22 have been shown to upregulate psoriasin and calprotectin in cells *in vitro*, and are abundantly produced cytokines in the Th-17-skewed immune response to Gc infection (Liang, Tan et al. 2006), (Rice 2005). Total zinc in the female reproductive tract is relatively low (~0.9-1.5μM) compared to serum levels (~12μM), and especially low compared to zinc levels in seminal fluid (~1mM) (Damjanovich, Sipos et al. 2020), (Takacs, Damjanovich et al. 2020), (Chuang, Lee et al. 2002), (Wong, Flik et al. 2001), (Kothari and Chaudhari 2016).

This led us to predict that, in order to establish infection, Gc has found a way to overcome the host methods of zinc restriction and is able to acquire zinc during infection of the female genital tract.

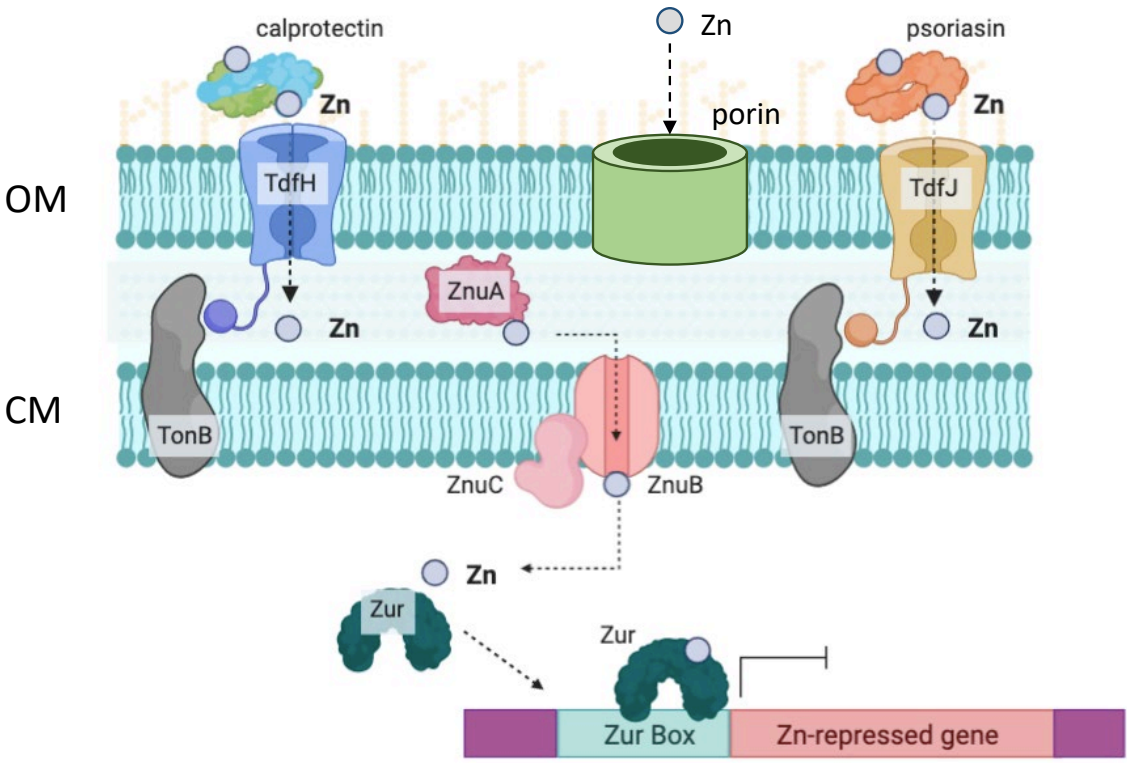
1.5 Zinc regulation and uptake in Gc

1.5.1 Zinc uptake:

Zinc must be transported through both the outer membrane and the inner membrane in order to be incorporated into metalloenzymes and used by Gc (Stork, Grijpstra et al. 2013). This process is illustrated in **Figure 1.5.1.1** and described below. The *Neisseria meningitidis* homolog of TdfJ was shown to transport free zinc ions (Calmettes, Ing et al. 2015). However, zinc in the female genital tract is likely limited, as described in **Section 1.4.5**, so free zinc would not be present. Instead, TdfH is able to bind and acquire zinc from the human protein calprotectin (S100 A8/A9) (Jean, Juneau et al. 2016), and TdfJ is able to bind and acquire zinc from human psoriasin (S100 A7) (Maurakis, Keller et al. 2019).

Fig. 1.5.1.1 Zinc import into Gc

Zinc crosses the outer membrane by active, TonB-dependent transport through TdfH from calprotectin, TdfJ from psoriasin, or diffusion through porin. Periplasmic zinc is prevented from diffusing out of the periplasm by high-affinity binding to ZnuA. ZnuA interacts with ZnuB (permease) and ZnuC (ATPase) to import zinc into the cytoplasm. Cytoplasmic zinc is incorporated into biomolecules, and high zinc levels saturate the zinc uptake regulator, Zur, leading to transcriptional repression of Zur-repressed genes, including TdfH, TdfJ, and ZnuABC.

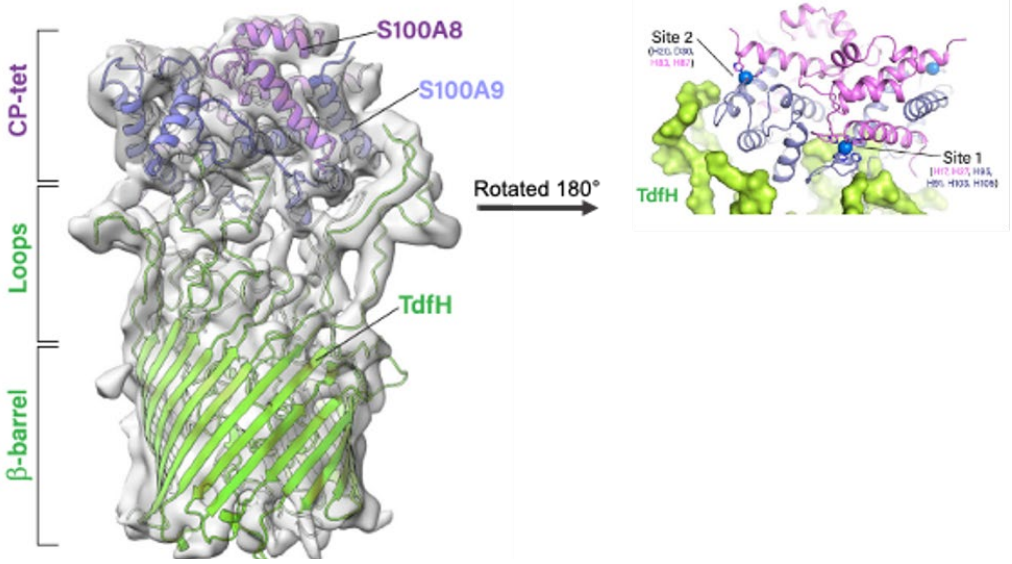


Jean *et. al* showed that Gc is able to bind calprotectin in a TdfH-dependent manner, and that growth of Gc with zinc-loaded calprotectin as the sole zinc source in chemically-defined media was dependent on TdfH expression (Jean, Juneau et al. 2016). Furthermore, Bera *et al* solved a crystal structure of calprotectin bound to TdfH, showing that TdfH interacts with site 1 of calprotectin (Bera, Wu et al. 2022) (**Figure 1.5.1.2**).

In 2019, Maurakis *et al* showed that Gc is able to use zinc-loaded psoriasin as a sole zinc source in a TdfJ- and TonB-dependent manner (Maurakis, Keller et al. 2019). Gc lacking either TdfH or TdfJ were able to grow in chemically-defined media when free zinc was supplemented, implying that zinc import may be possible through other means. When zinc is abundant, it likely diffuses into the periplasm through PorB, Gc's outer membrane porin (Stork, Grijpstra et al. 2013), (Kattner, Zaucha et al. 2013).

Once zinc has reached the periplasm, it is bound by ZnuA, the high-affinity shuttle protein component of the ZnuABC tripartite transporter. ZnuB and ZnuC are the permease and ATPase, respectively, which move zinc from the periplasm into the cytoplasm (Chen and Morse 2001).

Fig. 1.5.1.2 Crystal structure of TdfH in complex with tetrameric calprotectin ($S100A8_2S100A9_2$) TdfH binds directly to human calprotectin, and interacts preferentially with zinc-bound tetrameric calprotectin. TdfH is depicted in green. Both monomers of S100A8 are magenta; both monomers of S100A9 are indigo. Zinc ions are represented by blue spheres in the right panel only. Calcium ions are not pictured. Bera *et al* 2022.



1.5.2 Zinc regulation in Neisseriae

In Gc, zinc uptake is regulated by the Zinc Uptake Regulator (Zur) (Wu, Seib et al. 2006). Zur is a Fur-family metalloregulatory protein which exists as a dimer in solution (Ma, Gabriel et al. 2011), (Gaballa and Helmann 1998). Zur mRNA expression has not been reported to be regulated by zinc levels (Pawlik, Hubert et al. 2012), and I also did not observe differential expression of *zur* when Gc was grown under zinc-limited conditions **(Chapter 2)**. Instead, the affinity of Zur for its targets is directly regulated by the cytoplasmic zinc concentration (Shin and Helmann 2016). When free zinc is present in the cytoplasm, the two regulatory zinc binding sites of the Zur dimer are filled and the protein complex has high affinity for palindromic motifs on the DNA called “Zur boxes” (Pawlik, Hubert et al. 2012). The consensus Zur box palindrome in *Neisseria meningitidis* was determined to be TGTTATDNHATAACA, where D= *not C*, H= *not G*, and N= *any* (Pawlik, Hubert et al. 2012). When zinc levels in the cytoplasm drop, zinc ions dissociate from Zur and the demetalated protein loses affinity for DNA and dissociates, allowing transcription of genes that had been repressed (Mikhaylina, Ksibe et al. 2018).

In Gc, Zur was originally called PerR by Wu *et al* due to sequence similarity to manganese-binding PerR in *Bacillus subtilis* and *Campylobacter jejuni*. However, I will refer to this protein as Zur, with the following rationale. Wu *et al* insertionally disrupted Zur with a kanamycin resistance cassette and compared RNA expression of log-phase Gc cultures between wildtype and *Δzur*

Gc by microarray. Although 9 of the 11 transcripts differentially expressed in Gc *Δzur* were also found to be differentially expressed due to zinc starvation in *Neisseria meningitidis* by Pawlik et al (Pawlik, Hubert et al. 2012), none of these were differentially expressed by Gc in response to hydrogen peroxide (Stohl, Criss et al. 2005). Additionally, Jean *et al* showed that zinc-repression of TdfH and TdfJ in FA19 or FA1090 Gc is dependent on Zur (Jean et al 2016). Excess zinc inhibited protein expression of TdfH and TdfJ by wildtype but not *Δzur* Gc. Additionally, *Δzur* Gc expressed higher levels of TdfH and TdfJ relative to wildtype (Jean, Juneau et al. 2016). Overall, these results support a model where Zur is a zinc-responsive transcriptional regulator, rather than a manganese-dependent peroxide-responsive regulator.

Genes previously described as being zinc- or Zur- regulated in *Neisseria meningitidis* and Gc are listed in **Table 1.5.2.1**. (Pawlik, Hubert et al. 2012). Of these, *tdfH*, *tdfJ*, *znuABC*, and possibly *ngo_1049* are likely to be involved in zinc uptake. The genes *rpmE2* and *rpmJ2* are likely to be invoved in zinc sparing, while the function of the ORF *ngo_0474* is unknown.

Table 1.5.2.1 Zur- and zinc-regulated genes in the pathogenic *Neisseria*

N. gonorrhoeae FA1090 ID	Symbol	Description	*N. gonorrhoeae* Zur repressed?[a]	*N. meningitidis* Zinc repressed?[b]	*N. meningitidis* MC58	*N. gonorrhoeae* NCCP11945
NGO_1442	AdhP	Alcohol dehydrogenase	-3.4	-4.1	NMB0546	NGK_1179
N/A			N/A	-1.8	NMB0577	NGK_1654
NGO_1205	TdfJ	Psoriasin-binding TdT	5.5	4.4	NMB0964	NGK_0312
NGO_0931	RpmJ2	non-zinc binding ribosomal protein L36	4.8	2.7	NMB0941	NGK_0868
NGO_1049		Hypothetical periplasmic zinc-binding protein	4.7	3.6	NMB1475	NGK_0735
NGO_0168	ZnuA	Zinc ABC transporter shuttle protein	4.0	1.5	NMB0586	NGK_0220
NGO_0930	RpmE2	non-zinc binding ribosomal protein L31	3.9	2.2	NMB0942	NGK_0869
NGO_0952	TdfH	calprotectin-binding TdT	2.7	1.6	NMB1497	NGK_0844
NGO_0166		hypothetical periplasmic protein	2.6	*ns*	NMB0866	NGK_0219
NGO_0170	ZnuC	Zinc ABC transporter ATPase	2.2	1.4	NMB0588	NGK_0222
NGO_0169	ZnuB	Zinc ABC transporter permease	2.0	2.1	NMB0587	NGK_0221
NGO_0474		hypothetical protein	1.8	N/A	N/A	NGK_1463
NGO_1685	YhhQ	hypothetical integral membrane protein	*ns*	4.6	NMB0316	NGK_2074
NGO_1684	QueF	7-cyano-7-deazaguanine reductase	*ns*	3.4	NMB0317	NGK_2073
NGO_0129	QueC	7-cyano-7-deazaguanine synthase	*ns*	2.5	NMB0525	NGK_0175
N/A		hypothetical protein in *N. meningitidis*	N/A	2.0	NMB0817	N/A
N/A		hypothetical protein in *N. meningitidis*	N/A	1.9	NMB0818	N/A
N/A		hypothetical protein in *N. meningitidis*	N/A	2.3	NMB0819	N/A
N/A		hypothetical protein in *N. meningitidis*	N/A	1.6	NMB0820	N/A

[a]Log2 fold change in *Δzur* compared to WT Gc (Wu, Seib et al 2006)
[b]Log2 fold change in zinc-excess vs. zinc-limited conditions (Pawlik, Hubert et al 2012)
NGO, NMB, and NGK IDs for genes are provided to aid in comparisons with other data sets.

The process of gene regulation by Zur appears to be more nuanced than a simple on-off switch. Pawlik showed that the degree of gene regulation due to zinc starvation correlated with the degree of similarity of that gene's Zur box with the consensus sequence (Pawlik, Hubert et al. 2012). I searched the genomes upstream of the transcriptional start site of genes reported by Wu *et* al to be Zur-regulated. Zur boxes that were detected are listed in **Table 1.5.2.2**, along with their divergence from the consensus sequence established by Pawlik (Pawlik, Hubert et al. 2012).

A molecular mechanism for stepwise phases in Zur regulation was proposed by Shin and Helmann for *Bacillus subtilis* Zur. They showed that *B. subtilis* Zur DNA affinity differed depending upon whether zero, one, or two regulatory zinc ions were bound per Zur dimer (Shin and Helmann 2016). This was based upon previous work showing that binding of the second zinc ion in the *B. subtilis* Zur dimer is inhibited by binding of the first ion, leading to two distinct zinc-bound states depending upon levels of available zinc (Ma, Gabriel et al. 2011). *B. subtilis* Zur with point mutations enabling only one regulatory zinc ion to be bound had very low affinity for the operators of some Zur-repressed genes, but affinity similar to fully zinc-bound Zur for two of these operators (Shin and Helmann 2016). The authors did not comment on what, if any, differences existed between the sequence of those Zur boxes compared to the Zur boxes which required fully metalated Zur.

Table 1.5.2.2 Zur-repressed genes and their identified Zur box motifs.

FA1090 gene ID	Symbol	Location of Zur box[b]	Zur Box	# mismatches
Consensus[a]			**TGTTAT** DNH **ATAACA**	*consensus*
NGO_0166		May be co-transcribed with ZnuA[c]		
NGO_0168	ZnuA	(-)28	**TGTTAT** GTT **ATA*t*CA**	1
NGO_0169	ZnuB			
NGO_0170	ZnuC			
NGO_0474		(-)43	**T*tg*T*t*T** TGT **A*a*A*ga*A**	6
NGO_0930	RpmE2	(-)33	**TGTTAT** GTT **ATAACA**	0
NGO_0931	RpmJ2	May be co-transcribed with *rpmE2*[d]		
NGO_0952	TdfH	(+)47	**TGTTAT** GCT **ATA*t*CA**	1
NGO_1049		(-)49	**TGTTAT** AGC **ATAACA**	0
NGO_1205	TdfJ	(-)25	**TGTTAT** ATA **ATAACA**	0
NGO_1442	AdhP	(-)216	**TGTTAT** AAT **ATAACA**	0

[a] *Neisseria meningitidis* consensus Zur box bioinformatically determined by Pawlik, Hubert *et al* 2012

[b]Location of Zur box represents the number of base pairs between the 5' end of the Zur palindrome and the 5' end of the start codon as mapped to the FA1090 genome in STDgen.

[c]*ngo_0166* is downstream from *znuA* on the same DNA strand, so it is possible that they are co-transcribed. However, an ORF is present on the opposite DNA strand in the region between *znuA* and *ngo_0166*.

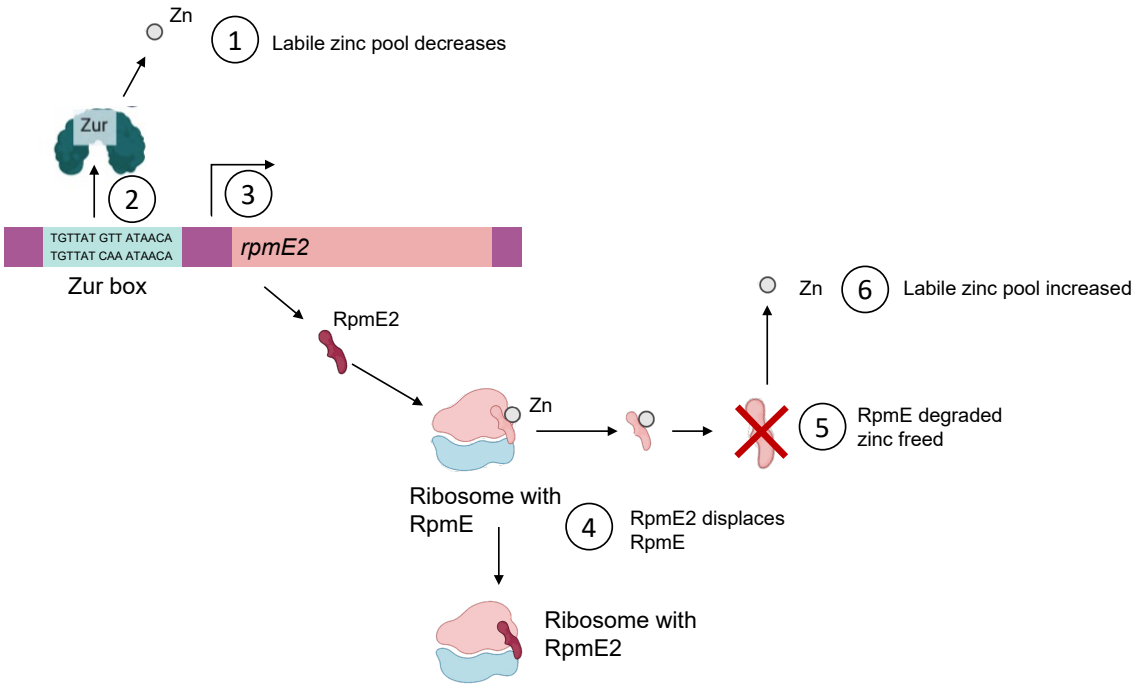
[d]*rpmJ2* start codon overlaps with *rpmE2* stop codon, and therefore is likely to be co-transcribed.

1.5.3 Zinc sparing:

Intracellular zinc dynamics within Gram-negative bacteria are poorly characterized, but only $1/10^6$ of the total zinc within the bacterium is estimated to be unbound (Capdevila, Wang et al. 2016). Zinc sparing encompasses the mechanisms to make zinc available for vital processes when zinc is scarce. While some bacteria, such as *Pseudomonads* and *Cyanobacteria* possess bacterial metallothioneins (MTs), Gc does not encode proteins homologous to known bacterial MTs (Thees, Pietrosimone et al. 2021). Instead, one potential store of zinc within a Gc cell is on the ribosomes. Each bacterium has a large number of ribosomes, each of which has, in times of zinc abundance, at least two potentially mobilizable zinc ions, bound to the L31 and L36 ribosomal proteins (model illustrated in **figure 1.5.3**) (Cheng-Guang and Gualerzi 2020).

Figure 1.5.3 Model of zinc mobilization through ribosomal protein cycling
rpmE (zinc-binding L31) is expressed consistently by bacteria regardless of zinc levels. Zur-repressed *rpmE2* (non-zinc-binding L31) is only expressed when zinc levels are low enough to desaturate Zur. Based upon studies in *E. coli* and *B. subtilis*, the following model emerges:

1- Labile zinc pool decreases and Zur loses zinc saturation
2- Zur dissociates from Zur box
3- *rpmE2* is transcribed and RpmE2 protein is produced
4- RpmE2 displaces RpmE on ribosomes due to its higher affinity. (RpmE2-ribosomes function equivalently to RpmE-ribosomes.)
5- RpmE is degraded and zinc is released
6- Labile zinc pool is increased



When zinc levels are low, Gc upregulates RpmE2 (L31) and RpmJ2 (L36), which are alternative forms of ribosomal proteins L31 and L36 (Wu, Seib et al. 2006), (Pawlik, Hubert et al. 2012). The steady-state versions of L31 (RpmE) and L36 (RpmJ) contain cysteine residues which coordinate one zinc ion per protein. The Zur-repressed paralogs do not contain these cysteine residues and therefore do not bind zinc. Based upon work in *E. coli* and *B. subtilis*, it is possible that RpmE2 and RpmJ2 function to displace RpmE and RpmJ, respectively, and liberate the zinc contained within these proteins (Gabriel and Helmann 2009), (Ueta, Wada et al. 2020), (Akanuma, Nanamiya et al. 2006). Due to the relative density of ribosomes to other zinc-requiring proteins in bacterial cells, this may be a significant source of intracellular zinc during times of zinc limitation (Cheng-Guang and Gualerzi 2020).

1.6 Basis for this study

Previously, Cynthia Cornelissen's lab established that TdfH and TdfJ were zinc- and zur- regulated TonB-dependent transporters (Jean, Juneau et al. 2016). Additionally, they demonstrated that Gc expressing TdfH were able to use zinc-loaded calprotectin as a lone zinc source (Jean, Juneau et al. 2016). Shortly after I began my thesis research, Stavros Maurakis in the Cornelissen lab demonstrated that TdfJ was required for Gc to use zinc-loaded psoriasin (S100A7) as a sole zinc source (Maurakis, Keller et al. 2019). I found that psoriasin was, according to the human protein atlas and BGee databases, most highly expressed in the ectocervix, tonsil, skin, and vagina of healthy human

subjects (Uhlen, Fagerberg et al. 2015), (Bastian, Roux et al. 2021). Targeted studies of psoriasin in the female reproductive tract had found high expression specifically in the ectocervix by immunohistochemistry (Mildner, Stichenwirth et al. 2010). Although the transition zone of the cervix is the primary site of Gc invasion (Yu, Wang et al. 2019), ectocervical epithelium is the first part of the cervix encountered by Gc and colonization of this stratified squamous epithelium is seen in patient biopsies (Edwards, Shao et al. 2000), (Edwards and Butler 2011), (Yu, Wang et al. 2019). To our surprise, we also found that S100A8 and S100A9, the two subunits that make up calprotectin, also have extremely high expression scores in the tissues of the ectocervix and vagina, in addition to immune tissues such as the tonsil and bone marrow (Bastian, Roux et al. 2021), (Uhlen, Fagerberg et al. 2015).

McClure *et al* examined the Gc transcriptome in four female cervical isolates (McClure, Nudel et al. 2015). *znuA*, *tdfJ*, *ngo_1049*, *tdfH*, and *rpmE2* were expressed by Gc isolated from all four subjects (McClure, Nudel et al. 2015). The remaining Zur-repressed genes were all detected in three of the four women: *znuB, znuC,* and *rpmJ2* were detected in all but Subject 2, and *ngo_0474* was detected in all but Subject 3 (McClure, Nudel et al. 2015). These data indicate that the majority of the Zur-repressed transcriptome was de-repressed in Gc during cervical infection, supporting a model where zinc is limited during infection.

For these reasons, we decided to study the role of TdfJ and TdfH during infection of the immortalized ectocervical cell line, Ect1 (Fichorova, Rheinwald et al. 1997). These cells are permissive to Gc infection, and resemble native ectocervical cells in their expression of cell surface markers and ability to induce membrane ruffling and cytokine production in response to Gc infection (Fichorova, Desai et al. 2001).

We confirmed that calprotectin and psoriasin can sequester zinc from *ΔtdfH* and *ΔtdfJ*, respectively, *in vitro.* We also sought to gain insight into how the conserved outer membrane proteins TdfH and TdfJ contributed to host colonization and resistance to nutritional immunity, specifically in the presence of calprotectin and psoriasin (Jean, Juneau et al. 2016, Maurakis, Keller et al. 2019). Surprisingly, I made the novel discovery that while TdfH and TdfJ were required for Gc growth grown in suspension when zinc was sequestered by calprotectin and psoriasin, the *ΔtdfH ΔtdfJ* mutant was able to survive these conditions if adherent to Ect1, as will be discussed in **Chapter 2**. Protection of adherent Gc from zinc sequestration was a phenomenon generalizable not only to other Gc genotypes and S100 proteins, such as WT from murine calprotectin, but also to adherence to other surfaces, including acid-washed glass. My work also provides new insight into the Gc transcriptional response to adherence in a zinc-dependent manner. We have a novel appreciation of ways that adherent Gc can overcome nutritional immunity at epithelial surfaces and establish productive

infections. My work also raises many new questions and provides new avenues for future research, as will be discussed in **Chapter 3**.

Chapter 2 RESULTS reported in Infection and Immunity 2022

Adherence Enables Neisseria gonorrhoeae to Overcome Zinc Limitation Imposed by Nutritional Immunity Proteins

Adapted from Ray JC, Smirnov A, Maurakis SA, Harrison SA, Ke E, Chazin WJ, Cornelissen CN, Criss AK. Adherence enables *Neisseria gonorrhoeae* to overcome zinc limitation imposed by nutritional immunity proteins [published online ahead of print, 2022 Feb 14]. *Infect Immun*. 2022;iai0000922. doi:10.1128/iai.00009-22
(Ray, Smirnov et al. 2022)

Jocelyn C. Ray: Cloned *ΔtdfH, ΔtdfJ, ΔtdfH ΔtdfJ, ΔznuA, and znuA*^{C} into OpaD WT background. Conducted all media growth assays, metal repletion experiments, Ect1 infections, conditioned media experiments, transwell filter experiments, dead Ect1 cell experiments, glass coverslip experiments, RNA extraction, qPCR, Western blots, RNA extraction for RNAseq, and sample preparation for ICP-OES. Conducted additional analysis of RNAseq data after significant genes had been called.

Asya Smirnov: Helped design media growth curve experiments

Stavros A. Maurakis: Creation of *ΔznuA* and *znuA*^{C} constructs that were transformed into OpaD+ WT. Oversaw production of guinea pig anti-ZnuA serum.

Simone A. Harrison: Cloning of murine calprotectin, production of psoriasin, calprotectin, and murine calprotectin.

Eugene Ke: RNAseq read trimming, alignment and analysis.

Walter J. Chazin: Design and oversight of recombinant S100 proteins

Cynthia Nau Cornelissen: Funding and conceptualization

Alison K. Criss: Funding, conceptualization, mentorship of JCR and experimental design

2.1 Abstract

Neisseria gonorrhoeae (Gc) must overcome limitation of metals such as zinc to colonize mucosal surfaces in its obligate human host. While the zinc-binding nutritional immunity proteins calprotectin (S100A8/A9) and psoriasin (S100A7) are abundant in human cervicovaginal lavage fluid, Gc possesses TonB-dependent transporters TdfH and TdfJ that bind and extract zinc from the human version of these proteins, respectively. Here we investigated the contribution of zinc acquisition to Gc infection of epithelial cells of the female genital tract. We found that TdfH and TdfJ were dispensable for survival of strain FA1090 Gc that were associated with Ect1 human immortalized epithelial cells, when zinc was limited by calprotectin and psoriasin. In contrast, suspension-grown bacteria declined in viability under the same conditions. Exposure to murine calprotectin, which Gc cannot use as a zinc source, similarly reduced survival of suspension-grown Gc, but not Ect1-associated Gc. We ruled out epithelial cells as a contributor to the enhanced growth of cell-associated Gc under zinc limitation. Instead, we found that attachment to glass was sufficient to enhance bacterial growth when zinc was sequestered. We compared the transcriptional profiles of WT Gc adherent to glass coverslips or in suspension, when zinc was sequestered with murine calprotectin or provided in excess, from which we identified genes whose expression was increased by zinc sequestration in adherent Gc. One of these, *znuA*, was necessary but not sufficient for survival of Gc under zinc-limiting conditions. These results show that

adherence protects Gc from zinc-dependent growth restriction by host nutritional immunity proteins.

2.2 Introduction

Neisseria species have the remarkable ability to overcome nutritional immunity by expressing TdTs that directly bind to and extract transition metals from human metal-binding proteins, ultimately transporting the metal into the cytoplasm for incorporation into biomolecules (Cornelissen 2018, Yadav, Noinaj et al. 2019). Recently, the Criss and Cornelissen labs showed that TdfH and TdfJ acquire zinc from the human zinc-binding proteins calprotectin and psoriasin, respectively (Jean, Juneau et al. 2016, Maurakis, Keller et al. 2019). Both TdfH and TdfJ bind to their respective human S100 protein ligand and extract and internalize the zinc using a process that is dependent on TonB (**Section 1.3.2**) (Jean, Juneau et al. 2016, Maurakis, Keller et al. 2019, Kammerman, Bera et al. 2020). *TdfH* and *tdfJ* are expressed by Gc during infection of symptomatic women, and *tdfJ* is more highly expressed in Gc infecting women than men (McClure, Nudel et al. 2015, Nudel, McClure et al. 2018). Neutrophils are both the first responders to Gc infection and a major source of calprotectin *in vivo* (Edgeworth, Gorman et al. 1991, Johnson and Criss 2011). The Criss and Cornelissen labs previously showed that Δ*tdfH* Gc had reduced survival in neutrophil extracellular traps, which contain abundant calprotectin (Jean, Juneau et al. 2016).

In conditions of zinc abundance, zinc ions likely freely diffuse through Gc outer membrane porins (Kattner, Zaucha et al. 2013, Stork, Grijpstra et al. 2013) and are captured in the periplasm by binding to the zinc shuttle protein ZnuA

(**Section 1.5.1**)(Chen and Morse 2001). ZnuA then delivers zinc to ZnuB and ZnuC, which transport the ion into the cytoplasm (Chen and Morse 2001). Zinc acquisition is regulated in part by the zinc uptake repressor, Zur, a zinc-responsive transcriptional repressor (**Section 1.5.2**) (Pawlik, Hubert et al. 2012) (initially called PerR by (Wu, Seib et al. 2006)).

Colonization of the cervical epithelium is an important step in both asymptomatic infection by Gc and symptomatic gonorrhea in women (**Section 1.2**)(Song, Yu et al. 2020). Gc forms microcolonies on the cervical epithelium in a pilus-dependent manner (Higashi, Lee et al. 2007), and subsequently invades the transition zone and endocervical epithelium in a manner that is influenced by expression of Opa adhesion proteins (Yu, Wang et al. 2019). Colonization puts the bacteria in close proximity to human cells and is anticipated to change the physiology of the bacteria (Du, Lenz et al. 2005). Calprotectin and psoriasin are found in the ectocervix, as assessed by histology and transcript abundance (Mildner, Stichenwirth et al. 2010, Uhlen, Fagerberg et al. 2015, Bastian, Roux et al. 2021). Moreover, calprotectin and psoriasin are among the most abundant proteins detected in the cervical secretions of healthy women, and increase in abundance during inflammation (Zegels, Van Raemdonck et al. 2009, Birse, Burgener et al. 2013, Borgdorff, Gautam et al. 2016). The effect of zinc sequestration by calprotectin and psoriasin on the ability of Gc to colonize human cervical epithelium is unknown.

In this work, we tested the hypothesis that Gc requires TdfH and TdfJ to acquire zinc and effectively colonize the surface of Ect1 human immortalized ectocervical cells (Fichorova, Rheinwald et al. 1997) in the presence of the zinc-sequestering proteins calprotectin and psoriasin. Gc lacking *tdfH* and *tdfJ* had a >3 log growth defect in medium containing calprotectin and psoriasin, which was rescued with excess zinc. To our surprise, *ΔtdfH ΔtdfJ* Gc that was adherent to Ect1 cells in the same medium did not exhibit this zinc- and S100 protein-dependent reduction in viability. Similarly, wild-type Gc that were adherent to Ect1 cells had significantly better survival over time in medium containing murine calprotectin, which Gc cannot use as a zinc source, compared with bacteria grown planktonically (Kammerman, Bera et al. 2020). We ruled out the possibility that Ect1 cells were providing zinc to adherent Gc. Instead, we found that attachment of Gc to abiotic surfaces was sufficient to protect Gc from this zinc sequestration. RNAseq analysis revealed a unique cohort of genes that are statistically significantly differentially expressed by bacteria that are both attached and zinc-limited. These findings indicate that Gc in an adherent state is better adapted to conditions in which zinc is sequestered, enhancing bacterial survival when confronted with nutritional immunity.

2.3 Results

2.3.1 TdfH and TdfJ are required when calprotectin or psoriasin bind zinc.

We previously showed that Gc uses TdfH to grow in a chemically defined medium where zinc-loaded calprotectin is the only source of zinc, and uses TdfJ for growth with zinc-loaded psoriasin (Jean, Juneau et al. 2016, Maurakis, Keller et al. 2019, Kammerman, Bera et al. 2020). Here, we sought to assess how TdfH and TdfJ contributed to the ability of Gc to overcome nutritional immunity when sufficient zinc for growth is sequestered by calprotectin and psoriasin. These studies used keratinocyte serum-free medium (KSFM), which contains 1.98 μM zinc as measured by ICP-MS in the presence of growth factors, or 0.48 μM zinc without growth factors (**Table 2.3.1**).

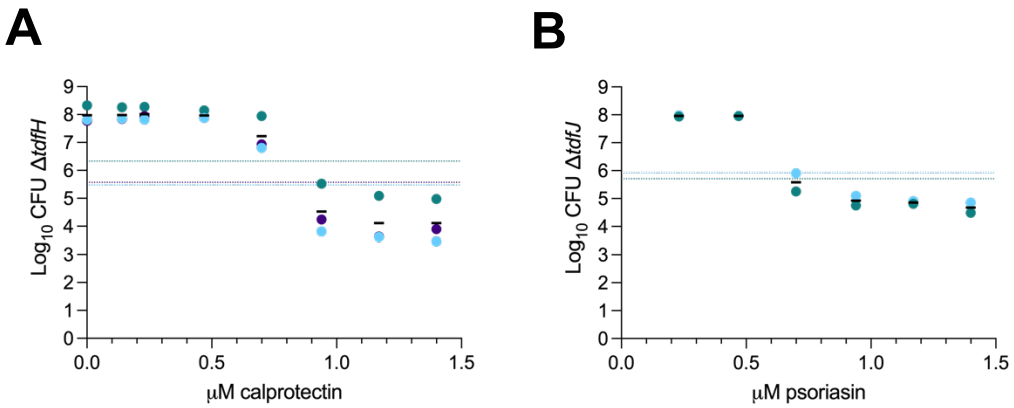
Table 2.3.1 Concentration of selected metals in KSFM by ICP-MS

	^{66}Zn µM	^{55}Mn µM	^{56}Fe µM	^{65}Cu µM
KSFM -GF	**0.48**	0.07	1.41	0.02
KSFM +GF	**1.98**	0.07	1.70	0.08

KSFM -GF: KSFM as supplied by the manufacturer
KSFM +GF: KSFM with added recombinant human epithelial growth factor and bovine pituitary extract.

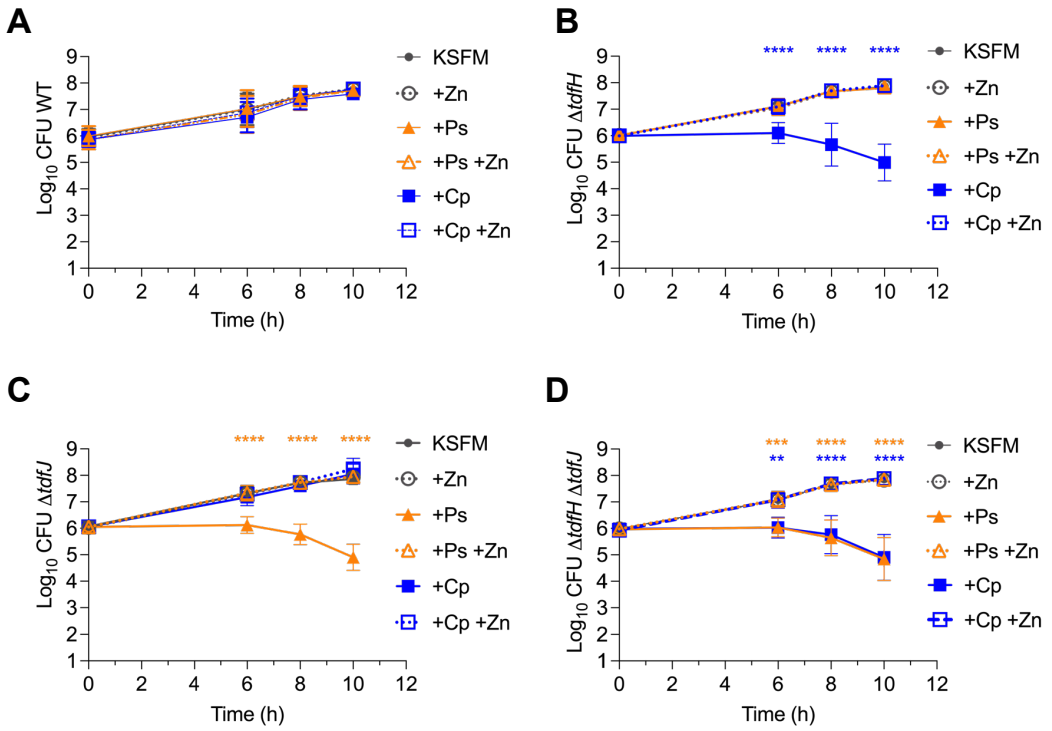
Complete KSFM containing growth factors was left untreated, or was supplemented with 1.4 µM each of recombinant human apo-calprotectin or apo-psoriasin to sequester zinc, with or without an additional 3 µM of $ZnSO_4$ to overcome zinc restriction by these proteins. 3 µM of $ZnSO_4$ was used because each S100 protein dimer contains two transition metal binding sites. 1.4 µM calprotectin and psoriasin were experimentally determined to be sufficient to achieve maximal growth restriction of *ΔtdfH* and *ΔtdfJ* Gc of strain FA1090 in KSFM containing growth factors, respectively **(Fig. 2.1.1)**.

Figure 2.1.1 Concentration dependence of the growth restriction of *ΔtdfH* by human calprotectin or *ΔtdfJ* by human psoriasin in KSFM with growth factors.
ΔtdfH (A) or *ΔtdfJ* (B) Gc (10^6 CFU/mL) was inoculated into KSFM with growth factors that was preincubated with the indicated concentration of calprotectin (A) or psoriasin (B). Data points are the Log_{10} transformation of CFU enumerated at 10 h. Dotted lines correspond to the inoculum for each biological replicate. Colors correspond to matched individual biological replicates; number of replicates: A, 3; B, 2.



Wild type (WT) Gc grew over time in KSFM, with CFU increasing ~60-fold over the 10 h time course **(Fig. 2.1.2A).** As expected, WT Gc grew similarly in KSFM, KSFM with added calprotectin or psoriasin, and KSFM with excess zinc, with or without calprotectin or psoriasin **(Fig. 2.1.2A)**. These results indicate: 1) the concentration of zinc in KSFM is sufficient to support WT Gc growth over time, 2) WT Gc can acquire metals from human nutritional immunity proteins in KSFM, and 3) the concentration of zinc added was not toxic for Gc. *ΔtdfH*, *ΔtdfJ*, and *ΔtdfH ΔtdfJ* Gc grew identically to WT in KSFM without psoriasin or calprotectin or with excess zinc **(Figs. 2.1.2B-D)**. The CFU of *ΔtdfH* Gc decreased ~10-fold relative to the inoculum after 10 h in KSFM containing calprotectin, but grew similarly to WT bacteria in medium without calprotectin or in KSFM containing the TdfJ ligand psoriasin **(Fig. 2.1.2B)**. Compared with conditions where the *tdfH* mutant could acquire zinc, *ΔtdfH* Gc in calprotectin-containing KSFM had a significant decrease in growth at 6, 8, and 10 h of incubation, with a ~2.8 log difference between these conditions at 10 h **(Fig. 2.1.2B).** Growth of *ΔtdfH* Gc in the presence of calprotectin was rescued when excess zinc was added **(Fig. 2.1.2B)**. Likewise, CFU of *ΔtdfJ* Gc significantly decreased over time in KSFM containing psoriasin, but not calprotectin or untreated medium, and psoriasin-restricted growth of *ΔtdfJ* Gc was rescued by adding excess zinc **(Fig. 2.1.2C)**. Moreover, compared with untreated KSFM, CFU of double mutant *ΔtdfH ΔtdfJ* Gc significantly declined in the presence of

Figure 2.1.2 TdfH and TdfJ are required for Gc to grow when zinc is sequestered by calprotectin and psoriasin.
A-D: Gc of the indicated genotype was inoculated in KSFM with growth factors (KSFM) alone (gray), or containing 1.4 μM human calprotectin (Cp; orange) or 1.4 μM human psoriasin (Ps; blue), with or without 3 μM $ZnSO_4$ (Zn) (solid lines, no Zn; dotted lines, + Zn). Genotypes are A, WT; B, *ΔtdfH;* C, *ΔtdfJ;* D, *ΔtdfH ΔtdfJ.* Data are presented as the log_{10} transformation of CFU recovered at the indicated time points. Error bars represent standard deviation. Asterisks indicate adjusted p-values from an ordinary two-way ANOVA with Tukey's multiple comparisons test, comparing the KSFM condition vs. +Cp (blue *) and/or +Ps (orange *). Data are from the following number of biological replicates: A, 3-5; B, 4-5; C, 4-7; D, 4-5. A-D: *=p<0.05; **=p<0.01; ***=p<0.001; ****=p<0.0001.

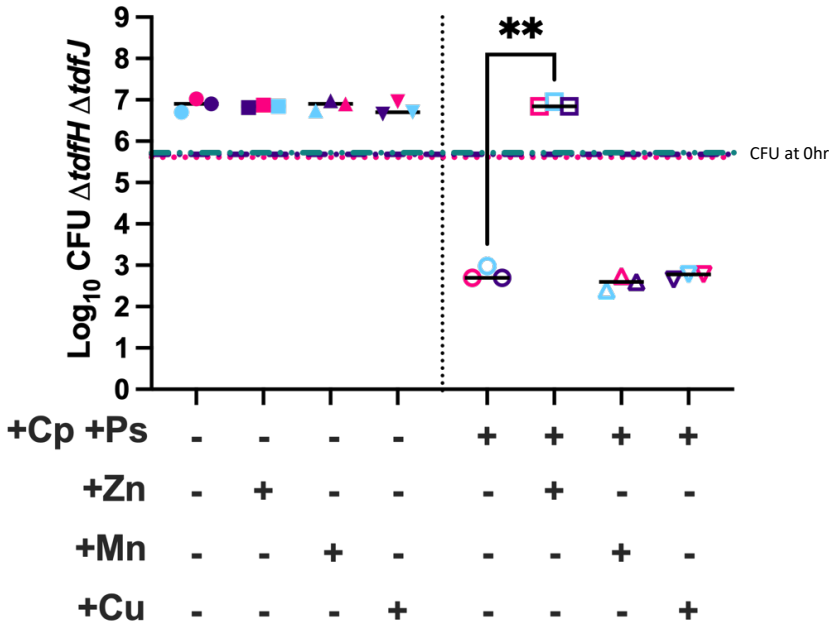


calprotectin or psoriasin, and was rescued in each case with the addition of excess zinc **(Fig. 2.1.2D)**.

These results are consistent with the hypothesis that Gc uses TdfH to overcome zinc sequestration by calprotectin, and TdfJ to acquire zinc sequestered by psoriasin. Growth of *ΔtdfH* Gc was not impaired in the presence

of psoriasin, and *ΔtdfJ* Gc was not impaired in the presence of calprotectin, which supports the concept that each TdT-ligand interaction is unique and specific. Calprotectin is known to bind metals other than zinc, specifically manganese and copper (Brophy, Nakashige et al. 2013, Diaz-Ochoa, Lam et al. 2016, Besold, Gilston et al. 2018). However, the reduction in *ΔtdfH ΔtdfJ* Gc CFU in KSFM containing calprotectin and psoriasin was only rescued with the addition of zinc, not manganese or copper **(Fig. 2.1.3)**.

Figure 2.1.3 Zinc, but not manganese or copper, rescues calprotectin- and psoriasin- mediated growth restriction of *tdfH tdfJ*.
E. *ΔtdfH ΔtdfJ* Gc was inoculated in KSFM with growth factors containing 1.4 μM Cp and 1.4 μM Ps (+Cp +Ps), alone or with 6 μM $ZnSO_4$ (+Zn), 6 μM $MnCl_2$ (+Mn), or 6 μM $CuCl_2$ (+Cu). Dotted line indicates the concentration of bacteria in the inoculum. CFU were enumerated 8 h later. Each color indicates one biological replicate. Asterisks indicate adjusted p-values from a one-way ANOVA with Šídák's multiple comparisons test. Data are from 3 biological replicates ****=p<0.0001.

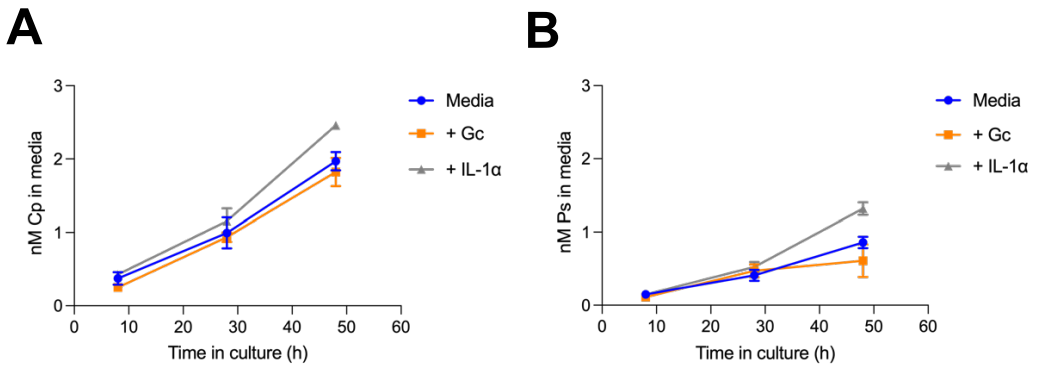


Taken together, these data show that Gc cultured in liquid medium depends upon TdfH and TdfJ to overcome calprotectin and psoriasin-mediated sequestration of zinc, respectively.

2.3.2 ΔtdfH ΔtdfJ Gc in contact with Ect1 cells is protected from calprotectin and psoriasin- mediated zinc sequestration.

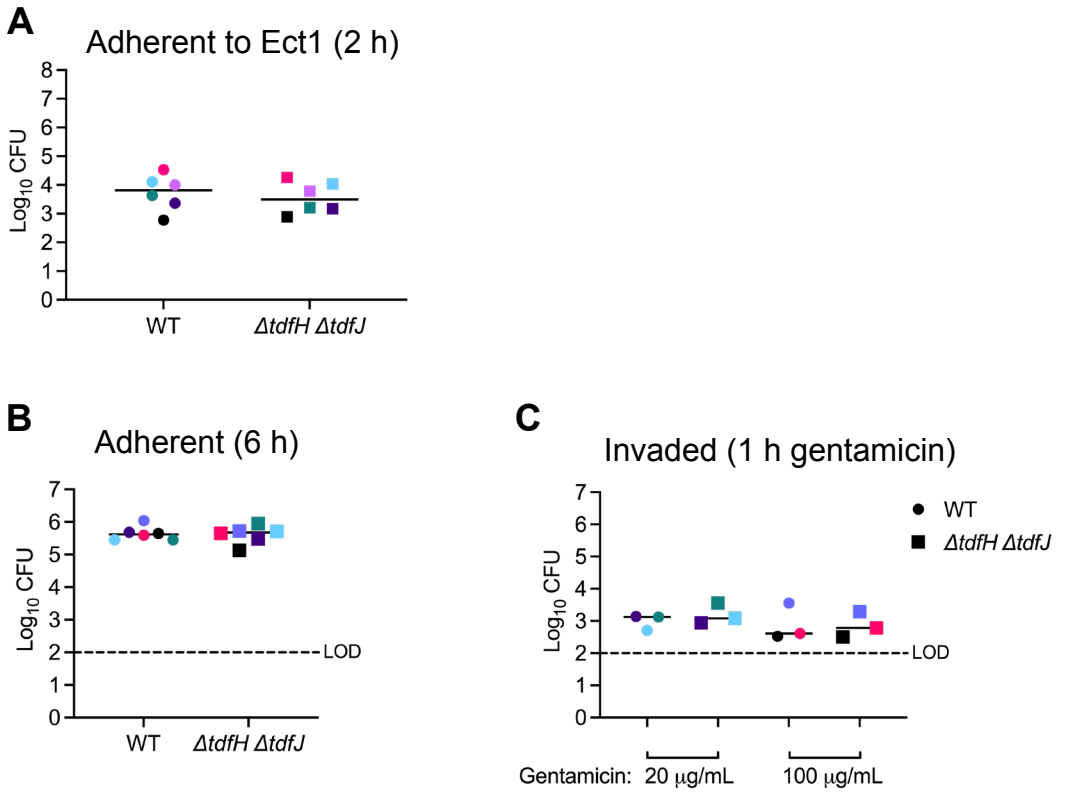
We next tested the hypothesis that Gc infection of lower female genital tract epithelial cells relies upon TdfH and TdfJ to acquire zinc from calprotectin and psoriasin. To do so, the immortalized ectocervical cell line Ect1 was infected with Gc (Fichorova, Rheinwald et al. 1997). Ect1 cells were selected because Gc has been shown to adhere to the apical membrane of Ect1 cells and grow in association with them (Fichorova, Desai et al. 2001), and cells of the human ectocervix, but not endocervix, express calprotectin and psoriasin (Mildner, Stichenwirth et al. 2010, Uhlen, Fagerberg et al. 2015, Bastian, Roux et al. 2021). However, Ect1 cells did not produce or secrete calprotectin or psoriasin to levels that would sequester the concentration of zinc that I measured in KSFM **(Fig. 2.2.1)**, and the concentration of these proteins released by Ect1 cells into conditioned medium was 1.5-2.5 logs lower than what has been reported in human cervicovaginal lavage fluid (Kunimi, Maegawa et al. 2006, Mildner, Stichenwirth et al. 2010). Addition of Gc or the proinflammatory cytokine IL-1α, which has been reported to increase production of calprotectin and psoriasin (Abtin, Eckhart et al. 2008, Bando, Hiroshima et al. 2010), did not increase secreted levels of these proteins **(Fig. 2.2.1).**

Figure 2.2.1 Limited secretion of calprotectin or psoriasin by Ect1 cells. Confluent Ect1 cells were grown in DMEM, alone (Media, blue), with infection by WT Gc (10^5 CFU/mL) (+Gc, orange), or with 30ng/mL IL-1α (+IL-1α, gray) for the duration of the time course. The concentration of calprotectin (Cp) (A) or psoriasin (Ps) (B) that was released into the media at 8, 24, and 48 h was quantified by ELISA. Values are shown as the mean +/- SD of three biological replicates.



To model the presence of calprotectin and psoriasin in human cervicovaginal secretions, we supplemented KSFM with calprotectin and psoriasin as in the *in vitro* growth assays above. Ect1 cells were infected with WT or *ΔtdfH ΔtdfJ* Gc in this medium for 2 h. Cells were washed to remove unbound bacteria, and CFU were enumerated from saponin lysates. There was no difference in the level of association between WT and *ΔtdfH ΔtdfJ* Gc after 2 h (**Fig. 2.2.2A**). To examine if the Gc was internalized by Ect1 cells, Gc were added to Ect1 cells for 6 h, then lysed to enumerate cell-associated CFU (adherent) or treated with gentamicin to kill extracellular Gc for an additional hour. At this time, on average 0.25% of cell-associated Gc were gentamicin-resistant (invaded) **(Fig. 2.2.2B, C),** and there was no difference in the number of adherent or invaded CFU between WT and *ΔtdfH ΔtdfJ* Gc (**Fig. 2.2.2B, C**). Thus, Gc colonizes but does not efficiently invade Ect1 cells; for this reason, cell-associated CFU will be referred to as “adherent.”

Figure 2.2.2 WT and *ΔtdfH ΔtdfJ* exhibit equivalent adherence to and invasion of Ect1 cells.
A. Confluent Ect1 cells were infected with 10^5 CFU/mL of WT (circles) or *ΔtdfH ΔtdfJ* (squares) Gc in KSFM with growth factors for 2 h. Data points indicate the Log_{10} transformed CFU enumerated that were associated with Ect1 cells at 2 h after washing. B-C. Ect1 cells ($5x10^5$/well) were infected with $2.5x10^6$ CFU of WT (circles) or *ΔtdfH ΔtdfJ* (squares) Gc in KSFM+GF. At 2 h, non-adherent Gc were washed away, and infection was allowed to proceed for 4 additional hours. At this time, nonadherent Gc were washed away and CFU were enumerated before (adherent; B) or after (invaded; C) 1 h of treatment with KSFM+GF containing 20 µg/mL or 100 µg/mL gentamicin. Colors indicate biological replicates conducted on the same day with same cell culture passage. Lines indicate the mean from the biological replicates (A, N=6; B, N=6; C, N=3). Differences were not significant by unpaired Student's t-tests.

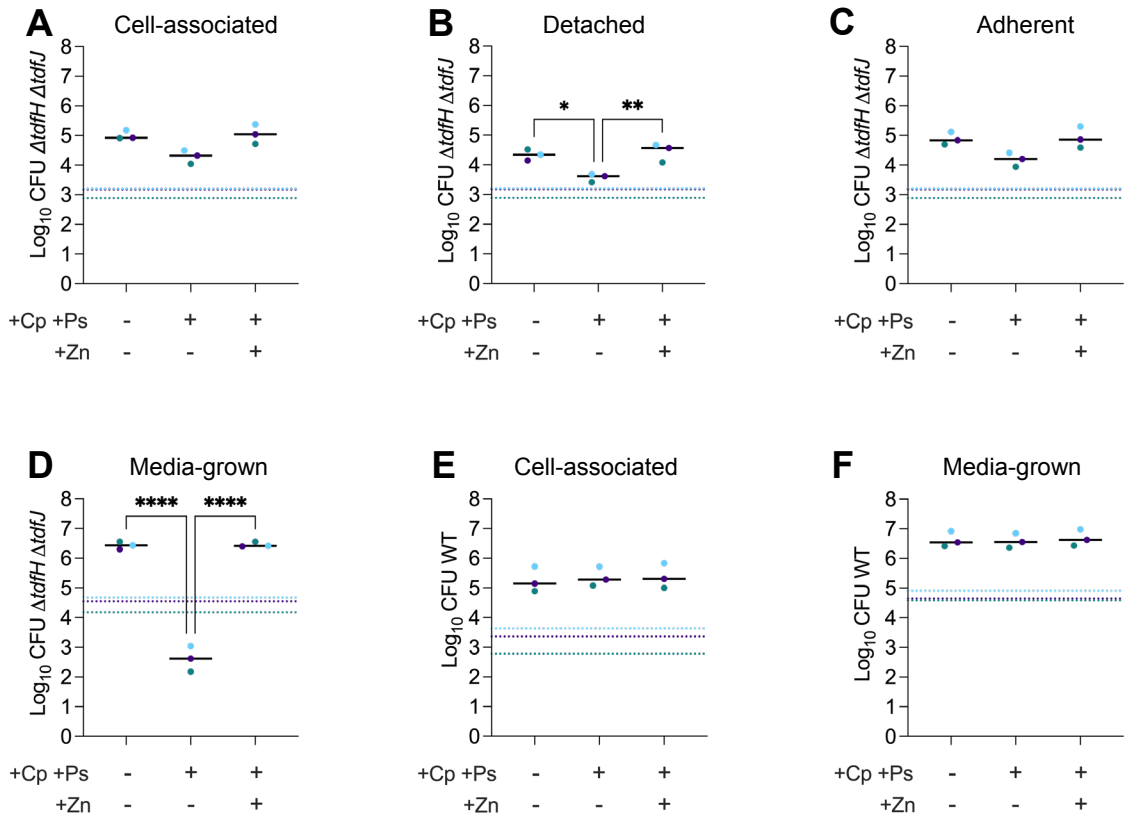


To examine how TdfH and TdfJ affected the ability of Gc to infect epithelial cells under zinc-limited conditions, Ect1 cells were infected with WT or *ΔtdfH ΔtdfJ* Gc in one of three conditions: in KSFM, KSFM with calprotectin and psoriasin, or KSFM with calprotectin, psoriasin, and $ZnSO_4$, using the conditions established for Gc in suspension in **Fig. 2.1.2**. After 2 h, cells were washed to remove non-adherent bacteria and replaced in medium with the same additives. The adherent Gc at 2 h served as the baseline against which outgrowth and growth decline were compared. After 9 h infection (2 h initial adherence plus 7 h incubation), the supernatant was collected (detached Gc) and the cells were lysed with saponin (adherent Gc), and CFU were enumerated from both. The sum of detached and adherent bacteria was termed “cell-associated Gc,” since the detached bacteria originated from the adherent bacteria at 2 h. The same Gc cultures were incubated in these media conditions without Ect1 cells, and CFU were enumerated after 9 h.

After 9 h infection, five-fold fewer cell-associated *ΔtdfH ΔtdfJ* Gc were recovered from Ect1 cells in the presence of calprotectin and psoriasin compared to infection in KSFM or with excess zinc, a statistically significant decrease **(Fig. 2.2.3A**). However, the numbers of *ΔtdfH ΔtdfJ* Gc were still 15-fold higher than after 2 h adherence (dotted lines, **Fig. 2.2.3A)**. The minimal decline of *ΔtdfH ΔtdfJ* Gc recovered from Ect1 cells in the presence of calprotectin and psoriasin occurred in both detached **(Fig. 2.2.3B)** and cell-adherent Gc **(Fig. 2.2.3C)**. In comparison, the CFU of *ΔtdfH ΔtdfJ* Gc in KSFM containing calprotectin and

psoriasin declined 70-fold compared to the inoculum, indicating overall death in the bacterial population under these zinc-limited conditions **(Fig. 2.2.3D).** Numbers of *ΔtdfH ΔtdfJ* Gc in KSFM containing calprotectin and psoriasin were 3.8 logs less than in KSFM or in KSFM with excess zinc, a statistically significant decrease **(Fig. 2.2.3D).** WT Gc grew similarly well in all conditions tested, regardless of association with Ect1 cells **(Fig. 2.2.3E, 2.2.3F)**. Thus, contrary to expectations, infection of Ect1 cells enhanced *ΔtdfH ΔtdfJ* Gc survival under conditions where zinc was sequestered by calprotectin and psoriasin.

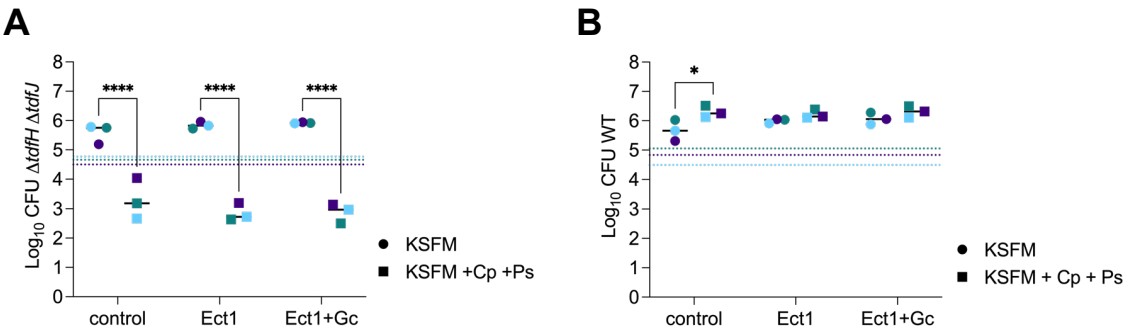
Figure 2.2.3 *ΔtdfH ΔtdfJ* Gc in contact Ect1 cells are protected from human calprotectin- and psoriasin- mediated zinc sequestration.
A-D: *ΔtdfH ΔtdfJ* Gc was inoculated into KSFM with growth factors alone, or with 1.4 μM human calprotectin (Cp) and 1.4 μM human psoriasin (Ps) with or without additional 3 μM $ZnSO_4$ (Zn). For A-C, Gc ($2.5x10^4$ CFU) was added to confluent Ect1 cells ($4x10^5$). After 2 h, unbound bacteria were washed away. For one set of wells, cells were lysed with saponin and CFU were enumerated from the lysate (dotted line). The remainder of the wells were replenished with fresh experimental media. After an additional 7 h, the media were collected (detached; B), the cells were lysed with saponin (adherent; C), and CFU were enumerated from both. Cell-associated (A) Gc is the sum of the detached and adherent Gc per experimental replicate. Each color represents an individual biological replicate completed on separate days. D, the same starting culture of *ΔtdfH ΔtdfJ* Gc was inoculated into the same batch of media in wells without Ect1 cells, where the bacteria do not attach (Media-grown). Each color indicates one biological replicate, and dotted line represents the inoculum for each replicate. E,F: The same conditions as in A and D were carried out, except using WT Gc. A-F: Asterisks indicate adjusted p-values from a one-way ANOVA with Tukey's multiple comparisons test; *= p<0.05; **=p<0.01; ****=p<0.0001.



2.3.3 Adherence is sufficient to protect *ΔtdfH ΔtdfJ* from zinc sequestration by human calprotectin and psoriasin. Live Ect1 cells are not required.

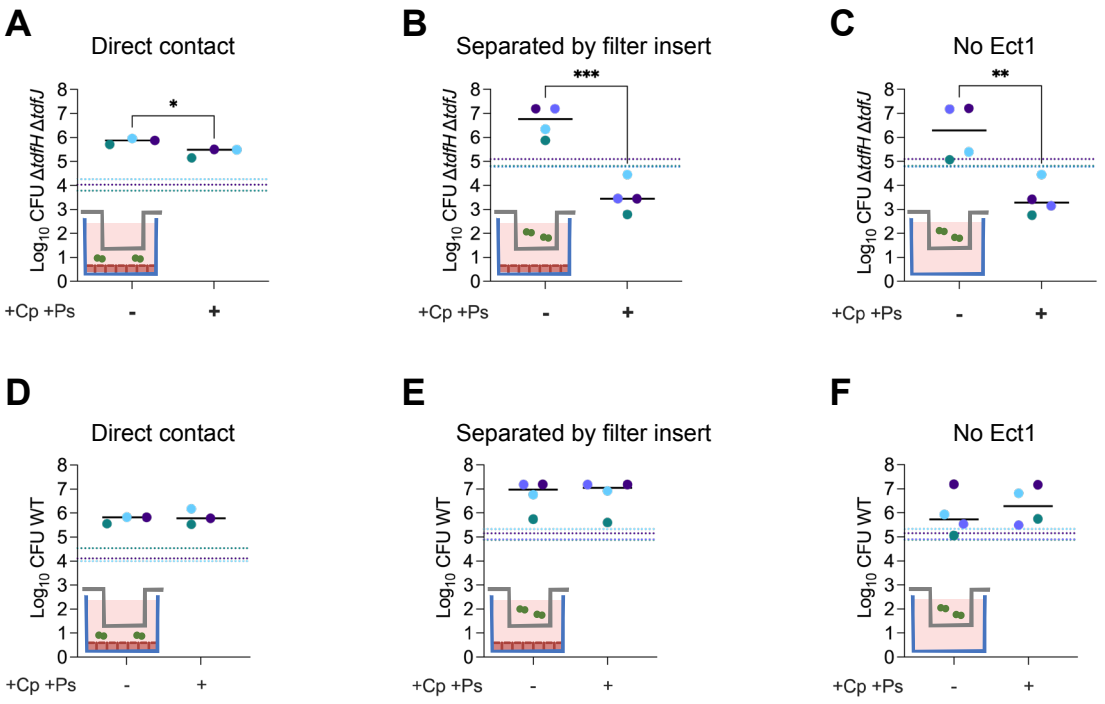
We investigated whether Ect1 cells played an active role in contributing to the increased survival of *ΔtdfH ΔtdfJ* Gc in the presence of psoriasin and calprotectin. To test if Ect1 cells altered the medium in such a way that supported Gc zinc-dependent growth, KSFM containing calprotectin and psoriasin was incubated at 37 °C for 7 h on Ect1 cells with or without *ΔtdfH ΔtdfJ* Gc infection, or without Ect1 cells for comparison. Medium without calprotectin and psoriasin incubated under these conditions was used as a control. Freshly grown *ΔtdfH ΔtdfJ* Gc was then inoculated into the cell-free media and CFU were enumerated at 7 h. *ΔtdfH ΔtdfJ* Gc significantly declined in all media conditions containing calprotectin and psoriasin, regardless of exposure to Ect1 cells, and was restored in unmodified media (**Fig. 2.3.1A**). WT Gc grew in all conditions over 7 h (**Fig. 2.3.1B**).

Figure 2.3.1 Protection of Gc from zinc sequestration is not replicated by Ect1-conditioned medium.
KSFM with or without 1.4 μM human calprotectin and 1.4 μM human psoriasin (+Cp +Ps) was incubated in wells without Ect1 cells (control), with confluent Ect1 cells (Ect1), or with confluent Ect1 cells infected with *ΔtdfH ΔtdfJ* Gc at an MOI of 0.5 (Ect1+Gc). After 7 h at 37 °C, 5% CO_2, the media were collected and sterile filtered. *ΔtdfH ΔtdfJ* (A) or WT (B) Gc (10^5 CFU/mL) was inoculated into each of the conditioned media (dotted line indicates inoculum), and CFU were enumerated after 7 h. Each color indicates one biological replicate. Asterisks indicate adjusted p-values from a one-way ANOVA with Šídák's multiple comparisons test. *= p<0.05****=p<0.0001



To further test the effect of Ect1 cells on the calprotectin- and psoriasin-mediated growth restriction of *ΔtdfH ΔtdfJ* Gc, Ect1 cells were grown in the lower reservoir of tissue culture wells containing 0.4 μm-pore Transwell filter inserts, and *ΔtdfH ΔtdfJ* Gc was inoculated in the upper reservoir. While direct contact with Ect1 cells protected *ΔtdfH ΔtdfJ* Gc from declining in CFU **(Fig. 2.3.2A, compare to Fig. 2.2.3A)**, *ΔtdfH ΔtdfJ* Gc separated from Ect1 cells by the semi-permeable membrane had a greater than 3-log reduction in CFU in the presence of calprotectin and psoriasin **(Fig. 2.3.2B)**. These results were nearly identical to *ΔtdfH ΔtdfJ* Gc in Transwell filter inserts when no Ect1 cells were present **(Fig. 2.3.2C)**. WT Gc was unaffected by the presence of calprotectin or psoriasin in each of these conditions, as expected **(Fig. 2.3.2D-F).** Taken together with the results with conditioned KSFM, these findings indicate that the medium from Ect1 cells is not sufficient to support growth of *ΔtdfH ΔtdfJ* Gc under zinc-limiting conditions imposed by calprotectin and psoriasin.

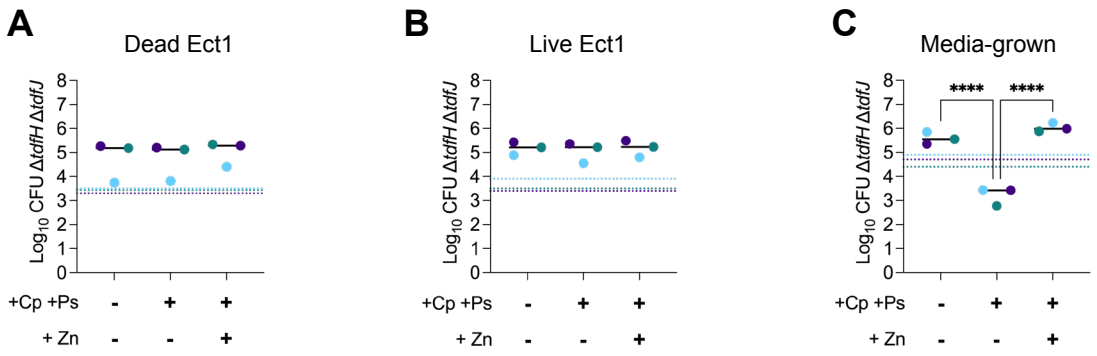
Figure 2.3.2 Protection of Gc from zinc sequestration requires Ect1 cell contact.
A-F: KSFM with or without 1.4 μM human calprotectin and 1.4 μM human psoriasin (+Cp +Ps) was added to wells containing confluent Ect1 cells (A,B,D,E) or no cells (C,F). 0.4 μm pore Transwell filter inserts were added to each well. *ΔtdfH ΔtdfJ* (A-C) or WT (D-F) Gc (10^5 CFU/mL) was inoculated into the lower (A,D) or upper (B,C,E,F) chamber of the filter insert. See diagram inserted in each graph that depicts the incubation conditions. For A and D, wells were washed and replaced with fresh media, using the conditions in **Figure 2.2.3**. CFU were enumerated 7 h later. Colors correspond to matched individual biological replicates, and dotted lines indicate the CFU present after 2 h (A,D) or the inoculum (B,C,E,F) for each replicate. Asterisks represent p-values from unpaired Student's t-tests. *= p<0.05**=p<0.01 ***=p<0.001



Based on these results, we hypothesized that contact with Ect1 cells is important for *ΔtdfH ΔtdfJ* Gc to overcome zinc limitation by calprotectin and psoriasin. To test whether the epithelial cells played an active role in this process, Ect1 cells were killed with paraformaldehyde and washed before infection with *ΔtdfH ΔtdfJ* Gc in KSFM. After 2 h of attachment, the cells were washed to remove unbound bacteria as in **Fig. 2.2.3,** and KSFM containing calprotectin and psoriasin, with or without excess zinc, or KSFM alone as a control was added for an additional 7 h. Gc adherent to paraformaldehyde-killed Ect1 cells did not decline in CFU over time in the presence of calprotectin and psoriasin **(Fig. 2.3.3A)**, which was similar to what was measured for the bacteria associated with live Ect1 **(Fig. 2.3.3B)**. In contrast, Gc in the same medium with calprotectin and psoriasin but without Ect1 cells declined over 2 logs in CFU compared with KSFM that was untreated or contained excess zinc **(Fig. 2.3.3C)**.

Together with the conditioned medium and Transwell experiments, we conclude that protection of *ΔtdfH ΔtdfJ* Gc from zinc limitation does not require an active contribution from the epithelial cells.

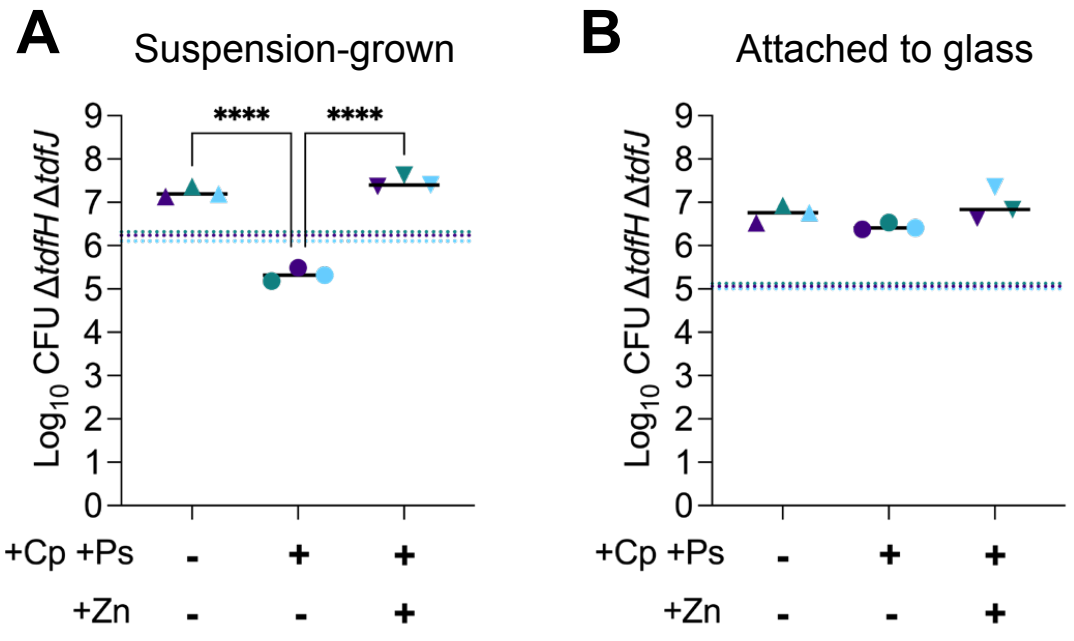
Figure 2.3.3 Adherence to dead Ect1 provides Gc with protection from zinc sequestration.
ΔtdfH ΔtdfJ (10^5 CFU/mL) was added to Ect1 cells that were alive (B) or killed with 4% paraformaldehyde (A). Cells were washed and replaced with fresh media after 2 h; dotted line indicates adherent CFU at 2 h. The media were KSFM alone, or with 1.4 μM human calprotectin and 1.4 μM human psoriasin (+Cp + Ps) with or without 3 μM $ZnSO_4$ (+Zn). For C, *ΔtdfH ΔtdfJ* (10^5 CFU/mL) was inoculated directly into the experimental media with no Ect1 cells; dotted line indicates the inoculum in the well. CFU were enumerated from cell lysates (A,B) or from the well (C) after 7 h. Colors correspond to matched individual biological replicates. Asterisks represent adjusted p-values from one-way ANOVA with Tukey's multiple comparisons test. ****=p<0.0001.



2.3.4 Gc attached to glass are protected from zinc sequestration.

To directly test whether the attachment-mediated protection of Gc from zinc limitation required epithelial cells, we measured the survival of *ΔtdfH ΔtdfJ* Gc in the presence of calprotectin and psoriasin when the bacteria were adherent to acid-washed glass coverslips (Merz, So et al. 2000). Here, WT Gc were allowed to adhere for 2 h to acid-washed glass coverslips, and non-adherent bacteria were washed away. After a further 7 h in the presence of experimental media, detached and adherent CFU were enumerated. As expected, *ΔtdfH ΔtdfJ* Gc grown in suspension when zinc was sequestered by calprotectin and psoriasin declined in CFU **(Fig. 2.4.1A)**. *ΔtdfH ΔtdfJ* Gc adherent to glass coverslips grew in the presence of calprotectin and psoriasin, and were equivalent in CFU to coverslip-associated bacteria in KSFM that was unmodified, or contained calprotectin, psoriasin, and excess zinc **(Fig. 2.4.1B)**. Zinc concentration in the medium did not increase with the addition of glass coverslips, suggesting that the glass was not a source of free zinc (by ICP-OES: 0.86 μM zinc when incubated in empty wells, 0.88 μM zinc when incubated with acid-washed glass coverslips). Taken together, these results show that Gc attached to surfaces, whether biotic (Ect1 cells) or abiotic (coverslips), do not demonstrate the reduction in viability that is imposed by zinc-binding proteins for bacteria grown in suspension.

Figure 2.4.1 Attachment to glass coverslips protects *ΔtdfH ΔtdfJ* Gc from zinc sequestration by human calprotectin and human psoriasin. A. *ΔtdfH ΔtdfJ* Gc (10^6 CFU/mL) was inoculated into experimental media: KSFM without growth factors, alone or with addition of 1.5 μM human calprotectin and 1.5 μM human psoriasin (+Cp +Ps) and/or 6 μM $ZnSO_4$ (+Zn) and grown without adherence for 7 h. dotted lines indicated inoculum CFU for each experiment. B. *ΔtdfH ΔtdfJ* Gc (10^6 CFU/mL) in KSFM was inoculated onto acid-washed glass coverslips for 2 h. Dotted lines indicate the CFU adherent after 2 h. Non-adherent Gc was washed away, and Gc was incubated an additional 7 h in experimental media. Each color indicates one biological replicate. Asterisks represent p-values from one-way ANOVA with Tukey's multiple comparisons test. ****=p<0.0001.

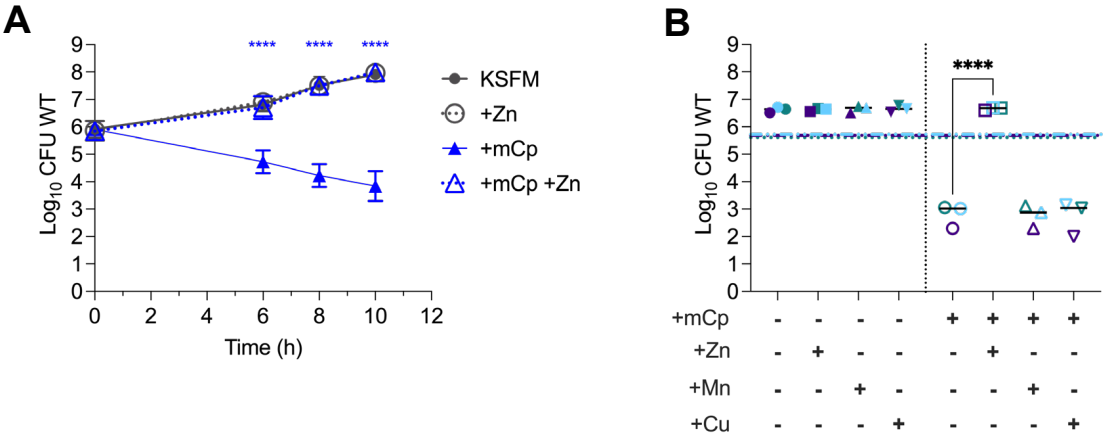


2.3.5 Murine calprotectin sequesters zinc from WT Gc.

The above results led us to propose a new hypothesis: that adherence enhanced the ability of Gc to survive in zinc-limiting conditions. We sought to test this hypothesis in a WT bacterial background in which potential mechanisms could be more easily screened than in *ΔtdfH ΔtdfJ* Gc with addition of human calprotectin and psoriasin. Previous work from our group showed that TdfH+ Gc can acquire zinc from human but not murine calprotectin (Kammerman, Bera et al. 2020). Therefore, we measured the effect of adding murine calprotectin on growth of WT Gc. The viability of WT Gc significantly declined over time when grown in suspension in KSFM in the presence of murine calprotectin compared to medium that was unmodified, or contained murine calprotectin and excess zinc, reaching a 4 log difference at 10 h post-inoculation (**Fig. 2.5.1A**). The reduction in CFU imposed by murine calprotectin was rescued by the addition of excess zinc, but not manganese or copper (**Fig. 2.5.1A-B**). Therefore, murine calprotectin restricts growth of WT Gc in a manner that phenocopies the effect of human calprotectin on *ΔtdfH* Gc **(Fig. 2.1.2B).**

Figure 2.5.1 Murine calprotectin restricts the zinc-dependent growth of wild type Gc in suspension

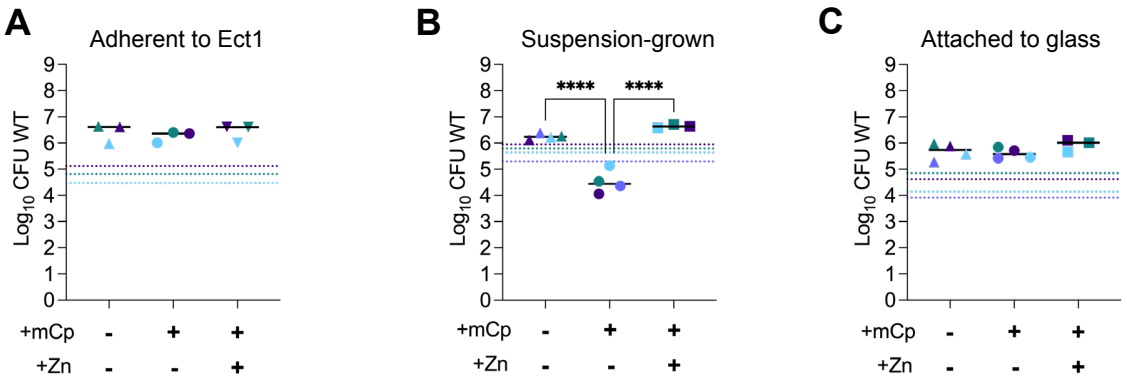
A: WT Gc (10^6 CFU/mL) was grown in KSFM containing growth factors, alone or with addition of 2 μM murine calprotectin (+mCp) and/or 6 μM $ZnSO_4$ (+Zn). Data are presented as the log_{10} transformation of CFU recovered at the indicated time point. Data points represent the mean of three independent experimental replicates. Asterisks represent adjusted p-values from a two-way ANOVA with Tukey's multiple comparisons test. KSFM condition with KSFM +mCp. B: WT Gc was grown as in A with or without 2 μM murine calprotectin (+mCp), 6 μM $ZnSO_4$ (+Zn), 6 μM $MnCl_2$ (+Mn), or 6 μM $CuCl_2$ (+Cu). Data points indicate the log_{10} CFU recovered after 8 h. Dotted line = inoculum. Asterisks represent adjusted p-values by one-way ANOVA with Šídák's multiple comparisons test. ****=p<0.0001.



We then tested whether WT Gc were protected from zinc limitation imposed by murine calprotectin during infection of Ect1 cells. Here, Ect1 cells were infected with WT Gc as described in **Fig. 2.2.3** (2 h infection, wash, 7 h chase), except that the KSFM contained murine calprotectin with or without excess zinc. WT Gc in the presence of murine calprotectin survived no differently after adherence to Ect1 cells than bacteria without calprotectin, or when excess zinc was added (**Fig. 2.5.2A**), while the same bacteria grown in medium containing murine calprotectin but without Ect1 cells showed an almost 2-log decrease in CFU (**Fig. 2.5.2B**). Furthermore, when WT Gc were allowed to adhere to acid-washed glass coverslips as described above in **(Fig. 2.4.1),** adherent Gc grew in the presence of murine calprotectin and were equivalent in CFU to coverslip-associated bacteria in KSFM that was unmodified or contained murine calprotectin and excess zinc **(Fig. 2.5.2C)**. These results show that the effect of adherence on Gc susceptibility to zinc restriction is not limited to *ΔtdfH ΔtdfJ* Gc incubated with human calprotectin and psoriasin. The ability of WT Gc to be rescued from zinc sequestration when adherent to either epithelial cells or glass coverslips suggests a broader, TdfH- and TdfJ-independent mechanism.

Figure 2.5.2 Murine calprotectin zinc restriction is overcome by adherence

A and C, WT Gc (10^6 CFU/mL) in KSFM was inoculated onto confluent Ect1 cells (A) or acid-washed glass coverslips (C) for 2 h. Dotted lines indicate the CFU adherent after 2 h. Non-adherent Gc was washed away, and Gc was incubated an additional 7 h in experimental media: KSFM with growth factors, alone or with addition of 3 μM murine calprotectin (+mCp) and/or 6 μM $ZnSO_4$ (+Zn). B, Gc was inoculated into experimental media without adherence and incubated for 7 h. For B, dotted lines indicated inoculum CFU for each well. Each color indicates one biological replicate. Asterisks represent p-values from one-way ANOVA with Tukey's multiple comparisons test. ****=p<0.0001.

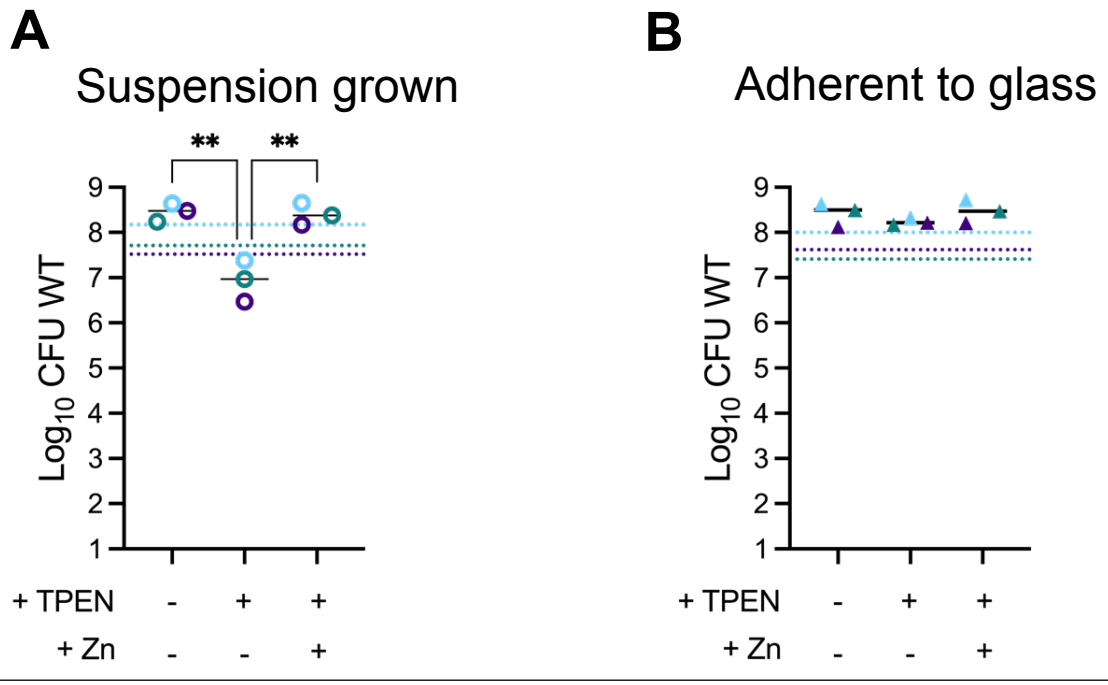


2.3.6 Gc total zinc content could not be conclusively quantified

We hypothesized that adherent bacteria might be more adept at taking up or retaining zinc than their suspension-grown counterparts. To test this, the assay was modified to prepare Gc for quantification of zinc by ICP-MS. The assay was scaled up to produce sufficient biomass for analysis, using a whole 6-well plate for each condition. To conserve murine calprotectin, the commercially available zinc chelator TPEN was used. WT Gc was added ($1x10^8$ CFU/mL) to 1 mL of KSFM with or without 2 μM TPEN, with or without 4 μM $ZnSO_4$.

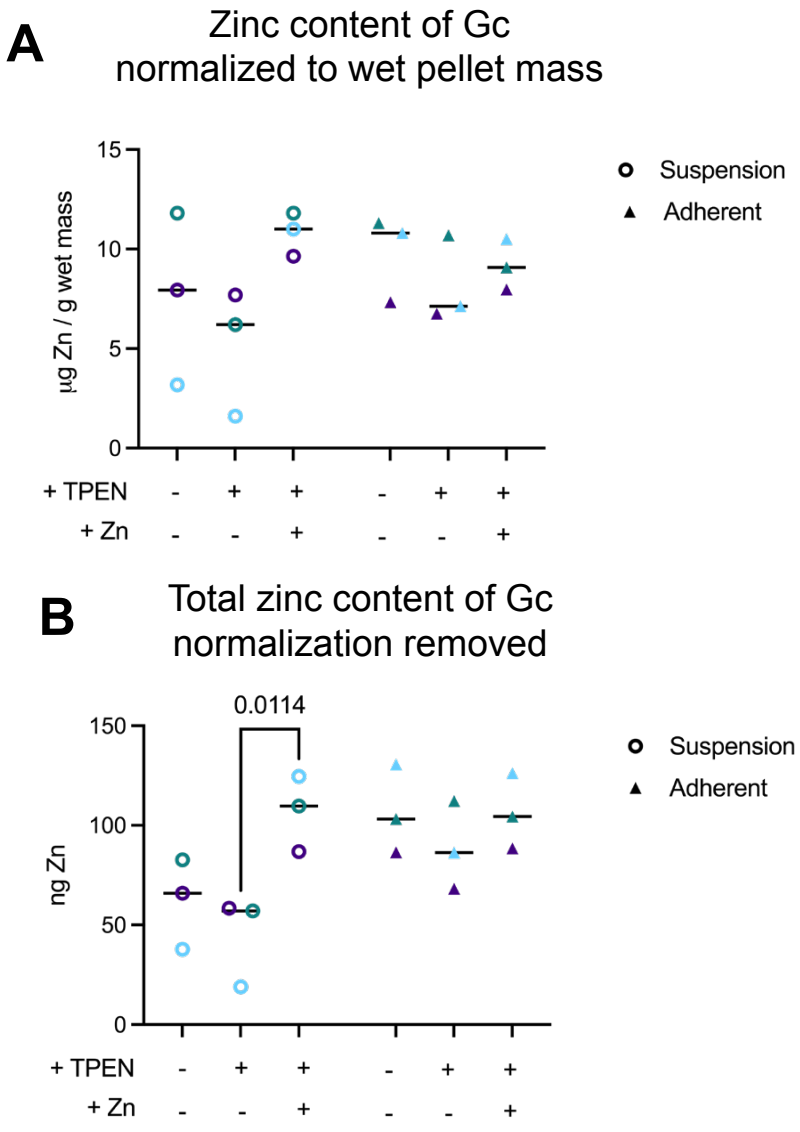
Importantly, suspension WT Gc died after 7 h of growth when zinc was limited by 2 μM TPEN and were rescued with the addition of excess zinc **(Figure 2.6.1A)**. By comparison, Gc that were allowed to adhere for 2 h to 25mm acid-washed glass coverslips survived 7 h in the presence of 2 μM TPEN **(Figure 2.6.1B)**.

Figure 2.6.1 Glass-adherent WT Gc are protected from TPEN-mediated zinc sequestration.
A. WT Gc (10^8 CFU/mL) was inoculated into experimental media: KSFM without growth factors, alone or with addition of 2 μM TPEN (+TPEN) and/or 4 μM $ZnSO_4$ (+Zn) and grown without adherence for 7 h. dotted lines indicated inoculum CFU for each experiment. B. WT Gc (10^8 CFU/mL) in KSFM was inoculated onto acid-washed glass coverslips for 2 h. Dotted lines indicate the CFU adherent after 2 h. Non-adherent Gc was washed away, and Gc was incubated an additional 7 h in experimental media. Each color indicates one biological replicate. Asterisks represent p-values from one-way ANOVA with Tukey's multiple comparisons test. A: **=p<0.1.



The suspension-grown and adherent Gc were collected after 4 h in their experimental media, pellets were washed with mili-Q H_2O, and pellets were sent for ICP-MS quantification of their zinc content, normalized to the wet mass of the pellet once all visible water was aspirated **(Figure 2.6.2A).** Normalization was done to account for potential differences in the biomass of the input, but accuracy of the pellet masses was low. To reflect this uncertainty, de-normalized total zinc content is also reported **(Figure 2.6.2B)**. In the normalized samples, there is no significant difference between any two conditions tested, and great variability between trials. Due to this variability, no definite conclusions could be made.

Figure 2.6.2 No significant differences were detected in zinc content between adherent and suspension Gc.
Suspension-grown and adherent Gc were grown as in **Fig. 2.6.1**, but collected after 4 h in experimental media. Pellets were washed and processed for ICP-MS quantification of zinc content. A: zinc content of Gc, normalized to pellet mass. B: total zinc content of Gc, without normalization. P-value represents adjusted p-value from ordinary one-way ANOVA with Tukey's multiple comparisons test.

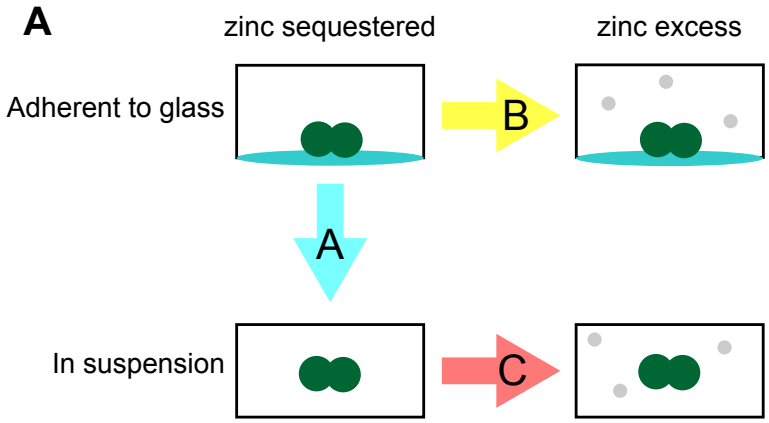


2.3.7 Adherent Gc gene expression changes under conditions of zinc sequestration.

To understand how attachment enables Gc growth under zinc-limited conditions, we took the unbiased approach of RNA-sequencing. RNA was collected from WT Gc that were adherent to glass coverslips (adh) or maintained in suspension (sus), using KSFM containing murine calprotectin (sequestered; seq) or KSFM containing murine calprotectin and excess zinc (excess) **(Fig. 2.7.1A)**. Glass coverslips were used instead of Ect1 cells so that there was no human RNA present. Suspension bacteria were inoculated in the experimental media and collected after 4 h. Adherent bacteria were allowed to attach to acid-washed glass coverslips for 2 h in unmodified KSFM. Coverslips were washed once with sterile PBS to remove nonadherent bacteria and the experimental media were added. After 4 h, adherent bacteria were collected and cell pellets were processed to purify RNA and processed for Illumina sequencing (see Materials and Methods). For each experiment, we confirmed that Gc exposed to murine calprotectin exhibited zinc-dependent growth decline over time, but CFU at the time of collection for RNA harvest was equivalent **(Fig. 2.7.2).**

Figure 2.7.1 Zinc- and adherence-dependent transcriptional responses of Gc.

A: Schematic of RNAseq experimental conditions and pairwise comparisons. WT Gc was inoculated into wells containing glass coverslips and allowed to adhere for 2 h (Gc adherent to glass; adh). Nonadherent CFU were washed away, and KSFM containing 3 μM murine calprotectin (zinc sequestered; seq), or 3 μM murine calprotectin with 6 μM $ZnSO_4$ (zinc excess; excess) was added. Alternatively, WT Gc was inoculated directly into wells without coverslips (Gc in suspension; sus) in the same media with sequestered and excess zinc. After 4 h, bacteria were collected, and RNA was extracted from all four conditions and subjected to RNAseq for each of 3 biological replicates. Comparison A indicates the 549 transcripts differentially expressed in adherent sequestered vs. suspension sequestered (blue). Comparison B indicates the 53 transcripts differentially expressed in adherent sequestered vs. adherent excess (yellow). Comparison C indicates the 90 transcripts differentially expressed in suspension sequestered vs. suspension excess (red). B: Diagram representing the transcripts that are differentially expressed in the same direction between the pairwise comparisons schematically represented in (A). transcripts that were not differentially expressed, or were differentially expressed in different directions between pairwise comparisons, are not represented.



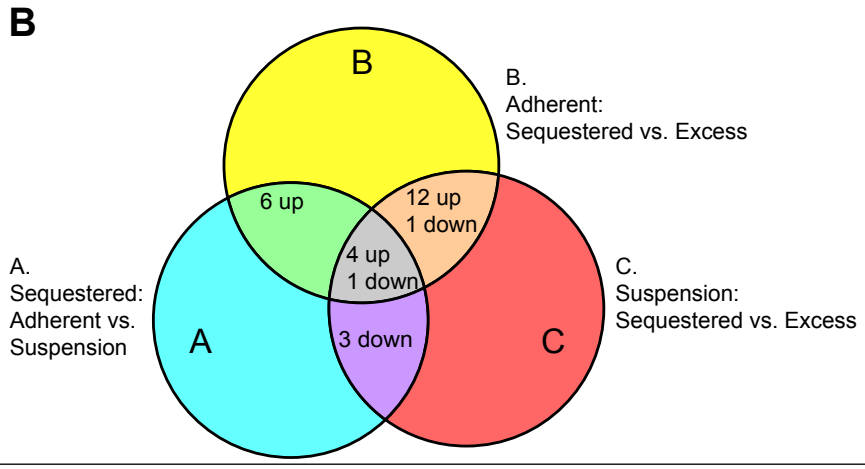
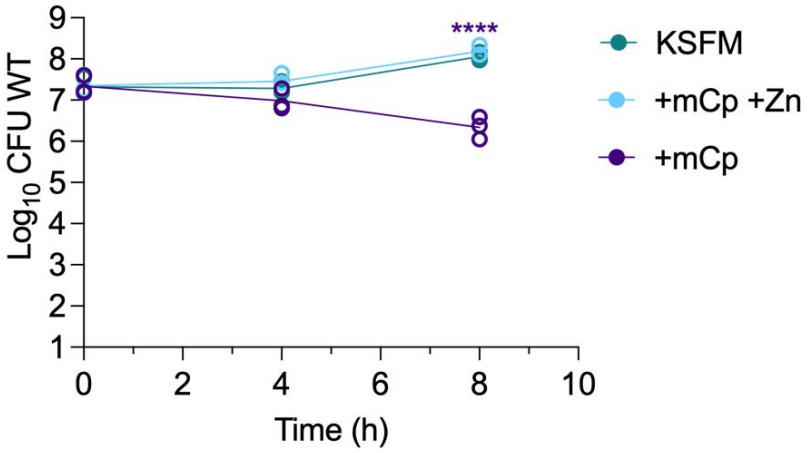


Figure 2.7.2 Growth curve control for RNAseq experiment
WT Gc was inoculated in 96-well round-bottom plates ($1x10^8$ CFU/mL) into KSFM-GF alone, with 3 μM mouse calprotectin (+mCp), or with 3 μM mouse calprotectin and 6 μM $ZnSO_4$ (+mCp +Zn). Gc was incubated at 37 °C in 5% CO_2. At 0 h, 4 h, and 8 h, CFU were enumerated. Samples for RNAseq were cultured in the same media with mCp and mCp +Zn on the same day. KSFM-GF alone was included as a control for overall viability of the cultures. Asterisks represent adjusted p-values from two-way ANOVA with multiple comparisons. ****=p<0.0001 comparing KSFM +mCp vs. KSFM +mCP +Zn.



Over 500 transcripts were differentially abundant between adherent and suspension-grown Gc in the presence of murine calprotectin **(Fig. 2.7.1B).** From these transcripts, we identified the transcripts that were associated with adherence as well as with zinc sequestration **(Table 2.3.7).** The differential expression of these transcripts was examined for three experimental comparisons: the effect of zinc limitation on adherent Gc (adh seq vs. adh excess); the effect of zinc limitation on suspension Gc (sus seq vs. sus excess); and the effect of adherence in zinc-limited conditions (adh seq vs. sus seq) **(Figure 2.7.1 A)**.

FA1090 gene ID[a]	Symbol[b]	Zn repressed[c]	L2FC[d] in A (adh seq vs. sus seq)	L2FC in B (adh seq vs. adh excess)	L2FC in C (sus seq vs. sus excess)	Definition[e]
Increased in A, B, and C						
NGO_0930	RpmE2	**(+)**	0.93	3.34	2.26	50S ribosomal protein L31
NGO_1049		**(+)**	0.60	2.33	1.46	hypothetical periplasmic zinc binding protein
NGO_0168	ZnuA	**(+)**	0.50	1.33	0.73	zinc ABC transporter substrate-binding protein
NGO_0217	FbpA		0.81	0.88	0.64	iron ABC transporter substrate-binding protein
Decreased in A, B, and C						
NGO_1442	AdhP	**(-)**	-0.81	-0.78	-1.42	alcohol dehydrogenase
Increased in A and B						
NGO_0340	CysK		1.97	0.55	*ns*	cysteine synthase
NGO_0374	GlnQ		1.47	0.62	*ns*	amino acid ABC transporter ATP-binding protein
NGO_1318	HemO		0.75	0.74	*ns*	heme oxygenase
NGO_0554			0.68	1.08	*ns*	hypothetical protein
NGO_1368	MtrF		1.53	0.52	*ns*	aminobenzoyl-glutamate transporter
NGO_0639	LldD		0.79	0.72	*ns*	L-lactate dehydrogenase
Increased in B and C						
NGO_1370			*ns*	2.51	1.68	membrane protein
NGO_1205	TdfJ	**(+)**	*ns*	2.03	1.53	TonB-dependent receptor protein
NGO_0166	HmcD	**(+)**	*ns*	1.42	1.16	hypothetical protein (possible periplasmic protein)
NGO_1378	ExbB		*ns*	1.05	0.94	biopolymer transporter ExbB
NGO_1496	TbpB		*ns*	1.00	0.78	transferrin-binding protein B
NGO_1495	TbpA		*ns*	0.82	0.63	transferrin-binding protein A
NGO_0322			*ns*	0.73	1.35	hypothetical protein
NGO_0704	RibB		*ns*	0.66	0.63	3,4-dihydroxy-2-butanone 4-phosphate synthase
NGO_1029	FumC		*ns*	0.64	0.72	fumarate hydratase
NGO_0753	NarX		*ns*	0.60	0.54	two-component system sensor kinase NarX/NarQ
NGO_1688	OmpU		*ns*	0.56	0.54	hypothetical protein
NGO_0952	TdfH	**(+)**	*ns*	0.88	*0.49*[f]	Ton-B dependent receptor protein
Not significantly changed in A, B, or C						
NGO_0129	QueC	**(+)**	*ns*	*ns*	*ns*	7-cyano-7-deazaguanine synthase
NGO_0169	ZnuB	**(+)**	*ns*	*ns*	*ns*	zinc ABC transporter permease
NGO_0170	ZnuC	**(+)**	*ns*	*ns*	*ns*	zinc ABC transporter ATP-binding protein
NGO_0931	RpmJ2	**(+)**	ND	ND	ND	50S ribosomal protein L36

Table 2.3.7 Annotated list of Gc open reading frames responsive to zinc and/or adherence as analyzed by RNA-seq.

[a] by alignment to ASM684v1.51
[b] Gene symbols from ASM684v1.51, (StdGen 2003), and literature search.
[c] according to (Wu, Seib et al. 2006) or (Pawlik, Hubert et al. 2012). **(+)** indicates Zn or Zur repressed, **(-)** indicates Zn or Zur induced.
[d] L2FC = Log_2 fold change. Positive values: higher in the first than the second condition. Negative values: lower in the first condition. Letters A, B, and C refer to comparisons described in **Figure 2.7.1**
ns = not significantly changed (adjusted p>0.05 or |L2FC|<0.5)
ND = not detected using the parameters of this study (read count was zero)
[e] (DeJong 2018) using genome: g2d_mirror/Neisseria_gonorrhoeae_FA_1090/ASM684v1_genomic
[f] TdfH had a L2FC less than the 0.5 cutoff in sus seq vs. sus excess

Four transcripts were more abundant in all three comparisons. Three of these transcripts are known to be zinc-repressed and regulated by Zur: the periplasmic zinc shuttle protein ZnuA (NGO_0168), a zinc-regulated hypothetical periplasmic protein (NGO_1049), and a zinc-independent ribosomal subunit RpmE2 (NGO_0930) (Wu, Seib et al. 2006, Pawlik, Hubert et al. 2012). One gene was reduced in abundance in all of the aforementioned comparisons, AdhP (NGO_1442), which has been previously described as induced under high zinc concentrations and in the presence of Zur (Wu, Seib et al. 2006, Pawlik, Hubert et al. 2012). Transcripts for an additional six ORFs were higher with adherence under zinc sequestered conditions (adh seq vs. sus seq) and with zinc sequestration when adherent (adh seq vs. adh excess), but not with zinc sequestration in suspension (sus seq vs. sus excess). None of these genes have previously been reported to be zinc regulated (Wu, Seib et al. 2006, Pawlik, Hubert et al. 2012). Finally, transcripts for 12 ORFs were increased with zinc sequestration when adherent (adh seq vs. adh excess) and when in suspension (sus seq vs. sus excess), but not significantly changed with adherence under zinc sequestered conditions (adh seq vs. sus seq). Three of these ORFs were previously identified as Zur-repressed in Gc (Wu, Seib et al. 2006): *tdfJ* (NGO_1205), *tdfH* (NGO_0952), and *hmcD* (NGO_0166). We had anticipated that all of the genes previously described as Zur-regulated would be differentially abundant in at least one of the zinc sequestered vs. excess conditions (Wu, Seib et al. 2006). However, three ORFs previously described as Zur-repressed in Gc

were not differentially expressed in sequestered vs. excess zinc: *znuB* (NGO_0169), *znuC* (NGO_0170), and *rpmJ2* (NGO_0931). Additionally, three ORFs previously described as zinc-repressed in *N. meningitidis* were also not differentially abundant when zinc was sequestered in these conditions: *queC* (NGO_0129), *queF* (NGO_1684), and NGO_1685. However, *queF* and NGO_1685 were increased with adherence under zinc sequestered conditions (adh seq vs. sus seq). These results indicate that the transcriptional response of Gc to zinc sequestration is distinct between suspension-grown and adherent bacteria, involving genes whose products are important for zinc acquisition as well as differential expression of metabolic genes not previously linked to zinc homeostasis. We next focused on investigating the effect of genes that were differentially expressed in adherent vs. suspension conditions when zinc was sequestered, and that were previously known to be involved in zinc acquisition. The primary candidate gene that fit these requirements was *znuA*.

2.3.8 ZnuA is not sufficient for Gc to survive under zinc-limiting conditions.

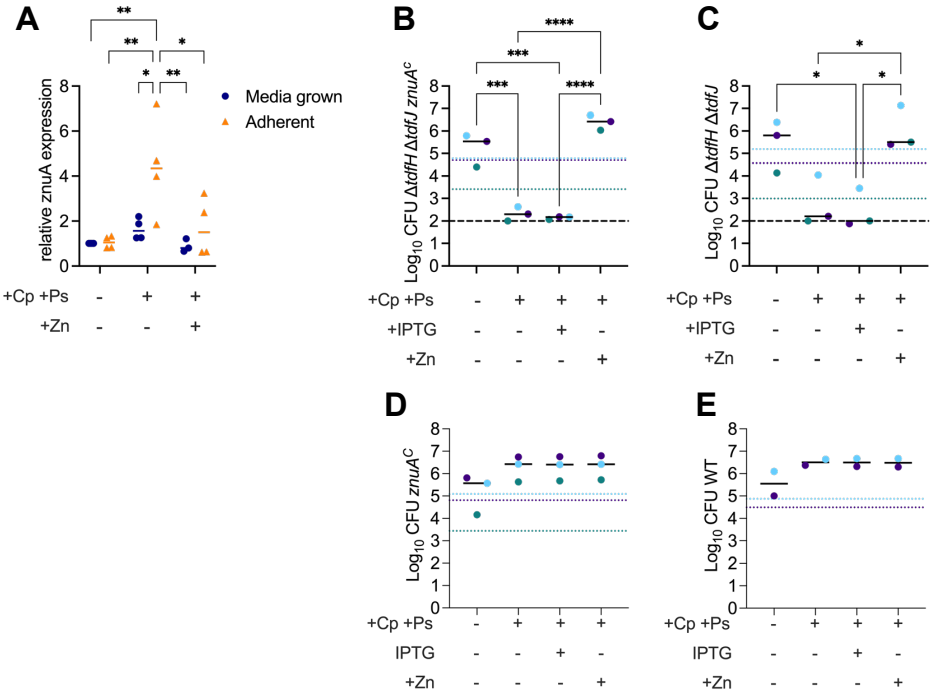
Transport of zinc into the cytoplasm in Gc is mediated by the tripartite ZnuABC system (Chen and Morse 2001). By RNA-seq, *znuA* was upregulated in both suspension and adherent Gc when zinc was sequestered, and was significantly higher in adherent than suspension zinc-sequestered bacteria. These findings were further investigated by qPCR using RNA harvested from Ect1 cells that were infected for 4 h with WT Gc in the presence of human calprotectin and psoriasin. Transcript abundance of *znuA,* normalized to 5S

rRNA, was significantly greater in adherent relative to media-grown Gc **(Fig. 2.8.1A)**. Since ZnuA or ZnuABC has been implicated in other pathogens as a primary means of overcoming calprotectin-mediated zinc sequestration (Liu, Jellbauer et al. 2012, D'Orazio, Mastropasqua et al. 2015, Hesse, Lonergan et al. 2019), these observations led us to hypothesize that adherent Gc increases production of ZnuA as a way to overcome zinc limitation.

Figure 2.8.1 *znuA* overexpression is not sufficient for Gc in suspension to overcome zinc-limitation

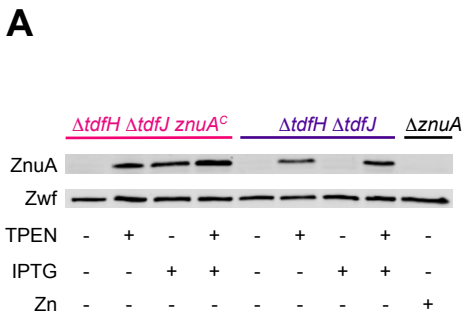
A: WT Gc was inoculated into media for 4 h (Media grown, blue), or added to Ect1 monolayers for 2 h, washed, and further incubated for 4 h as in Figure 2 (Adherent; orange). The media used were KSFM alone, or with 1.4 μM human calprotectin and 1.4 μM human psoriasin (+Cp + Ps), with or without 3 μM $ZnSO_4$ (+Zn). RNA was extracted and qRT-PCR was conducted using primers specific for *znuA* and 5S rRNA. ZnuA expression was calculated as $2^{-\Delta\Delta CT}$, normalized to 5S rRNA, and is expressed relative to KSFM suspension-grown bacteria. Asterisks represent adjusted p-values from an ordinary two-way ANOVA with Šídák's multiple comparisons test.

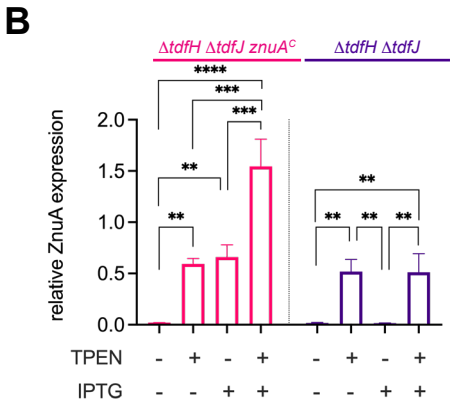
B-C: *ΔtdfH ΔtdfJ* Gc with an IPTG-inducible *znuA* complement (*ΔtdfH ΔtdfJ znuA*^{C}) (B) and *ΔtdfH ΔtdfJ* Gc (C) were inoculated into KSFM alone, or with 1.4 μM human calprotectin and 1.4 μM human psoriasin (+Cp + Ps), 3 μM $ZnSO_4$ (+Zn), and/or 1 mM IPTG (+IPTG) as indicated, and CFU were enumerated after 9 h. Dotted lines indicate the inoculum of each biological replicate, indicated by a different color; limit of detection (LOD) is indicated by the black dashed line. Asterisks represent adjusted p-values from an ordinary one-way ANOVA with Tukey's multiple comparisons test. D-E: WT Gc with (D) or without (E) an IPTG-inducible *znuA* complement (*znuA*^{C}) was inoculated into KSFM alone, or with 1.4 μM human calprotectin and 1.4 μM human psoriasin (+Cp + Ps), 3 μM $ZnSO_4$ (+Zn), and/or 1 mM IPTG as indicated. Dotted line indicates inoculum for each biological replicate. CFU were enumerated after 9 h. Number of biological replicates: D, 3; F, 2. Differences in D were not significant by two-way ANOVA with Tukey's multiple comparisons test.



To determine if upregulation of *znuA* is sufficient to protect Gc from zinc limitation imposed by human calprotectin and psoriasin, an IPTG-inducible *znuA* complement construct (*znuA*^{C}) was introduced into the *ΔtdfH ΔtdfJ* Gc background (see Materials and Methods). This strain retained its native *znuA* gene in addition to the inducible construct. *ΔtdfH ΔtdfJ znuA*^{c} Gc was grown in suspension in medium containing psoriasin and calprotectin, with or without IPTG to induce *znuA* expression (**Fig. 2.8.1B**). CFU were enumerated after 8 h. As a control, *ΔtdfH ΔtdfJ* Gc without the inducible *znuA* construct was grown in the same media conditions (**Fig. 2.8.1C**). Overexpression of *znuA* did not rescue suspension-grown *ΔtdfH ΔtdfJ znuA*^{C} Gc from growth restriction by calprotectin and psoriasin **(Fig. 2.8.1B)**. Overexpression of *znuA* in a WT Gc background using the same complement construct did not affect bacterial zinc-dependent growth **(Fig. 2.8.1D-E).** Induction of the complementation construct increased ZnuA protein levels approximately 3-fold in Gc treated with the zinc chelator TPEN ((N,N,N',N'-tetrakis(2pyridinylmethyl)-1,2,ethanediamine) alone **(Fig. 2.8.2A, quantified in B)**. Since overexpression of *znuA*, at least to the levels achievable in this inducible complementation system, is not sufficient to rescue the survival defect of suspension-grown Gc under zinc-limited conditions, this suggests that the upregulation of *znuA* in adherent Gc does not fully explain bacterial protection from zinc limitation.

Figure 2.8.2 ZnuA expression is increased in *ΔtdfH ΔtdfJ znuA^C*
A-B: *ΔznuA*, *ΔtdfH ΔtdfJ,* or *ΔtdfH ΔtdfJ znuA^C* Gc was grown for 18 h on GCB agar plates with the specified additions (TPEN= 10 μM TPEN, IPTG= 1 mM IPTG, Zn= 25 μM $ZnSO_4$). Lysates of Gc were separated by 4-20% gradient SDS-PAGE, transferred to nitrocellulose, and Western blotted for ZnuA with Zwf as loading control. A is one representative of three biological replicates. B: band intensity for each replicate was quantified, and displayed as the relative intensity (ZnuA/Zwf) for each condition. Asterisks represent p-values from ordinary one-way ANOVA with Šídák's multiple comparisons test.

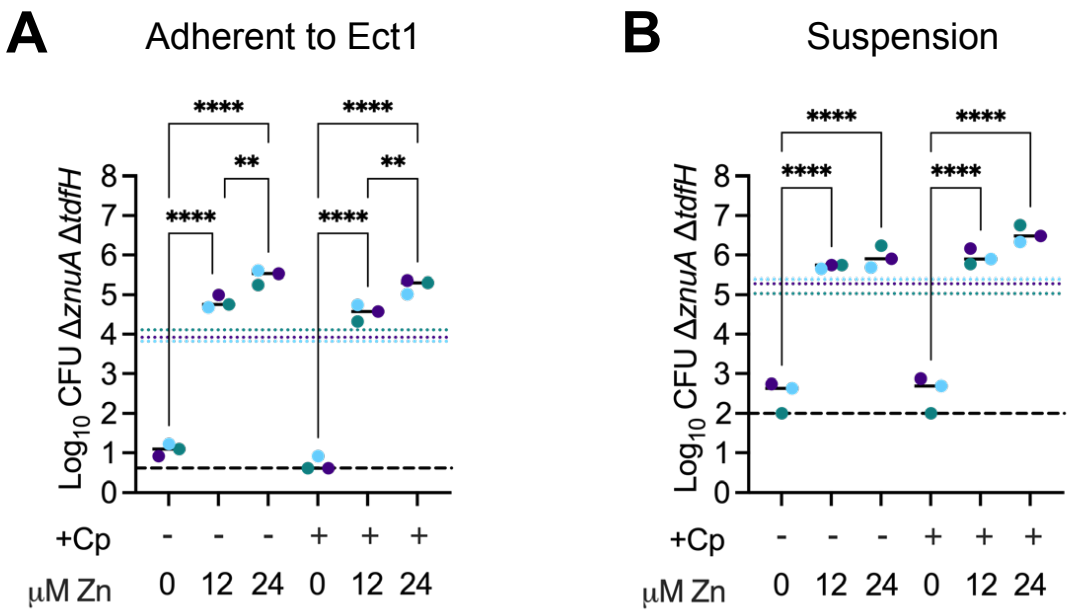




2.3.9 ZnuA is necessary for rescue of adherent Gc from zinc sequestration

Although ZnuA overproduction did not rescue the survival defect of zinc sequestered Gc in suspension, we sought to determine whether ZnuA was necessary to protect attached Gc under zinc-restricted conditions. To test this, a *znuA* deletion was introduced into *ΔtdfH* Gc, and the resulting double mutant was inoculated onto Ect1 cells or into KSFM without adherence, as described in **Fig. 2.2.3.** *ΔznuA ΔtdfH* Gc declined >3 logs over the inoculum in KSFM without excess zinc, even in the absence of calprotectin (**Fig. 2.9.1A**). This is consistent with previous experience with the *znuA* mutant, which requires additional zinc to grow even in rich medium (Maurakis, Keller et al. 2019). Adherence to Ect1 cells did not rescue the growth defect of *ΔznuA ΔtdfH* Gc and appeared similar to the double mutant in suspension (**Fig. 2.9.1A-B**). Full restoration of *ΔznuA ΔtdfH* Gc growth required addition of 24 μM $ZnSO_4$, compared to 6 μM that was used in experiments with bacteria carrying an intact ZnuA (**Fig. 2.1.3**). The addition of human calprotectin did not significantly affect the recovered CFU in either suspension-grown or adherent *ΔznuA ΔtdfH* Gc **(Fig. 2.9.1A-B)**. We conclude that while overexpression of ZnuA is not sufficient to rescue suspension-grown Gc from zinc limitation, ZnuA is necessary for Gc growth in any conditions other than highly abundant zinc, whether the bacteria are adherent or in suspension.

Figure 2.9.1 ZnuA is required for adherent Gc to be protected from zinc sequestration.
A-B: *ΔtdfH ΔznuA* Gc (10^5 CFU/mL) was added to Ect1 cells in KSFM (A) or inoculated in experimental media into wells without Ect1 cells (B). Experimental media were KSFM alone, or with 1.4 μM human calprotectin (+Cp) with or without 12 μM or 24 μM $ZnSO_4$ as indicated (Zn). For A, cells were washed and experimental media were added after 2 h; dotted line indicates adherent CFU at 2 h. For B, dotted line indicates the inoculum in the well. CFU were enumerated from cell lysates (A) or from the well (B) after 7 h. Colors correspond to matched individual biological replicates. Asterisks represent adjusted p-values from an ordinary one-way ANOVA with Tukey's multiple comparisons test. For simplicity, only significant comparisons within each condition with or without calprotectin are depicted. A-G: *=p<0.05; **=p<0.01; ***=p<0.001; ****=p<0.0001



2.4 Discussion

Gc is able to overcome nutritional immunity by expressing specific transporters that extract transition metals from human metal-sequestering proteins. Zinc is required for Gc growth *in vitro*, and our previous work showed that TdfH and TdfJ are required for Gc to acquire zinc from calprotectin and psoriasin, respectively (Jean, Juneau et al. 2016, Maurakis, Keller et al. 2019, Kammerman, Bera et al. 2020). The goal of this project was to determine the contribution of TdfH and TdfJ to Gc infection of mucosal epithelial cells of the female genital tract, specifically from the ectocervix, where calprotectin and psoriasin are detected (Mildner, Stichenwirth et al. 2010, Uhlen, Fagerberg et al. 2015). To our surprise, we found *ΔtdfH ΔtdfJ* Gc that was adherent to ectocervical cells was able to grow in the presence of human calprotectin and psoriasin, conditions that restricted the zinc-dependent growth of Gc in suspension. We further determined that this increased survival could be replicated in a reductionist system using WT Gc adherent to glass coverslips in the absence of ectocervical cells, and in the presence of murine calprotectin, which Gc cannot use as a zinc source. Comparative analysis of the transcriptional profiles of adherent and suspension-grown Gc under conditions of low available zinc indicated that adherent bacteria express relatively more of certain genes implicated in the response of *Neisseria* to low zinc concentrations. These findings reveal a new way in which adherence is critical to Gc

pathogenesis, by enhancing bacterial survival when confronted with limited amounts of the essential micronutrient transition metal, zinc.

This study was initiated to examine the role of TdfH and TdfJ in mucosal infection by Gc. Because calprotectin and psoriasin are present in the female genital tract and expressed by ectocervical but not endocervical cells (Mildner, Stichenwirth et al. 2010, Uhlen, Fagerberg et al. 2015, Bastian, Roux et al. 2021), we used the immortalized human ectocervical cell line Ect1 E6/E7 to study the adherence and invasion of Gc. However, Ect1 did not secrete abundant calprotectin and psoriasin; thus additional psoriasin and calprotectin was added to sequester the zinc in the growth medium, as would be found in female genital secretions. Intriguingly, TdfJ was found to be 4.86-fold more highly expressed in Gc in the female genital tract than in the male urethra (Nudel, McClure et al. 2018). This finding may be connected to the observation that male seminal fluid contains approximately 1000-fold more zinc than the 0.9-1.5 μM zinc reported in female cervical secretions (Wong, Flik et al. 2001, Chuang, Lee et al. 2002, Costello and Franklin 2016, Kothari and Chaudhari 2016, Damjanovich, Sipos et al. 2020). The concentrations of calprotectin and psoriasin used in this study to sequester the low μM zinc concentrations in KSFM are in keeping with the estimates of these S100 proteins in female genital secretions. Calprotectin and psoriasin are among the most abundant proteins detected by multiple unbiased proteomics studies on cervicovaginal lavage fluid or cervical mucus from human patients (Zegels, Van Raemdonck et al. 2009, Birse, Burgener et al. 2013,

Borgdorff, Gautam et al. 2016). One study on undiluted cervical mucus reported a mean calprotectin concentration of 996 nM (Kunimi, Maegawa et al. 2006) and a study of cervicovaginal lavage fluid reported mean psoriasin concentrations of 62 nM in 2 mL saline lavage (Mildner, Stichenwirth et al. 2010). The presence of these proteins extracellularly in the female genital tract mucosa supports our use of exogenous recombinant protein in the absence of sufficient calprotectin and psoriasin production by cultured Ect1 cells, especially since Gc was predominantly extracellular in this Ect1 model.

Zinc-loaded calprotectin and psoriasin can serve as zinc sources for Gc in chemically defined medium, in a TdfH- and TdfJ-dependent manner, respectively (Jean, Juneau et al. 2016, Maurakis, Keller et al. 2019, Kammerman, Bera et al. 2020). Here, we found that apo-calprotectin and psoriasin, when added to zinc-containing medium, limited the growth of Gc in a TdfH- and TdfJ-dependent manner, and could be overcome by adding excess zinc. These findings are important because they demonstrate that these S100 proteins can sequester zinc and limit growth of Gc if the bacteria's dedicated outer membrane transporters are absent. The decline in CFU for Gc lacking TdfH and/or TdfJ under zinc-limiting conditions began to manifest after 4 h of incubation (see **Fig. 2.1.2** and **Fig. 2.7.2**). While the zinc-storage capacity for Gc has not been described, these findings suggest depletion of Gc zinc reserves takes several hours.

The most surprising finding in this study was that Δ*tdfH* Δ*tdfJ* Gc survived and replicated in association with Ect1 cells in medium containing human

calprotectin and psoriasin in a zinc-dependent manner, conditions that led to overall death for the same Gc in suspension. Initially we considered that Ect1 cells released zinc (free or bound to proteins) into the medium, or that the cells were internalizing or degrading calprotectin and psoriasin to increase extracellular availability of free zinc. However, by using conditioned media and filter supports to physically separate Gc from Ect1 cells, the mechanism of protection was instead found to be contact-dependent. We then considered that Gc might acquire zinc directly from the cell surface, but this possibility was ruled out when paraformaldehyde-killed and washed Ect1 cells were found to support growth of Δ*tdfH ΔtdfJ* Gc in zinc-limited conditions. The final piece of data suggesting that Ect1 cells were not necessary for Gc to be protected from zinc limitation was that adherence of Gc to the abiotic surface of acid-washed glass was sufficient to support bacterial enhanced survival under zinc-limited conditions. We conclude that adherence alone is sufficient to enhance survival of Gc under zinc-restricted conditions. We envision at least three nonexclusive ways in which Gc accomplishes this feat: it is more efficient at zinc uptake, it is more efficient at retaining zinc intracellularly, or it physiologically adapts to require less zinc. Findings presented here with *znuA* as well as other genes differentially expressed by RNA-seq provide preliminary support for each of these possibilities. We attempted to quantify the total zinc within adherent and suspension Gc when zinc was chelated by TPEN, but the data were highly variable (**Figure 2.6.2A)**. One culprit may be the method of normalizing the zinc

quantities measured. To account for differences in the number of bacteria recovered between conditions and between days, the raw zinc concentration measured in the digested and diluted pellets is normalized to the wet mass of the pellet when all visible liquid had been aspirated. We have low confidence in the accuracy of this measurement, however, because the masses of the pellets were frequently below the accuracy range of the balances available. In the future, we will test other methods of normalization to validate zinc concentrations, such as cellular sulfur content as described in (Hesse, Lonergan et al. 2019). We expected to see significantly lower internal zinc in the TPEN-treated suspension-grown Gc as compared to the zinc-replete conditions. The fact that we did not observe this may indicate that TPEN, which is known to be cell-permeable (Hyun, Sohn et al. 2001), could be gaining access to the inside of the Gc. In that case, the bacteria would be zinc-starved because TPEN keeps the zinc unavailable for use, but ICP-MS only measures the quantity of the element, not its availability, and would find no difference. Therefore, when these studies are repeated, zinc will be restricted using murine calprotectin or other extracellular methods. Future studies that measure zinc uptake and release by Gc are needed to determine how Gc adapts to zinc limitation when adherent to epithelial cells.

We were surprised to find that the protection of adherent Gc from zinc sequestration was not directly related to the ability of TdfH and TdfJ to acquire zinc from human calprotectin and psoriasin. In fact, addition of murine calprotectin, which Gc cannot use as a zinc source (Kammerman, Bera et al.

2020), restricted the zinc-dependent growth of WT bacteria. The use of murine calprotectin was preferable to the small molecule TPEN (N,N,N',N'-tetrakis(2pyridinylmethyl)-1,2,ethanediamine), which is commonly used to chelate zinc in biological systems (Sigdel, Easton et al. 2006). TPEN is membrane-permeable and not completely selective for zinc (Chai, Truong-Tran et al. 2000, Hyun, Sohn et al. 2001, Cho, Lomeda et al. 2007). We found that KSFM containing TPEN killed Ect1 cells (not shown), in keeping with reports that TPEN causes cell death and DNA damage in cell culture (Chai, Truong-Tran et al. 2000, Mendivil-Perez, Velez-Pardo et al. 2012). While TPEN was not used in most of these studies, we validated that the addition of TPEN to suspension-grown Gc results in fewer CFU, and this is rescued with excess zinc (**Figure 2.6.1A)**. Additionally, adherence to glass coverslips prevents this decline in CFU **(Figure 2.6.2B)**. The ability of adherent Gc to grow in the presence of calprotectin or psoriasin, in the absence of any specific TonB-dependent transporters, may be due to occasional release of zinc from these S100 proteins, which have a relatively lower affinity for zinc compared to that of transferrin for iron (K_d ~ 10 pM for calprotectin and 400 pM for psoriasin, vs 10^{-20} M for transferrin (Aisen, Leibman et al. 1978, Brophy, Hayden et al. 2012, Cunden, Brophy et al. 2017).

Neisseria and many other bacteria respond to changes in zinc availability through the zinc uptake regulator, Zur (NGO_0542), which senses cytoplasmic zinc concentrations and, when zinc is abundant, represses expression of genes

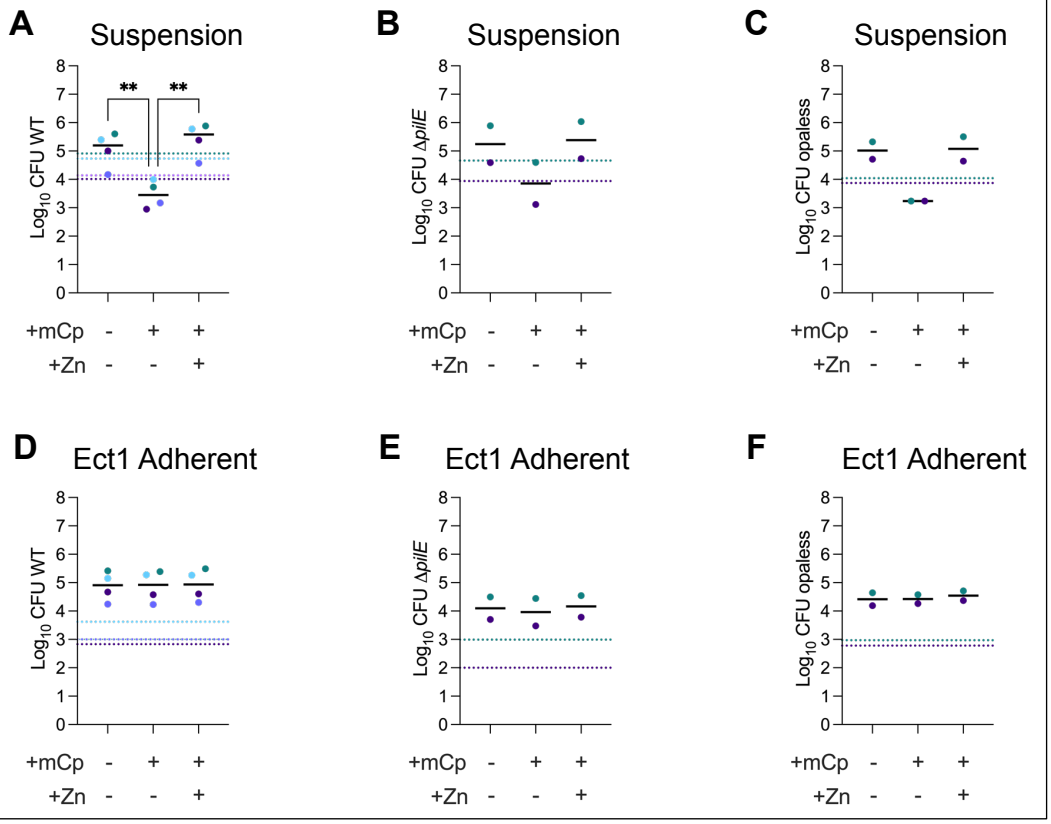
involved in zinc uptake (Wu, Seib et al. 2006, Pawlik, Hubert et al. 2012). Zur was not differentially expressed as a result of adherence or zinc sequestration in this study. Transcripts for six of the ten genes reported to comprise the Gc Zur regulon were upregulated in Gc under zinc sequestration by murine calprotectin. Of the six, transcripts for three were more abundant in adherent Gc than suspension-grown Gc when zinc was sequestered: *znuA, ngo1049,* and *rpmE2*. These three ORFs may represent genes involved in the response to zinc limitation that are also modulated by adherence. ZnuA has been implicated in other pathogens as a primary means of overcoming calprotectin-mediated zinc sequestration, by trapping any zinc that enters the periplasm and shuttling it to the ZnuBC machinery for import into the cytoplasm (Hood, Mortensen et al. 2012, Liu, Jellbauer et al. 2012, D'Orazio, Mastropasqua et al. 2015, Hesse, Lonergan et al. 2019). Here we found that ZnuA was necessary for adherent Gc to survive in the presence of calprotectin, underscoring its importance to zinc acquisition in Gc. However, ZnuA overexpression was not sufficient to enable growth of suspension-grown Gc when zinc was limited (**Fig. 2.8.1**). NGO_1049 is bioinformatically predicted to be localized to the periplasm and contain a metal-binding motif (I. Liyayi and A.K.C., unpublished results), while RpmE2 is a zinc-independent ribosomal protein which replaces its zinc-dependent paralogue RpmE (NGO_2126) under conditions of zinc starvation (Nanamiya, Akanuma et al. 2004). The remaining transcript that was increased by both zinc sequestration and adherence was FbpA (NGO_0217), which has been reported to be regulated

by iron but not zinc(Forng, Ekechukwu et al. 1997). FbpA is a periplasmic protein, which binds iron and transfers it to inner membrane proteins for transport into the cytoplasm(Strange, Zola et al. 2011). Future studies will examine the contribution of these gene products to the increased survival of adherent Gc under zinc-limited conditions.

A related set of genes of interest were those whose transcripts were increased with zinc sequestration that was specific to the adherent bacteria (increased in adherent sequestered vs. excess Gc and adherent vs. suspension zinc sequestered, but not in suspension sequestered vs. excess conditions). None of these genes have been previously described as zinc- or Zur-regulated, but each has been reported to be either repressed in anaerobic conditions or induced in the presence of hydrogen peroxide, and many of these genes were previously described as iron-repressed. For instance, CysK (NGO_0340) was induced in strain MS11 Gc by adherence to cervical cells (Du, Lenz et al. 2005) and contributes to epithelial colonization by *N. meningitidis* (Capel, Zomer et al. 2016) and LldD is a L-lactate dehydrogenase that is important for intracellular survival of Gc under microaerobic conditions (Atack, Ibranovic et al. 2014). GlnQ (NGO_0374), a predicted amino acid transporter, is upregulated in a constitutively piliated *pilT* mutant (Friedrich, Arvidson et al. 2007). The increase in expression of these genes suggest a metabolic shift in adherent, zinc-sequestered Gc that either results from or facilitates bacterial survival. We did not find a role for expression of type IV pili or opacity-associated (Opa) proteins in

the enhanced survival of zinc-limited, adherent Gc (**Fig. 2.10.1**), suggesting a more general way in which Gc “senses” adherence to induce this protective gene expression profile. Notably, the adherent, zinc-sequestered Gc regulon did not overlap with the regulon of strain 1291 Gc in mature biofilms established on glass under flow conditions (Falsetta, Bair et al. 2009, Phillips, Steichen et al. 2012). We did not directly test for biofilm components, and previous studies of Gc biofilms required at least 24 hours to allow biofilm formation (Greiner, Edwards et al. 2005, Anderson, Byerly et al. 2016). However, over the four hours of adherent growth prior to RNA collection, it is likely that Gc organized into microcolonies (Anderson, Byerly et al. 2016). While these bacteria are transcriptionally distinct from Gc in canonical biofilms, inter-bacterial interaction and colony structure may play a role in the protection of adherent Gc and will be investigated in the future.

Figure 2.10.1 Type IV pili and Opa proteins are dispensable for the enhanced survival of Gc that are associated with Ect1 cells when zinc is sequestered by mouse calprotectin.
A-C: WT (A), *ΔpilE* (B), or opaless (C) Gc were inoculated (~10^5 CFU/mL) into KSFM-GF, alone or with addition of 3 μM mouse calprotectin (+mCp) and/or 6 μM $ZnSO_4$ (+Zn). Data are presented as the log_{10} transformation of CFU recovered after 7 h. Dotted line indicates the inoculum CFU. D-F: Confluent Ect1 cells (4x10^5 cells/well) were infected with WT (A), *ΔpilE* (B), or opaless (C) Gc (~2.5x10^4 CFU/well) in KSFM-GF for 2 h. Non-adherent Gc were washed away, and Gc was incubated an additional 7 h in the same media conditions described in A-C. Media was collected to enumerate detached CFU, and cells were lysed to enumerate adherent CFU. Data are presented as the log_{10} transformation of the sum of detached and adherent CFU (Cell-associated) 7 h after washing. Dotted lines indicate the CFU adherent at 2 h. Each biological replicate is indicated by a different color, with the same starting culture used for A and D, B and E, and C and F. Asterisks in A represent adjusted p-values from ordinary one-way ANOVAs with Tukey's multiple comparisons test, **=p<0.01.



Transcripts for the remaining three genes in the Zur regulon were induced by zinc sequestration, but were unaffected by adherence (Wu, Seib et al. 2006, Pawlik, Hubert et al. 2012): *tdfJ, hmcD, and tdfH* (**Table 2.3.7**). *tdfH* fell just below the 0.5 fold-change cutoff in suspension sequestered vs. excess (0.49), but this change was statistically significant so it has been included in this subset of genes. Since these genes were not significantly differentially expressed between adherent and suspension Gc under zinc sequestration, they are unlikely to be responsible for the increased survival of adherent Gc under zinc-limited conditions. However, they may work with the other differentially expressed genes described above to enable enhanced Gc survival, and may indicate a more general cross-talk between zinc and iron regulons for Gc during infection.

Four genes that are repressed in excess zinc in *N. meningitidis* were not differentially expressed in any of the comparisons examined: *znuB* (NGO_0169), *znuC* (NGO_0170), *rpmJ2* (NGO_0931) and *queC* (NGO_0129) (Wu, Seib et al. 2006, Pawlik, Hubert et al. 2012). It is particularly surprising that *znuC* and *znuB* were unchanged, because they are the first two ORFs of the operon containing *znuA*. This observation suggests additional regulatory mechanisms for the *znuCBA* operon that are relevant in the context of bacterial adherence, which will be a subject of future studies. The inability of zinc sequestration by murine calprotectin in KSFM to derepress the full Zur operon could indicate that the concentration of murine calprotectin that was used is not sufficient to fully sequester zinc, the concentration of excess zinc used does not fully saturate Zur,

and/or a more nuanced regulation of zinc-regulated genes based on the similarity of each promoter's Zur binding sites to the canonical Zur binding motif, as was reported in *N. meningitis* by (Pawlik, Hubert et al. 2012). In addition to the transcriptional studies reported here, post-transcriptional gene regulatory mechanisms may also be responsible for the increased survival of adherent Gc under zinc-limited conditions, a topic for future investigation.

There is a continuous interplay between the need for a pathogen to acquire zinc during infection and the restriction of zinc bioavailability by the infected and inflamed host (Abtin, Eckhart et al. 2008, Wan, Latter et al. 2014, Borgdorff, Gautam et al. 2016). The healthy female genital tract possesses high levels of calprotectin and psoriasin, which we predict would increase during the neutrophilic inflammation that characterizes Gc infection. Other S100 proteins that are known to bind zinc have been detected in the female genital tract (Zegels, Van Raemdonck et al. 2009, Murray, Tonkin et al. 2012, Birse, Burgener et al. 2013, Gilston, Skaar et al. 2016). Additionally, epithelial cells import zinc using ZIP transporters and sequester zinc internally using metallothioneins (Kimura and Kambe 2016), which could create a local environment of lower zinc near cell surfaces. Therefore, we anticipate that the ability to acquire zinc from human proteins, as well as the ability to survive despite zinc sequestration by proteins Gc cannot directly use, are both important for Gc to effectively colonize human epithelia. In this light, the ability of Gc to proliferate when adherent when zinc is sequestered suggest Gc has an adaptive defense against zinc limitation

during infection. Targeting the necessity for zinc acquisition in Gc during infection is a potential therapeutic approach for drug-resistant gonorrhea, for instance by inhibiting the binding of zinc by ZnuA or by developing vaccines against TonB-dependent transporters that are expressed in infected individuals (Cornelissen and Hollander 2011, McClure, Nudel et al. 2015). Taken together, the multifaceted ways in which Gc resists human nutritional immunity exemplify how Gc has evolved to evade and circumvent the human immune response to infection in order to become the urgent public health threat it is today.

2.5 Materials and Methods

2.5.1 Bacterial strain construction.

Wildtype (WT*)* for these studies is piliated Opaless FA1090 with locked-on OpaD (OpaD+, also called $OpaD^{+}_{nv}$) (Ball and Criss 2013). The Opa and PilE genes of this strain are not phase variable, which removed potential confounding effects from variability of these important bacterial adhesins. *ΔtdfH* Gc was generated by spot transforming *wt* Gc with genomic DNA from strain MCV955 (Opaless FA1090 *tdfH::kan)* ((Jean, Juneau et al. 2016)) as described in (Dillard 2011) and selection on GCB (BD Difco 228920) containing 30 μg/mL kanamycin. *ΔtdfJ* Gc was generated by spot transforming WT with genomic DNA from strain MCV928 (FA19 *tdfJ::Ω*) (Strange, Zola et al. 2011), with selection on GCB containing 50 μg/mL spectinomycin, followed by two additional rounds of backcrossing into WT. *ΔtdfH ΔtdfJ* Gc was generated by transforming gDNA from *ΔtdfH* into *ΔtdfJ* and selecting on plates containing both 50 μg/mL spectinomycin

and 30 μg/mL kanamycin. *ΔznuA* was generated by transforming WT with gDNA from MCV951 (FA19 *znuA::Ω*) on GCB plates supplemented with 25 μM $ZnSO_4$, 25 μM $MnCl_2$, 12 μM $Fe(NO_3)_3$, 5 mM D-mannitol, with selection on GCB plates with these supplements and 50 μg/mL spectinomycin, followed by backcrossing twice into WT (Maurakis, Keller et al. 2019). All mutants were confirmed by PCR and/or sequencing **(Table 2.5).** *znuA*^{c} was generated by transforming WT with gDNA from MCV951 transformed with pVCU234 containing *znuA*^{c}, selecting on 0.5 μg/mL chloramphenicol, and backcrossing twice into WT Gc (Maurakis, Keller et al. 2019). *ΔtdfH ΔtdfJ znuA*^{C} was generated by transforming *ΔtdfH ΔtdfJ* Gc with gDNA from *znuA*^{c}. In both cases overexpression of ZnuA was confirmed by Western blot with a ZnuA-specific antibody (see **Fig. 2.8.2**). Opaless and OpaD$^+_{nv}$ *ΔpilE* have been previously described (Ball and Criss 2013, Stevens, Gray et al. 2018).

Unless otherwise noted, Gc was prepared for experiments by inoculating stocks from -80 °C onto GCB and culturing for 24 h at 37 ˚C, 5% CO_2. Gc was then passaged to GCB containing 10 μM of the zinc chelator TPEN (N,N,N',N'-tetrakis(2pyridinylmethyl)-1,2,ethanediamine) (Sigma P4413) and grown for 18 h at 37°C, 5% CO_2. This concentration of TPEN induced robust TdfJ expression by Western blot without restricting growth of Gc (data not shown). *ΔznuA* Gc was grown on GCB supplemented with 25 μM $ZnSO_4$, because this mutant does not grow on GCB without supplemental zinc (data not shown).

Table 2.5 Primers used in this study

Primer name	Sequence	Application
5s rRNA F	CGGCCATAGCGAGTTGGT	qRT-PCR
5s rRNA R	TTGGCAGTGACCTACTTTCG	qRT-PCR
ZnuA F qPCR	TCCGCAGTGCAAAACTCGTC	qRT-PCR
ZnuA R qPCR	TGGTGGTGTCCTTCGTGGTC	qRT-PCR
ZnuA F	CCCTTATTGCCGCATTGCTGGCCAC	confirmation
ZnuA R	TTGCTTCATCGCGTTGGTCAAGGCTTTG	confirmation
TdfJ F	CCCCTCCTCGCCCAAGCGCATGAAACTGAG	confirmation
TdfJ R	GGCGTGGCAATCATCGTATTCGTGGCTGTG	confirmation
TdfH F	GGCAGCGAGGCGCAGATACAGGTTTTGGAA	confirmation
TdfH R	GCGCCAGGTTTTCCCGCCAGCTTTTATCAT	confirmation
OpaD F	AAGAAGGAATGCCCGAACCG	confirmation
OpaD R	AACCAATGTTACGTCGTGGCG	confirmation

2.5.2 Recombinant S100 protein preparation.

Human and murine calprotectin were produced as previously described (Kehl-Fie, Chitayat et al. 2011, Kammerman, Bera et al. 2020). Human psoriasin was sub-cloned into a pET22b vector and purified as described (Maurakis, Keller et al. 2019) using a protocol developed in the Grötzinger laboratory (Michalek, Gelhaus et al. 2009). In all cases, the molarity of the recombinant proteins is presented based upon the molecular weight of the dimer (calprotectin: S100A8/S100A9 heterodimer; psoriasin: S100A7 homodimer).

2.5.3 Media growth assay.

Keratinocyte serum free medium (KSFM) was obtained from Gibco (17005042). KSFM with growth factors (abbreviated KSFM+GF in figure legends) was prepared by adding bovine pituitary extract to 0.05 mg/mL and human recombinant EGF to 0.1 ng/mL final concentrations (both provided with KSFM by the manufacturer), and $CaCl_2$ (Fisher M-5133) to a final concentration of 400 μM. KSFM with growth factors was incubated for 20 min at 37 ˚C with increasing concentrations of S100 proteins, with or without 3 μM $ZnSO_4$ (Sigma-Aldrich 204986). Based on these results **(Fig. 2.1.1),** the subsequent experiments used 1.4 μM human calprotectin, 1.4 μM human psoriasin, or 2 μM murine calprotectin. WT or *tdf* mutant Gc (1 x 10^6 CFU/ml) was inoculated into the experimental media in round-bottom 96-well plates (Sarstedt 82.1582.001). No adherence of Gc to the wells was observed in any experiment. At the experiment start (time 0 h) and the indicated timepoints, wells were mixed by pipetting, and

20 µl of the sample was removed for serial dilution and plating on GCB. CFU were enumerated 20-24 h later. Results are presented as the $\log_{10}$ transformation of CFU recovered at a given timepoint. Statistics are by two-way ANOVA with Tukey's multiple comparisons test.

2.5.4 Metal repletion experiments.

Media growth experiments were performed as above but with the following changes. KSFM with growth factors was incubated for 20 minutes at 37 ˚C with 2 µM murine calprotectin, or 1.4 µM human calprotectin and/or 1.4 µM human psoriasin, with or without 6 µM $ZnSO_4$, 6 µM $MnCl_2$ (Sigma M3634), or 6 µM $CuCl_2$ (Sigma C3279). Statistics are adjusted p-values from a one-way ANOVA with Šídák's multiple comparisons test. The comparisons reported are for each S100 protein with and without additional metal.

2.5.5 Ect1 infection.

Ect1 E6/E7 immortalized ectocervical cells (ATCC CRL-2614) were acquired from ATCC and maintained in KSFM with growth factors plus antibiotics (1x antibiotic-antimycotic Gibco 15240062) at 37 °C in 5% CO_2. For experiments, cells were seeded at $5x10^4$ cells/well in 24-well flat-bottom tissue-culture treated plates and grown to confluency (approximately $4x10^5$ cells/well). Two days before the experiment, cells were changed to medium without antibiotics. On the day of the experiment, KSFM with growth factors was pre-incubated with 1.4 µM human calprotectin and 1.4 µM human psoriasin for 20 min, with or without 3 µM $ZnSO_4$. WT or *ΔtdfH ΔtdfJ* Gc ($2.5x10^4$ CFU) were inoculated on Ect1 cells for an MOI of

~0.6 CFU/cell. After 2 h, cells were washed to remove non-adherent Gc. One set of cells was lysed with 1% saponin for 10 min at 37 ˚C (Fluka analytical 47036), diluted to 0.2% saponin with GCBL, serially diluted, and plated to enumerate cell-associated CFU. After 9 h total (7 h after wash) supernatants were collected, diluted, and plated for CFU to enumerate detached bacteria. Cell-associated bacteria were collected by treating the wells with 1% saponin to lyse the Ect1 cells, and then diluting and plating for CFU as above. Media-grown bacteria were inoculated at 10^5 CFU/mL and grown on the same day and in the same KSFM conditions as the adherent Gc, for 9 h total. For each adherent Gc experiment, the CFU associated with Ect1 cells at 2 h is plotted as horizontal lines in colors corresponding to experimental replicates. For the media-grown condition, the measured inoculum CFU is plotted as horizontal lines. CFU at the experimental endpoint is plotted as the $\log_{10}$ transformation of recovered CFU. Statistics are from one-way ANOVA with Tukey's multiple comparisons test.

2.5.6 Conditioned media.

KSFM with 400 μM $CaCl_2$ and lacking growth factors (abbreviated KSFM-GF in figure legends) was incubated for 20 min at 37 °C with or without 1.4 μM human calprotectin and 1.4 μM human psoriasin. These media were added to wells of a 24-well plate containing confluent Ect1 cells, Ect1 cells infected with *ΔtdfH ΔtdfJ* Gc following the protocol described above, or no Ect1 cells, and incubated at 37 °C in 5% CO_2. After 7 h, media were removed, sterile filtered, and stored at 4 °C overnight. The next day, the media were warmed to 37 °C and

used for growth assays using *ΔtdfH ΔtdfJ* or WT Gc as described in the media growth assay methods. After 7 h, samples were diluted and plated on GCB to enumerate CFU. Results are presented as $\log_{10}$ transformed CFU, and statistics were calculated by ordinary one-way ANOVA with Šídák's multiple comparisons test.

2.5.7 Separation of bacteria and Ect1 cells with filter inserts.

Ect1 cells were maintained and seeded as described above. Three conditions were employed, using KSFM without growth factors with or without 1.4 μM human calprotectin and 1.4 μM human psoriasin (see **Figure 2.3.2** for schematics). 1) Contact-dependent infection: 10^5 CFU/mL *ΔtdfH ΔtdfJ* or WT Gc was inoculated into wells containing Ect1 cells and incubated for 2 h in KSFM without growth factors, washed to remove non-adherent bacteria, and fresh media replaced (KSFM +/- calprotectin and psoriasin) for an additional 7 h as described above, except that a Transwell filter insert (6.5 mm, 0.4 μm pore polyester; Corning Costar) was also added to the well. Dotted lines indicate the CFU adherent at 2 h in each experimental replicate. 2) Exposure without Ect1 contact: Ect1 cells were incubated for 2 h in KSFM without Gc and containing Transwell filter inserts, then washed. At the time of medium replacement, *ΔtdfH ΔtdfJ* or WT Gc was inoculated onto the Transwell filter, which separated the bacteria from the Ect1 cells that were seeded in the wells, and incubated an additional 7 h. Dotted lines indicate the inoculum for each experimental replicate. 3) No Ect1 cells: *ΔtdfH ΔtdfJ* or WT Gc was inoculated onto Transwell filter

inserts in wells that did not have Ect1 cells for 7 h. Dotted lines indicate the inoculum for each experimental replicate. Results are presented as the $\log_{10}$ transformed CFU for each condition. Statistics were calculated by unpaired Student's t-test within each experimental condition.

2.5.8 Bacterial growth after adherence to live vs. dead Ect1 cells.

ΔtdfH ΔtdfJ Gc (10^5 CFU/mL) was inoculated into wells containing Ect1 cells that were alive, or killed by treatment with 4% paraformaldehyde (Electron Microscopy Sciences 15714) for 20 min at 37 °C (cells were washed four times to remove fixative prior to addition of Gc). Infection was allowed to proceed as described above for Ect1 infection, with cells washed at 2 h and experimental media added. Media conditions were KSFM without growth factors alone, or containing 1.4 μM human calprotectin and 1.4 μM human psoriasin, with or without 6 μM $ZnSO_4$. One set of cells was lysed at 2 h after washing, and the other at 9 h total, from which CFU were enumerated. Gc inoculated into wells containing these media conditions without Ect1 cells served as a control. Results are presented as $\log_{10}$ transformed CFU, and statistics were calculated by ordinary one-way ANOVA and Tukey's multiple comparisons test.

2.5.9 Bacterial attachment to coverslips.

12 mm diameter round glass coverslips (Fisher Scientific 12-545-80P) were acid-washed by heating in 1 N HCl at 60 °C for 4 h, followed with successive washes of distilled water, 70% ethanol, and 100% ethanol. Gc (10^6 CFU/mL) was inoculated onto coverslips in wells of a 24-well plate in 250 μL

KSFM without growth factors. After 2 h, the medium was removed and coverslips were washed once with sterile PBS+ 5 mM $MgSO_4$. CFU were enumerated from one set of wells that were resuspended in KSFM by vigorous pipetting and serial dilution. Experimental media were KSFM alone or containing 2 μM murine calprotectin, with or without 6 μM $ZnSO_4$. Experimental media were added to the other set of wells and incubated for an additional 7 h, at which time detached and adherent Gc were collected and CFU were enumerated. For each experimental replicate, CFU were simultaneously collected from infection of Ect1 cells or inoculation into wells lacking coverslips (suspension-grown) as described above. Data are presented as the $\log_{10}$ transformation of the CFU recovered at the experiment endpoint, and asterisks represent significant differences by ordinary one-way ANOVA with Tukey's multiple comparisons test.

2.5.10 RNA extraction and qPCR of Gc from Ect1 infection.

Ect1 cells were infected as described above, in KSFM without growth factors, using a higher inoculum ($5x10^7$ CFU/mL) and for a shorter time (2 h + 4 h after wash). For comparison, Gc were grown for 4 h under the same conditions, in wells without Ect1. At the experimental endpoint, the supernatant was removed and adherent Gc were collected in 200 μL per well of RNAprotect cell reagent (Qiagen 76526). Cells from 4 replicate wells per condition were pooled, pelleted, resuspended in 200 μL RNAprotect cell reagent, and stored at -80 °C

until processed to purify RNA. 4 wells of uninfected Ect1 cells in RNAprotect cell reagent were spiked into the bacteria grown without Ect1.

Samples were lysed according to the “Enzymatic Lysis and Proteinase K Digestion of Bacteria” protocol from Qiagen before proceeding with RNeasy Plus mini kit (Qiagen 74134) per manufacturer’s instructions. qPCR was conducted using a Thermo Fisher QuantStudio3 instrument, Power SYBR Green PCR master mix (Thermo Fisher Scientific 4368577), RNAse inhibitor (Life Technologies N8080119), and Multiscribe Reverse Transcriptase (Life Technologies 4311235) and qRT-PCR primers (**Table 2.5**). Data were normalized to a 5S rRNA reference control. Data are expressed as relative expression ($2^{-\Delta\Delta CT}$) relative to the KSFM medium-grown condition. Statistics were calculated by ordinary two-way ANOVA with Šídák’s multiple comparisons test.

2.5.11 Generation of guinea pig anti-ZnuA antiserum.

FA1090 ZnuA peptide Ac-CSYAEATKGIQPLKAEE-amide was synthesized by Vivitide (formerly New England Peptide) and confirmed by HPLC (>85% purity by percent area) and mass spectral analysis (calculated mass within 0.1% of MW: 1879 Da). 3 mg of peptide was fused to keyhole limpet hemocyanin and utilized in four immunizations of two guinea pigs, which were ultimately exsanguinated yielding ~15mL serum per guinea pig. Optimal dilution factor for antiserum was determined by Western analysis of whole cells lysates of GC overexpressing or not expressing ZnuA. To remove nonspecific reactivity,

antiserum was preincubated with a nitrocellulose membrane onto which a lysate from *ΔznuA* Gc had been transferred.

2.5.12 ZnuA overexpression and bacterial growth in suspension.

KSFM without growth factors was incubated for 20 min at 37 °C with no additives, with 1 mM IPTG (for induction of *znuA* complement expression), or with 1.4 μM human calprotectin and 1.4 μM human psoriasin, with or without 1 mM IPTG or 3 μM $ZnSO_4$. Gc (10^5 CFU/mL) was inoculated into each medium condition and incubated at 37 °C in 5% CO_2. CFU were enumerated from the inoculum and after 9 h incubation as described above for media growth. Data is presented as Log_{10} CFU recovered, and statistics represent p-values from ordinary one-way ANOVA with Tukey's multiple comparisons test.

To prepare lysates for Western blot, Gc of the indicated strains was cultured for 24 h on GCB at 37 ˚C, 5% CO_2. Gc was then passaged to the following GCB plates: +Zn: 25 μM $ZnSO_4$; +TPEN: 10 μM TPEN; +IPTG: 1 mM IPTG; +TPEN+IPTG: 10 μM TPEN and 1 mM IPTG. After 18 h, bacteria were collected using polyester swabs (Fisher Scientific 25-806) and resuspended in PBS+5 mM $MgSO_4$. Samples were normalized to equal optical densities and resuspended in sample buffer containing 12 mM Tris pH 6.8, 5% glycerol, 0.4% SDS, 1% β-mercaptoethanol, and 0.02% bromophenol blue. Samples were boiled for 5 min and sheared with a syringe and 22-gauge needle (BD 305156).

2.5.13 Western blot.

Bands were resolved using a 4-20% Criterion TGX precast gel (5671094, Bio-Rad) and transferred in tris-glycine methanol buffer (0.3% Tris-HCl, 1.4% glycine, 20% methanol) to nitrocellulose. Blots were blocked with 2.5% BSA (Fisher BP1600) in TBST (0.24% Tris-HCl, 1% NaCl, 0.01% Tween-20, pH 7.6) for 1 h, and primary and secondary antibodies were diluted in TBST + 2.5% BSA. Blots were incubated with guinea pig anti-ZnuA antiserum (1:1,000), followed by donkey anti-guinea pig 800CW (1:15,000). Rabbit anti-Zwf antiserum (1:10,000) (Wierzbicki, Zielke et al. 2017) and goat anti-rabbit 680H+L (1:15,000) were used as loading control. The blot was imaged on a LI-COR Odyssey CL-X and relative band intensities were quantified using Image Studio software. ZnuA protein levels were normalized to the Zwf loading control and statistics were calculated by ordinary one-way ANOVA with Šídák's multiple comparisons test. Blot image was cropped and rearranged for ease of interpretation, but not otherwise altered.

2.5.14 RNA extraction for RNAseq.

Adherent Gc: WT Gc (10^8 CFU/ml in 500 µL KSFM without growth factors) were grown as described above and allowed to adhere to 18 mm diameter acid-washed glass coverslips (12-545-100P) for 2 h, then washed to remove non-adherent Gc. The experimental media (KSFM without growth factors containing 3 µM mCp, with or without 6 µM $ZnSO_4$) were added and the infection was allowed to proceed for another 4 h at 37 °C in 5% CO_2, at which time the supernatant was removed and 1 volume (500 µL) KSFM without growth factors and 2

volumes (1 mL) RNAprotect bacteria reagent (Qiagen 76506) were added. Gc was collected by scraping, and cells from 8 wells were pooled together in a 15mL conical tube, incubated at room temperature for 5 min, and pelleted at 4,000 x g for 11 min. Suspension-grown Gc: WT Gc was prepared in the same experimental media as described above, inoculated at $1x10^8$ CFU/mL into 96-well round-bottom plates, and grown for 4 h at 37 °C in 5% CO_2. Gc was harvested by pooling 2 mL of culture per condition, adding 2 volumes RNAprotect bacteria reagent to the pooled cultures and centrifuging as above. In all growth conditions, pellets were processed and RNA was purified as described for qPCR. DNAse treatment was completed using a TURBO DNAfree kit (Invitrogen AM1907).

Illumina RNA sequencing was conducted by the Genomics Resource Center of the Institute of Genome Sciences at the University of Maryland. Ribosomal RNA reduction was completed using NEBNext rRNA Depletion Kit (Bacteria) (NEB E7850), and RNA library prep was done using NEBNext Ultra II Directional RNA Library Prep Kit (NEB E7760). Sequencing was performed using NovaSeq S2 on the the NovaSeq6000 platform, with >5M 150bp paired-end read pairs per sample.

Reads were checked for quality using FASTQC (Andrews 2010) (v0.11.8), trimmed using BBMAP (Bushnell 2014) (v3.8.16b), and aligned to the *Neisseria gonorrhoeae* FA1090 genome with Ensembl annotations (ASM684v1.51) using STAR (Dobin, Davis et al. 2013) (v2.7.1a). Transcripts per million calculations

were performed by RSEM (Li and Dewey 2011) (v1.3.1), the results of which were imported into R (v4.0.2) (R_Core_Team 2020) and Bioconductor (Huber, Carey et al. 2015) (v3.12) using tximport (Soneson, Love et al. 2015) (v1.18.0). Significant genes were called using DESeq2 (Love, Huber et al. 2014), using fold change cutoffs and pvalue cutoffs of 0.5 and 0.05 respectively. RNA-seq data are deposited at Gene Expression Omnibus (GEO) database under accession number GSE191020.

2.5.15 ICP-MS and ICP-OES.

Quantification of zinc and other metals was conducted by the Center for Applied Isotope Studies at the University of Georgia. KSFM with and without growth factors was acidified to a final concentration of 2% nitric acid, diluted 10-fold, and analyzed by ICP-MS. KSFM without growth factors was incubated for 7 h in wells of a 96-well round-bottom plate (incubated in empty wells) or with acid-washed glass coverslips in wells of a 24-well plate (incubated with glass coverslips). Media were removed from wells, like wells were pooled, and samples were acidified to 2% nitric acid and analyzed by ICP-OES.

Adherent Gc: WT Gc (10^8 CFU/ml in 1 mL KSFM without growth factors) were grown as described above and allowed to adhere to 25 mm diameter acid-washed glass coverslips (Fisher Scientific 12-546-2p) for 2 h, then washed to remove non-adherent Gc. The experimental media (KSFM without growth factors alone or containing 2 μM TPEN, with or without 4 μM $ZnSO_4$) were added and the infection was allowed to proceed for another 4 h at 37 °C in 5% CO_2. After 4

h the supernatants were discarded and adherent Gc were collected in 1 mL KSFM per well, with 6 mL of like conditions pooled together in pre-weighed metal-free 15 mL conical tubes (Labcon 3134-345-001). Gc were pelleted, washed once with milli-Q water, and pelleted again. All supernatant was aspirated and pellets were weighed and sent to CAIS for digestion in nitric acid and analysis by ICP-MS. Suspension-grown Gc: WT Gc was prepared as described above and inoculated at $1x10^8$ CFU/mL into the same experimental media described for adherent Gc. After 4 h of growth in ultra-low attachment 6-well plates (Corning 3471) at 37 °C in 5% CO_2, Gc was harvested by pooling 6 mL of culture per condition in pre-weighed metal-free conical tubes and pelleted and processed as described for adherent Gc.

2.5.16 Figure generation and statistical analysis.

All graphical figures and statistical analyses were generated using Graphpad Prism 9 for macOS Version 9.2.0, GraphPad Software, San Diego, California, USA, www.graphpad.com. Specific statistical tests used are noted for each figure.

Chapter 3 DISCUSSION

3.1 Perspective

Neisseria gonorrhoeae is an urgent public threat and the causative agent of the second-most frequent bacterial STI in the United States, gonorrhea. Understanding the mechanisms by which Gc overcomes nutritional immunity are valuable for elucidating pathogenesis and improving experimental models. Although the zinc-sequestering proteins psoriasin and calprotectin are abundant in the female reproductive tract, the importance of zinc acquisition for Gc infection of mucosal surfaces was not known.

When this project was initiated, it was known that Gc can acquire essential metals from human proteins through specific TonB-dependent transporters (TdTs) (Cornelissen, Biswas et al. 1992). Furthermore, TdfH, a TdT, is able to bind calprotectin, and Gc can acquire zinc from calprotectin in a TdfH-dependent manner (Jean, Juneau et al. 2016). This was given biological context by the discovery that a *tdfH* mutant had reduced survival when exposed to NETs, which are decorated with calprotectin (Jean, Juneau et al. 2016). My research trajectory was initially influenced by what was known about ZnuD, the TdfJ homolog in *Neisseria meningitidis*. ZnuD had been shown to be able to acquire free zinc (Calmettes, Ing et al. 2015) and to be involved in heme utilization when expressed in *E. coli* (Kumar, Sannigrahi et al. 2012). A major leap forward occurred when preliminary data from Stavros Maurakis in Cynthia Cornelissen's lab showed that WT Gc could use zinc-loaded psoriasin as a zinc source, while a

ΔtdfJ mutant Gc could not (Maurakis, Keller et al. 2019). In this discussion, I highlight some early directions of my research (**3.2**).

Literature evidence showed that calprotectin and psoriasin were both produced and expressed in ectocervical cells (Mildner, Stichenwirth et al. 2010), (Wilkinson, Busuttil et al. 1988, Coleman and Stanley 1994, Uhlen, Fagerberg et al. 2015) and present in significant quantities in cervicovaginal lavage fluid (Birse, Burgener et al. 2013), (Zegels, Van Raemdonck et al. 2009), (Borgdorff, Gautam et al. 2016), which is known to be low in total zinc compared to other body fluids such as serum and seminal fluid (Damjanovich, Sipos et al. 2020), (Takacs, Damjanovich et al. 2020), (Chuang, Lee et al. 2002), (Wong, Flik et al. 2001), (Kothari and Chaudhari 2016). Therefore, I hypothesized that TdfH and TdfJ would be required for Gc infection of Ect1 epithelial cells in the presence of calprotectin and psoriasin, but found this not to be the case. Instead, I discovered that adherence to a surface enables Gc to grow under conditions of zinc sequestration which prevented survival in suspension-grown Gc. This change in survival correlated with a change in gene expression, with approximately 25% of the known Gc genome differentially regulated between adherent and suspension-grown Gc experiencing zinc sequestration. While ZnuA was significantly upregulated in adherent over suspension zinc-sequestered Gc, overexpression of ZnuA was not sufficient to enable enhanced survival of suspension-grown bacteria and recapitulate the phenotype of adherent Gc.

This study is the first to show that adherence enables a bacterium to overcome nutritional immunity, and generates a number of exciting new questions to explore. In the following chapters, I discuss the potential of additional gene regulation for the periplasmic zinc shuttle protein ZnuA (**section 3.3**) and what is known about differential gene expression in Gc adherent to surfaces or in suspension (**section 3.4**). I discuss whether the interplay between adherence and nutritional immunity might be applicable to other conditions of zinc-limitation (**section 3.5**), other anatomical sites (**section 3.6**) or other metals (**section 3.7**). I also discuss questions arising from the RNAseq analysis of genes expressed in adherent vs. suspension Gc in low- or high-zinc conditions, including a surprising finding in the PCA clustering of the RNAseq conditions (**section 3.8**).

Finally, I have generated three non-exclusive hypotheses to explain the mechanism by which adherent Gc are protected from the decline in CFU experienced by suspension Gc when zinc is sequestered. Adherent Gc may require less zinc to survive in their niche relative to suspension-grown (**3.9.1**), or Gc may have more capacity for zinc uptake (**3.9.2**) or mobilization of internal zinc stores (**3.9.3**). I discuss the support for each hypothesis and describe ways in which they might be tested.

3.2 Early hypotheses and exploration of other models

Early on in my thesis work, we did not yet know that psoriasin was the ligand for TdfJ. Therefore, we explored other contexts where TdfJ might be

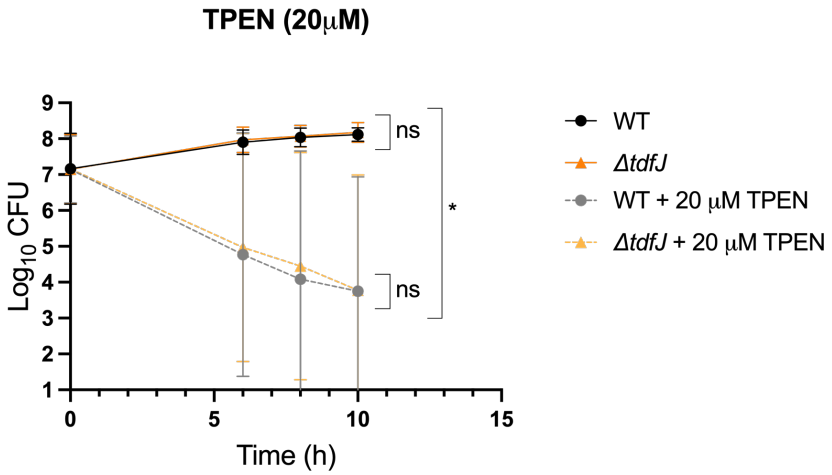
important for zinc uptake. Studies in *Neisseria meningitidis* reported that the homolog of TdfJ, ZnuD, was able to acquire free zinc (Calmettes, Ing et al. 2015). Additionally, Jean *et al* showed that in Gc FA19, *tdfJ* mutant was impaired for growth in GCB + TPEN relative to WT, even when supplemental zinc was added (Jean, Juneau et al. 2016). Once I created a Δ*tdfJ* mutant in FA1090 OpaD+$_{nv}$, Dr. Asya Smirnov tested whether Δ*tdfJ* Gc were impaired when zinc was sequestered by the zinc chelator TPEN. When grown in KSFM, WT and Δ*tdfJ* Gc were equally robust in zinc-rich media and equally sensitive to TPEN-mediated zinc chelation, suggesting that in FA1090, TdfJ is not required for uptake of free zinc (**figure 3.2.1**).

Figure 3.2.1 TdfJ does not enhance Gc survival when zinc is chelated by TPEN

OpaD+$_{NV}$ and OpaD+$_{NV}$ ΔtdfJ grow equivalently KSFM and are equally inhibited by excess TPEN. Log-phase Gc cultures were back-diluted into GCBL + 20 μM TPEN to an OD of 0.07 and cultured for 2.5 h before inoculation at 1*10^7 CFU/mL in 200 μL KSFM + growth factors. Cultures were incubated at 37°C 5% CO_2 in 96-well plates. At inoculation and at the indicated time intervals, 20 μL of culture was serially diluted and plated to determine CFU.

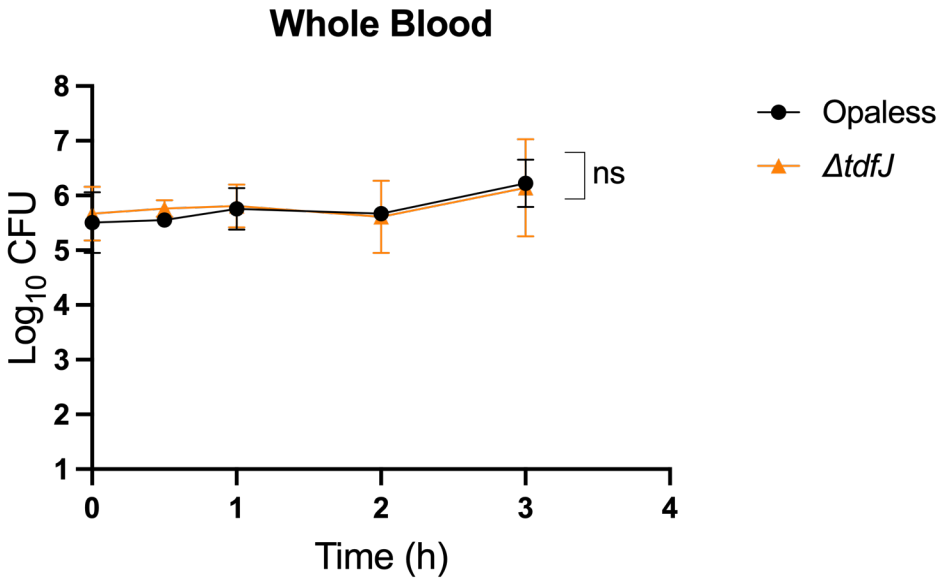
Assay was conducted in duplicate wells on four separate occasions. Ordinary two-way ANOVA with Tukey's multiple comparisons test was conducted in Graphpad Prism. *= p<0.05 ns: p> 0.05

Data courtesy of Asya Smirnov, PhD.



I next explored the possibility that TdfJ might support Gc dissemination and growth in whole blood, given that ZnuD had been reported to enable heme uptake when expressed in *E. coli* (Kumar, Sannigrahi et al. 2012). Again, I found that WT (opaless) and Δ*tdfJ* grew equally well in whole blood (**figure 3.2.2**).

Figure 3.2.2 TdfJ is not required for Gc growth in whole blood
Δopa and Δopa ΔtdfJ grow equivalently in heparinized human blood. Log-phase Gc cultures were back-diluted into GCBL + 20 μM TPEN to an OD of 0.07 and cultured for 2.5 h before inoculation at $1*10^5$ CFU/mL in 200 μL heparinized whole blood recently collected from a healthy human donor. Donors with known prior history of gonorrhoea infection were excluded from study criteria. Cultures were incubated at 37°C in microtubes with continuous rotation to prevent coagulation. At inoculation and at the indicated time intervals, 20 μL of culture was serially diluted and plated to determine CFU.
Assay was conducted in triplicate tubes on four separate occasions. Ordinary two-way ANOVA was conducted in Graphpad Prism. ns: p> 0.05



When Stavros Maurakis showed that the zinc-binding S100 protein psoriasin provided zinc to Gc in a TdfJ- and TonB- dependent manner, I hypothesized that TdfJ is *only* essential for Gc growth when zinc is limited through zinc binding to psoriasin.

Maurakis showed that, in zinc-free media, Gc require TdfJ to use zinc-loaded psoriasin as a zinc source. By contrast, my experiments at the beginning of Chapter 2 tested the hypothesis that psoriasin is capable of sequestering zinc already present in media, and WT Gc can overcome this limitation because they express functional TdfJ. These results are complementary to, but distinct from, Maurakis' experiments because they establish a role for psoriasin as a mediator of zinc-limitation at mucosal surfaces, and demonstrate that Gc expression of TdfJ is involved in overcoming this limitation.

This model has held true and can be extrapolated to apply to TdfH and zinc binding by calprotectin. This was a major paradigm shift in our understanding of TdfJ in Gc and was the foundation for the remainder of the experiments described in **Chapter 2**.

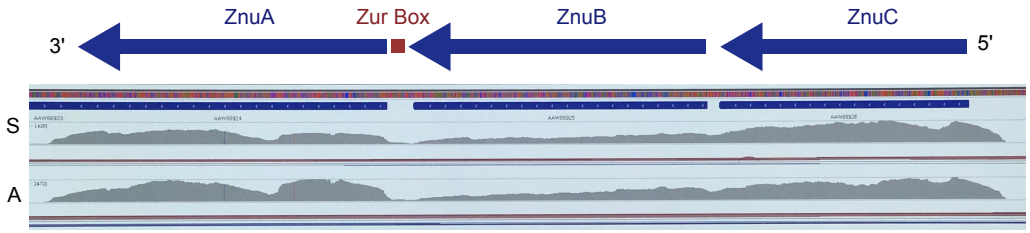
3.3 Observations and speculation about znuA regulation

Four genes emerged from the RNAseq analysis in **Chapter 2** as zinc regulated and also adherence- regulated. These were *rpmE2*, *ngo_1049*, *znuA*, and *fbpA*. Zinc sequestration in both adherent and suspension (comparisons B and C), and adherence when zinc was sequestered (comparison A), induced *znuA*, but not *znuB* or *znuC* **(Figure 2.7.1)**. Despite being the most 3' in the

series of three genes, *znuA* possesses its own promoter and Zur box. Therefore, independent regulation of *znuA* relative to *znuB* and *znuC* is plausible. When sequencing reads were mapped to the Gc genome, the observed pattern is consistent with two separate transcription initiation sites: one upstream of *znuC*, and a second between *znuB* and *znuA* **(figure 3.3.1).**

Figure 3.3.1 RNAseq reads mapping to ZnuC, ZnuB, and ZnuA support the hypothesis that ZnuA is regulated independently from ZnuCB.

Histogram of RNAseq reads mapped to *znuA, znuB,* and *znuC* in the FA1090 Gc genome. Samples: S= suspension zinc sequestered (top) and A= adherent zinc sequestered (bottom). Non-quantitative visualization showing that mapping frequency decreases steadily from the 5' to 3' end of the *znuBC* transcript, followed by an increase in mapping frequency at the 5' end of *znuA*. Additionally, relative frequency of reads mapped to *znuA* increases in Adherent as compared to Suspension zinc sequestered.



Wu *et al* found Zur-dependent regulation of *znuA, znuB,* and *znuC* in Gc strain 1291 (Wu 2006), **Table 1.5.2.1**. However, there is no obvious Zur box within 300bp upstream of *znuC* in the FA1090 genome (**Table 1.5.2.2**). It is therefore possible that, in Gc strain FA1090, *znuA* alone of the three genes is Zur-regulated.

The majority of the previously-reported Gc Zur regulon was differentially expressed due to zinc sequestration in both suspension and adherent Gc. The only genes reported by Wu as differentially expressed which were not detected in this RNAseq were *znuB, znuC, rpmJ2,* and *ngo_0474*. The lack of differential expression of *rpmJ2* was particularly surprising because its start codon overlaps with the stop codon of *rpmE2*, which was highly differentially expressed. In actuality, the signal for *rpmJ2* was likely lost during analysis, because *rpmJ2* is only 126bp, which is less than the 150 bp cutoff used during early quality control steps. Therefore, in the future we will assess *rpmJ2* expression by qPCR to determine whether it is differentially expressed in response to zinc limitation and adherence.

Of the Zur regulon, only *znuA, ngo_1049,* and *rpmE2* were also differentially regulated as a result of adherence to glass. None of the conditions in the RNAseq experiment resulted in differential gene expression of *zur* (NGO_0542). This raises the possibility of additional regulatory mechanisms beyond Zur which may be affecting these transcripts.

3.4 Transcriptional regulation beyond Zur

As introduced in **Chapter 1.2**, attachment of Gc to epithelial cell surfaces is essential for productive infection, regardless of the anatomical site of infection. Few studies have examined the effect of attachment to epithelial cells on Gc gene expression. Notable examples are a report by Du, Lenz, and Arvidson on the role of sigma factors in regulating Gc strain MS11 interactions with the epithelial carcinoma cell line A431 (Du, Lenz et al. 2005), and by Falsetta *et al* on Gc strain 1291 gene expression in biofilms formed on glass under continuous flow conditions (Falsetta, Bair et al. 2009). Interestingly, there is very little overlap in the genes detected as significantly differentially expressed by these two reports. The only gene that was significantly differentially expressed both in one of these prior reports and in this study was NGO_0340 *cysK*, a cysteine synthase which was upregulated in Gc adherent to A431 cells according to Du *et al* and which was higher in adherent vs. suspension Gc and zinc-regulated only in adherent Gc. Du *et al* also observed significant upregulation of the alternative sigma protein *rpoH* (NGO_0288) and of genes with promoters recognized by RpoH, but in this study *rpoH* was not differentially regulated in any of the adherent vs. suspension conditions (Du, Lenz et al. 2005). Of the 13 ORFs previously reported to be upregulated when RpoH was overexpressed, only four (and none of the top 5) were significantly increased in adherent vs. suspension when zinc was limited (Gunesekere, Kahler et al. 2006). These were: *lon* (NGO_0775), *secB* (NGO_0116), *groES* (NGO_2094), and *creA* (NGO_0570).

Besides RpoH, the only other alternative sigma factor characterized in Gc is Ecf (NGO_1944) (Gunesekere, Kahler et al. 2006). When zinc was sequestered, *ecf* was L2FC= 0.4 higher in adherent vs. suspension Gc, and one of its four other target genes, NGO_1947, was upregulated L2FC= 1.37 (Gunesekere, Kahler et al. 2006). However, none of the other Ecf-regulated genes were differentially expressed. For context, the six aforementioned genes that have been reported to be recognized by alternative sigma factors RpoH or Ecf are six out of over 700 genes significantly differently detected between adherent and suspension Gc when zinc was sequestered. Taken together, it is unlikely that an alternative sigma factor is responsible for the majority of the differential expression observed in these studies.

Gc is a facultative anaerobe, and Falsetta *et al* reported that biofilm formation under continuous flow conditions led to induction of genes involved in the anaerobic response, such as *aniA* (NGO_1276), *norB* (NGO_1275), and *ccp* (NGO_1769) (Falsetta, Bair et al. 2009). We therefore hypothesized that adherent Gc might experience anaerobic growth conditions, and that genes involved in the anaerobic response might also lead to increased resistance to zinc sequestration. In fact, we saw precisely the opposite, with adherent Gc expressing significantly lower levels of *aniA* (L2FC Adherent Excess vs. Suspension Excess = -5.32 padj= $1*10^{-12}$). Levels of *aniA* were unaffected by zinc sequestration, however, and therefore do not appear in the table of zinc- and adherence- regulated genes.

The type 4 pilus and Opa proteins are two major adhesion factors in Gc. Hu *et al* recently reported that nonpiliated Gc are more susceptible to killing by streptonigrin, an antibiotic which required intracellular free iron to be active (Hu, Yin et al. 2020). This link between an adhesion factor and metal homestasis was intriguing, however both PilE and Opa proteins seem not to be necessary for resistance to zinc sequestration by adherent Gc **(Figure 2.10.1)**.

Enterohemhorragic *E. coli* senses its mechanical environment (including attachment to stiff surfaces) through GrlA which is membrane-bound until sufficient membrane stress is detected (Sirisaengtaksin, Odem et al. 2020). Gc has no homolog of GrlA, but this model is an example of attachment-mediated gene expression that is not signaled through the adhesion factors themselves. Overall, there is no known explanation for the transcriptional response of Gc to attachment in my RNAseq gene expression experiments. This will be a fascinating subject for future studies.

3.5 Site specific calprotectin and other zinc chelators

As described in **Chapter 1**, calprotectin has two asymmetrical transition metal-binding sites: Site 1 is composed of 6 histidines and binds zinc, copper, or manganese with high affinity. Site 2 has 3 histidines and one asparagine to bind zinc and copper but not manganese **(figure 1.4.3)** (Damo, Kehl-Fie et al. 2013). Kammerman *et al* reported that calprotectin binding by TdfH was affected by point mutations at Site 1 or Site 2. WT human calprotectin had biphasic binding with a 4nM binding coefficient, while Site 1 knockout (S1KO) bound at 1.2 μM

and Site 2 knockout (S2KO) bound at 66nM. The double knockout (dKO Cp) fit a single-site model, binding at 13μM **(Table 3.5.1)**. The binding affinity data showed that Site 1 contributed more strongly to binding between TdfH and calprotectin than did Site 2.

The Site 1 knockout (S1KO) was generated using His-Asn substitutions of the 4 conserved His residues that coordinate zinc at Site 1 (S100A8 H17 and H27; S100A9 H91 and H95). Kammerman *et al* showed that WT Gc could not use S1KO Cp as a sole zinc source. I examined whether S1KO Cp could sequester zinc from WT and *ΔtdfH* Gc grown in suspension (**Figure 3.5.1 A and B**). CFU of both WT **(A)** and *ΔtdfH* **(B)** Gc were decreased when media contained 2μM S1KO Cp, and this decrease was reversed with addition of excess zinc. Zinc-loaded S1KO Cp was unable to support growth of WT Gc in zinc-free CDM, but could lead to reduction of WT CFU in the absence of excess zinc (Kammerman, Bera et al. 2020). Taken together, these results suggest that S1KO Cp retains the ability to bind zinc, but cannot be used by TdfH for zinc acquisition.

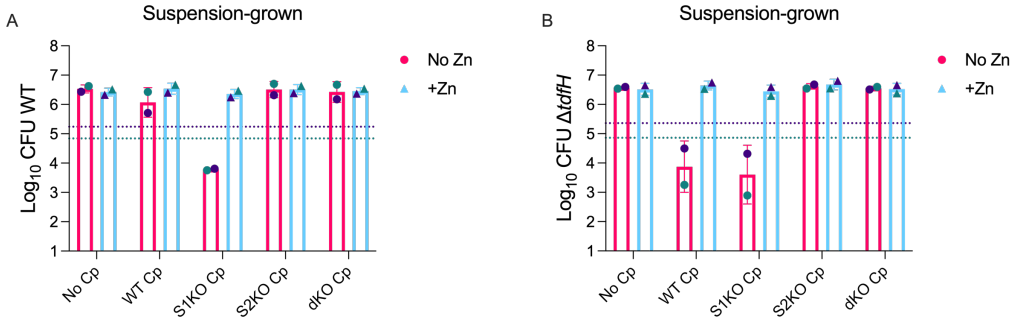
The site 2 KO (S2KO) has 3 His-Asn substitutions for the conserved His residues and an Asp-Ser substitution for the conserved Asp residue of the binding site (S100A8 H83 and H87; S100A8 H20 and D30) (Damo, Kehl-Fie et al. 2013). WT Gc were able to use zinc-loaded WT and S2KO Cp as zinc sources, but not S1KO or dKO (Kammerman, Bera et al. 2020)(**Table 3.5.1**).

Table 3.5.1 Comparing the ability of mutated calprotectin to support Gc growth and sequester zinc.

	Binding to TdfH[a]		Support WT Gc[b]	Restrict WT Gc[c]	Restrict *ΔtdfH* Gc[d]
	High affinity site	Low affinity site	(sole Zn source)	In suspension	In suspension
WT hCp	$4.0x10^{-9}$ M	$3.5x10^{-5}$ M	**Yes**	No	Yes
S1KO hCp	$1.2x10^{-6}$ M	$6.8x10^{-5}$ M	No	**Yes**	**Yes**
S2KO hCp	$6.6x10^{-8}$ M	$6.8x10^{-5}$ M	**Yes**	No	No
dKO hCp[e]	$1.3x10^{-5}$ M		No	No	No
mCp	$7.2x10^{-7}$ M	$5.1x10^{-5}$ M	No	**Yes**	**Yes**

[a]Binding coefficents as reported in Kammerman *et al* 2020. All but dKO hCp best fit a dual-binding site model. "High affinity site" and "low affinity site" simply refer to the higher and lower K_d values, they do not necessarily correspond consistently with Site 1 and Site 2.
[b]Ability to support WT Gc growth as a sole zinc source is based upon zinc-loaded and dialyzed calprotectin provided to WT FA19 Gc grown in suspension in CDM.
[c]Ability to restrict the growth of WT *Gc* in suspension, as described in **Figure 3.5.1A**
[d]Ability to restrict the growth of *ΔtdfH Gc* in suspension, as described in **Figure 3.5.1B**
[e]dKO hCp best fits and individual site model, so only one K_d is reported.

Figure 3.5.1 Site 1 knockout Cp restricts the growth of both WT and *ΔtdfH* suspension-grown Gc in a zinc-dependent manner.
WT (A) or *ΔtdfH* (B) Gc (10^5 CFU/mL) was inoculated into experimental media, KSFM with the following additions: No Cp= KSFM, WT Cp= 1.4 μM human calprotectin, S1KO= 3 μM Site 1 (6his) knockout, S2KO= 3 μM Site 2 knockout, dKO = 3 μM double knockout, +Zn= 6 μM $ZnSO_4$. Gc were grown without adherence for 7 h. Dotted lines indicated inoculum CFU for each experiment. CFU were enumerated from the inoculum (horizontal lines) and cultures after 7 hours. Data from two independent experiments are shown.



Based on these results, I predicted that S2KO Cp would affect WT and Δ*tdfH* Gc similarly to WT calprotectin. Intact TdfH of WT Gc would be able to interact with intact Site 1 on S2KO Cp to acquire zinc, but Δ*tdfH* Gc would have no way to interact with calprotectin. To my surprise, S2KO Cp had no effect on growth of WT or Δ*tdfH* Gc (**Figure 3.5.1**). The disparity between these data and the observations by Kammerman *et al* warrant further investigation. Hypotheses to explain these data include: S2KO Cp may have reduced affinity for zinc at site 1 due to changes in the protein resulting from the loss of Site 2. This looser binding could be adequate to allow increased zinc uptake through TdfH-independent pathways and enable the survival of Δ*tdfH* Gc. Second, the batch of S2KO Cp we received might be defective—the protein could have denatured or the proteins could already contain zinc on arrival and therefore be unable to bind zinc in our media. Calprotectin can also sometimes precipitate out of solution, so the actual concentration in the liquid phase of the protein may have been inaccurate. Through either reduced affinity or partially denatured protein, more S2KO Cp might be required to fully sequester all zinc in the media, and therefore these experiments should be repeated with titration to increase the S2KO Cp concentration.

The double knockout (dKO Cp) contains all 7 His-Asn and 1 Asp-Ser substitutions, eliminating both transition metal binding sites (Kammerman, Bera et al. 2020). Consistent with the predicted inability of this protein to bind zinc, it

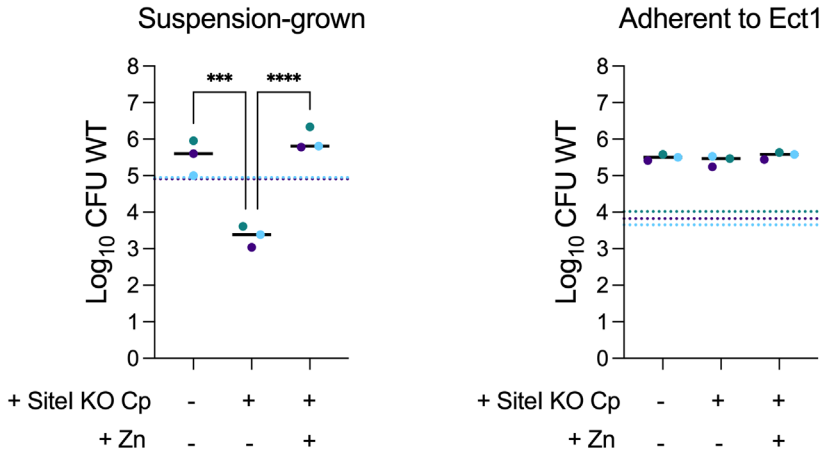
could neither support WT Gc growth in CDM (Kammerman, Bera et al. 2020) nor inhibit WT or Δ*tdfH* Gc growth in KSFM (**Figure 3.5.1**).

A 6.1 Å resolution crystal structure of calprotectin tetramer with TdfH was recently published by Bera *et al* and shows that while both Site 1 and Site 2 interact with TdfH, Site 1 interacts at a location closer to the plug domain (Bera, Wu et al. 2022). I hypothesize that both Site 1 and Site 2 are involved in binding between TdfH and calprotectin, but zinc acquisition by TdfH occurs from Site1.

S1KO Cp, like murine calprotectin, has the ability to sequester zinc from the medium but cannot be used by Gc TdfH as a zinc source. Because S1KO Cp lacks the 6-histidine binding motif required for manganese binding, it is more zinc-specific than WT human or murine calprotectin.

I next tested the effect of zinc sequestration by S1KO Cp on WT Gc when adherent to Ect1 cells. While WT Gc recoverable CFU declined in the presence of S1KO in suspension (**figure 3.5.2A)**, the CFU recovered adherent to Ect1 was not significantly different when S1KO Cp was added or in the presence of excess zinc **(figure 3.5.2B)**.

Figure 3.5.2 WT Gc are protected from zinc sequestration by Site 1 KO calprotectin when adherent to Ect1 cells.
A. WT Gc (10^5 CFU/mL) was inoculated into experimental media: KSFM without growth factors, alone or with addition of 3 μM S1KO Cp (+S1KO Cp) and/or 6 μM $ZnSO_4$ (+Zn) and grown without adherence for 7 h. dotted lines indicated inoculum CFU for each experiment. B. WT Gc (10^5 CFU/mL) in KSFM was inoculated onto confluent Ect1 cells for 2 h. Dotted lines indicate the CFU adherent after 2 h. Non-adherent Gc was washed away, and Gc was incubated an additional 7 h in experimental media described in A. Each color indicates one biological replicate. Asterisks represent p-values from one-way ANOVA with Tukey's multiple comparisons test. ***=p<0.001, ****=p<0.0001.



Overall, the effect of adherence on the ability of Gc to survive zinc sequestration applied to WT when zinc was sequestered by S1KO human calprotectin, murine calprotectin, or TPEN, as well as Δ*tdfH ΔtdfJ* when zinc was sequestered by calprotectin and psoriasin. These results, taken together, suggest that the effect of adherence is not specific to any of these chelators, but may be generalizable to other low-zinc contexts. Further questions include: what is the effect of adherence when Gc are grown in media that has little to no zinc, instead of in the presence of a zinc chelator? Ian Liyayi, a graduate student in the Criss Lab, has pioneered chelex-treated chemically defined medium for Gc growth in our lab. Chelex-100 is an ion-exchange resin with high affinity for transition metals, and can be used to remove transition metals such as zinc, iron, and manganese from the medium (Maurakis, Keller et al. 2019). Chemically-defined medium is preferable to typical tissue culture media because it contains no metal-binding proteins that could retain metal despite performing cation exchange. While this medium may not support Ect1 cell infection, it could be used to assess WT Gc growth in suspension vs. adherent to acid-washed glass coverslips.

3.6 Adherence and zinc restriction at other sites of infection

The female cervix is one of at least 5 distinct mucosal sites where Gc can initiate infection in humans. The others include: urethral epithelium (usually in males), rectal epithelium, pharyngeal epithelium, and the ocular sclera (Workowski, Bachmann et al. 2021). Zinc availability and nutritional immunity

proteins differ at each of these sites, but attachment to epithelial surfaces is an important first step in all infections. The applicability of the results in this thesis to other infection sites is not certain. Many Gc-epithelial cell interactions are cell type-specific due to differing receptor expression (Edwards, Brown et al. 2001, Edwards and Apicella 2004, Yu, Wang et al. 2019) but Gc protection from zinc sequestration was seen when adherent to an abiotic surface. Therefore, I speculate that interaction with specific human cell receptors is not required for this phenotype.

Because seminal plasma contains high levels of free zinc, it could be assumed that Gc during urethral infection experience a zinc-replete environment. Metal content of the normal urethral mucosa is less well known, but high zinc levels typical of seminal fluid could be a transient state to be survived by Gc, rather than the norm. Intriguingly, glandular epididymis tissue stained very highly for psoriasin in immunohistochemical studies and psoriasin is detected in urine of healthy subjects of both sexes, although psoriasin in the male reproductive tract remains relatively unstudied (Uhlen, Fagerberg et al. 2015, Bastian, Roux et al. 2021).

In a study by Nudel *et al,* the only Zur-repressed gene that was differentially expressed between Gc isolated from urethral and cervical specimens was *tdfJ* (Nudel, McClure et al. 2018). Gc isolated from the female cervix contained 5x more reads mapping to *tdfJ* than Gc from male urethral swabs, but *tdfJ* was still detected in all three isolates from male patients. The

remaining Zur-repressed genes were not differentially expressed between samples from the two sexes, and total levels were not reported in the manuscript (Nudel, McClure et al. 2018). However, an earlier paper from the same group reported that *znuA, tdfJ, ngo_1049, tdfH,* and *rpmE2* were detected in Gc isolated from four female subjects (McClure, Nudel et al. 2015) (discussed in **section 1.6**). Because *tdfJ* is iron-induced in addition to being zinc-repressed (Jean, Juneau et al. 2016), its increased expression in women compared to men could be due to increased free iron levels in the female genital tract, rather than a difference in zinc availability to Gc.

In the oral mucosa and tonsils, psoriasin and calprotectin are highly abundant (Meyer, Harder et al. 2008, Uhlen, Fagerberg et al. 2015, Bastian, Roux et al. 2021). Additionally, many pharyngeal commensal *Neisseria* species possess and express homologs of TdfJ, suggesting that zinc acquisition from psoriasin may be important for colonization of the pharynx (Cornelissen and Hollander 2011). Other S100 proteins are able to bind zinc, including A1, A2, A3, A5, A6, A12, A16, and B (Gilston, Skaar et al. 2016). The role that these proteins may play in zinc sequestration, both in the female reproductive tract and at other sites of infection, are unknown but provide interesting avenues for future research. Stavros Maurakis in the Cornelissen lab showed that WT Gc could grow in zinc-free chemically defined media when zinc-loaded S100A2, A4, A6, A12, psoriasin, or calprotectin was the sole zinc source (Stavros Maurakis and Cynthia Cornelissen, unpublished data). S100A12 (also called calgranulin C) has

been crystallized in complex with copper and with zinc, and has been reported to be antimicrobial against *Helicobacter pylori* through zinc sequestration (Moroz, Antson et al. 2003),(Moroz, Burkitt et al. 2009),(Jackson, Little et al. 2017).Gc without functional *tonB* were still able to use zinc-loaded S100A12 as a sole zinc source in zinc-free media (Maurakis, Keller et al. 2019). These results suggest that zinc is acquired from S100A12 in a TonB-independent manner, and further study will be required to elucidate the mechanism. Therefore, other zinc-binding S100 proteins may actually serve as additional zinc resources for Gc, rather than as sources of zinc sequestration.

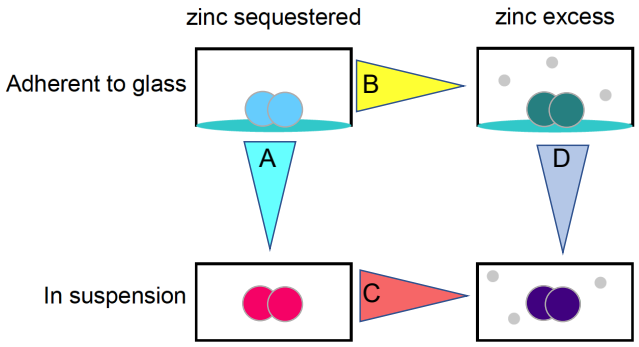
3.7 Does adherence enhance survival when other metals are sequestered?

While Gc survival in the presence of murine calprotectin was not rescued by addition of manganese or copper, it is possible that adherence may affect Gc's susceptibility to sequestration of other metals, such as iron. Many of the genes upregulated in adherent Gc have been previously identified as iron-repressed, including the periplasmic iron shuttle protein FbpA (**Table 3.7.1**).

Table 3.7.1 Known regulation of differentially expressed ORFs

Gene ID	Symbol	Description	Anaerobic down[a]	H_2O_2 up[b]	Fe[c]	Zn[d]	Adherent up[e]	Other[f]
Increased in A, B, C, and D								
NGO_0217	**FbpA**	iron ABC periplasmic shuttle	(+)		(+)			Lrp
Decreased in A, B, C, and D								
NGO_1442	**AdhP**	alcohol dehydrogenase				(-)		
Increased in A, B, and C								
NGO_0930	**RpmE2**	50S Ribosomal protein L31			(+)	(+)		
NGO_1049		hypothetical protein				(+)		
NGO_0168	**ZnuA**	zinc ABC periplasmic shuttle			(+)	(+)		
Increased in A, B, and D								
NGO_0340	**CysK**	cysteine synthase	(+)				(+)	FNR
NGO_0374	**GlnQ**	amino acid ABC transporter ATPase	(+)					FNR FarR
NGO_0639	**LldD**	L-lactate dehydrogenase	(+)		(+)			
Increased in A and B								
NGO_1368	**MtrF**	aminobenzoyl-glutamate transporter	(+)					MtrR FarR
NGO_1318	**HemO**	Heme oxygenase	(+)	(+)	(+)			
NGO_0554		hypothetical protein	(+)	(+)	(+)			

[a] Downregulated in anaerobic conditions (Isabella and Clark 2011)
[b] Upregulated in response to hydrogen peroxide (Stohl, Criss et al. 2005)
[c] Downregulated in response to iron excess (Jackson, Ducey et al. 2010)
[d] Downregulated in response to zinc excess (Pawlik, Hubert et al. 2012) or (Wu, Seib et al. 2006)
[e] Upregulated in response to adherence to A431 cervical carcinoma cells (Du, Lenz et al. 2005)
[f] Other known regulatory factors (Isabella and Clark 2011)

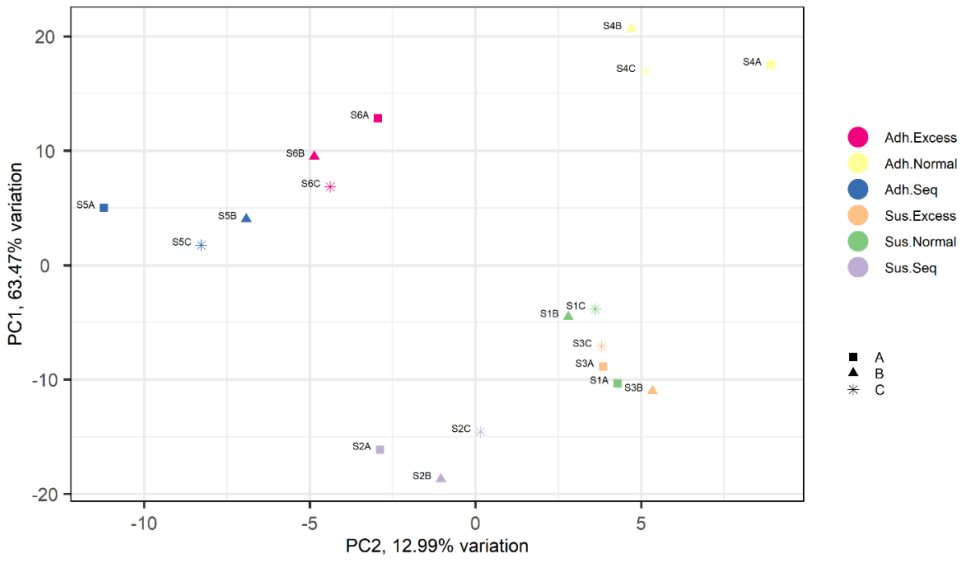


The effect of adherence on iron sequestration can be tested by sequestering iron with bovine transferrin (which Gc cannot use as a zinc source) and determining whether survival is enhanced in adherent Gc compared to suspension-grown Gc. One complication of this method is that the formulation of KSFM contains human transferrin, which will likely enable Gc to continue to acquire iron. In order to circumvent this problem, we can conduct these assays in chemically defined, chelex-treated medium with a known quantity of iron and either bovine or human transferrin added.

3.8 PCA clustering of adherent zinc sequestered and adherent zinc excess

When we analyzed the RNAseq dataset (**Chapter 2**), we compared only the zinc-sequestered (+ murine calprotectin) and zinc-excess (+ murine calprotectin + zinc) conditions. However, we also tested “normal” (untreated KSFM) conditions for both adherent and suspension Gc. There were no significantly differentially regulated genes between the suspension-grown “normal” and “zinc-excess” conditions, which led us to conclude that “zinc-excess” indeed provided enough zinc for normal growth. However, there were 83 differentially regulated ORFs between adherent normal and adherent zinc-excess. Significant genes were used to generate a principal component analysis and all samples were plotted against PC1 and PC2 **(figure 3.8.1)**.

Figure 3.8.1 Principal component analysis plot
Principal component analysis (PCA) plot illustrating relationships between differentially expressed genes in all samples analyzed. PC1, which accounts for the majority of the variability (~63%) appears to be associated with adherence status.
Adherent: Gc were allowed to adhere to acid-washed glass for 2 h before nonadherent Gc were washed away and adherent Gc were allowed to grow in experimental media for another 4 h. **Suspension**: Gc were grown in suspension in 96-well plates, with 250 µL experimental media per well, and grown for 4 h. Experimental media conditions: **Excess**: "Excess zinc": zinc added in molar excess of mCp. KSFM without growth factors + 3 µM mCp + 6 µM $ZnSO_4$ **Normal**: "Normal" media without additives
KSFM without growth factors, **Seq.**: "Sequestered zinc:" mCp was added to sequester free zinc. KSFM without growth factors + 3 µM mCp
Figure generated by Eugene Ke.

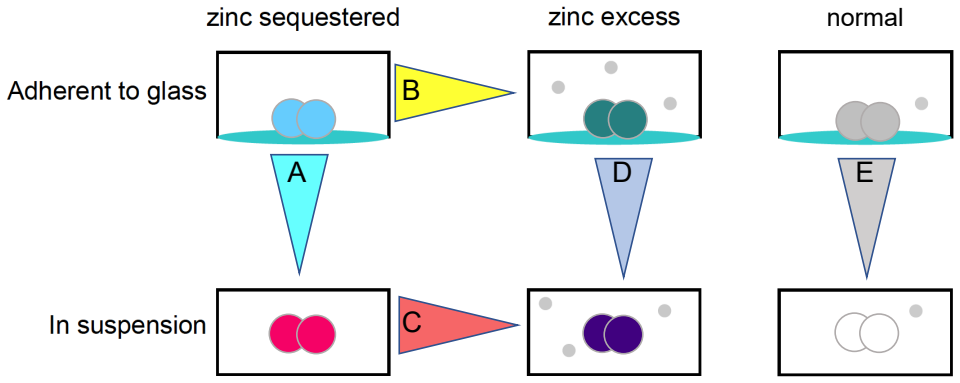


Adherent Excess and Adherent Sequestered clustered together, with Adherent Normal mapping farthest away **(figure 3.8.1).** By contrast, Suspension Normal and Suspension Excess plotted so closely together that they were intermingled.

Some genes whose transcripts were statistically significantly differentially regulated between adherent excess and suspension excess were no longer significant between adherent and suspension in “normal” media, and vice-versa **(Table 3.8.1)**.

Table 3.8.1 Differential gene regulation of in all media conditions

Ensembl_gene	Symbol		L2FC A	L2FC B	L2FC C	L2FC D	L2FC E
Increased in A, B, C, D, and E							
NGO_0217	**FbpA**	iron ABC transporter substrate-binding protein	0.81	0.88	0.64	0.58	0.59
Decreased in A, B, C, and D							
NGO_1442	***AdhP***	*alcohol dehydrogenase*	*-0.81*	*-0.78*	*-1.42*	*-1.40*	*ns*
Increased in A, B, and C							
NGO_0930	**RpmE2**	50S Ribosomal protein L31	0.93	3.34	2.26	*ns*	*ns*
NGO_1049		hypothetical periplasmic zinc-binding protein	0.60	2.33	1.46	*ns*	*ns*
NGO_0168	**ZnuA**	zinc ABC transporter substrate-binding protein	0.50	1.33	0.73	*ns*	*ns*
Increased in A, B, D, and E							
NGO_0340	**cysK**	cysteine synthase	1.97	0.55	*ns*	0.83	3.20
NGO_0639	**LldD**	L-lactate dehydrogenase	0.79	0.72	*ns*	0.53	0.39
Increased in A, B, and D							
NGO_0374	**glnQ**	amino acid ABC transporter ATP binding protein	1.47	0.62	*ns*	0.67	*ns*
Increased in A, B and E							
NGO_1368	**MtrF**	**aminobenzoyl-glutamate transporter**	1.53	0.52	*ns*	*ns*	2.23
NGO_0554		**hypothetical protein**	0.68	1.08	*ns*	*ns*	0.91
Increased in A and B							
NGO_1318	**HemO**	**Heme oxygenase**	0.75	0.74	*ns*	*ns*	*ns*



A: Log_2 fold change adherent zinc sequestered vs. suspension zinc sequestered
B: Log_2 fold change adherent zinc sequestered vs. adherent zinc excess
C: Log_2 fold change suspension zinc sequestered vs. suspension zinc excess
D: Log_2 fold change adherent zinc excess vs. suspension zinc excess
E: Log_2 fold change adherent normal vs. suspension normal
“normal”: KSFM without growth factors
“sequestered”: KSFM with mCp
“excess”: KSFM with mCp and $ZnSO_4$
ns = not significantly differentially expressed (p_{adj} >0.05)

I also made the observation that Gc appeared to be less tightly adherent to the coverslips in both conditions where murine calprotectin was present (sequestered and excess) compared with normal medium. This led me to conclude that murine calprotectin was affecting the ability of Gc to adhere to the coverslips. A similar reduction in adherence was seen when Gc were allowed to adhere in KSFM to which bovine pituitary extract was added, suggesting that additional protein in the media affects the chemistry of the glass and reduces Gc binding efficiency. This reduction in binding in the presence of mCp was not seen in Gc adherent to Ect1 cells, as evidenced by **(Figure 2.2.3)**, where no more detached Gc were recovered in KSFM+Cp+Ps or KSFM+Cp+Ps+Zn than in KSFM alone.

One way to address this potential confounding factor would be to conduct the experiments described in **section 3.5**, using media with extremely low zinc content and determining whether adherence rescues zinc-dependent bacterial death in this context. If this is the case, then RNAseq could be repeated using Gc in suspension or adherent, with or without excess zinc, with no need for zinc-sequestering proteins.

3.9 Three hypotheses for enhanced survival of adherent Gc in zinc-limited conditions

I showed that adherent Gc are able to tolerate zinc sequestration by mammalian nutritional immunity proteins and the zinc-chelator TPEN. Additionally, when zinc was sequestered, adherent Gc demonstrated significantly

different gene expression compared to suspension-grown bacteria. However, the precise mechanism by which adherent Gc are protected from zinc sequestration remains elusive. I propose three non-exclusive hypotheses that can be tested to further understand this phenomenon. First, adherent Gc may occupy a niche that places lower zinc demands on the bacteria. Second, adherent Gc may be able to take up zinc more efficiently when extracellular free zinc levels are low. Third, adherent Gc may have an enhanced ability to mobilize intracellular zinc stores or reallocate zinc to important processes.

3.9.1 Do adherent Gc occupy a niche with lower zinc demands?

One possible explanation for enhanced growth of adherent Gc in the face of zinc sequestration is that they simply require less zinc. Adherent growth in a microcolony presents a new niche, distinct from planktonic growth, which may have different stressors that place less demand on the bacterium for a robust zinc supply. This model is indirectly supported by upregulation of metabolic genes transcripts such as *cysK, glnQ*, and *LldD* which occur in adherent over suspension Gc regardless of zinc status (**Table 3.8.1**) but are more highly expressed in zinc sequestered than excess adherent Gc.

Data which argue against this hypothesis include **figure 2.2.3**, where neither adherent nor detached Gc experienced a reduction of CFU in the presence of zinc-binding proteins, suggesting that formerly attached Gc may retain the protective phenotype that was gained during attachment.

A way to test whether the microenvironment of attachment is responsible for enhanced tolerance for zinc sequestration would be to determine the effect of disrupting colony structure on continued survival in media where zinc is limited.

In the preceding experiments, Gc were always allowed to adhere for two hours to the surface before the Gc that remained in suspension were washed away and the remaining adherent Gc were allowed to proceed through the assay. Gc adherent to coverslips attach loosely enough that scraping and vigorous pipetting is sufficient to detach them. Gc adherent for 2 hours could be detached and transferred to grow in suspension with zinc restriction, and compared to Gc that remain adherent or Gc that grew in suspension for those two hours. If microcolony environment is required to protect adherent Gc, then disrupted colonies should have the same susceptibility to zinc sequestration as suspension-grown.

3.9.2 Do adherent Gc have a greater capacity for zinc uptake than suspension Gc?

We do not currently know whether adherent Gc have an enhanced ability to take up zinc compared to their suspension-grown counterparts. Initially, we attempted to assess comparative zinc uptake using dye reporters that fluoresce when coordinated with zinc, such as Fluozin, which has been used successfully in the Gram-positive bacterium *Streptococcus pneumoniae* previously (Thermo Scientific F24195)(Chimalapati, Cohen et al. 2012). However, we found no staining of Gc with these dyes, regardless of the amount of excess zinc added.

We anticipate that Fluozin staining was unsuccessful because the double-membrane and efflux pumps of Gc prevented the dye from accessing the cytoplasm.

I attempted to instead assess zinc content by ICP-MS (**Chapter 2)** but the data were inconclusive. Zinc content was normalized to bacterial pellet mass, which was unreliable and should be replaced in the future by normalization to sulfur content or some other measure. Additionally, no distinction could be made between zinc that was present prior to the assay vs. zinc that was accumulated in the presence of metal binding proteins while adherent vs. growing in suspension. Bacteria keep their cytoplasmic zinc levels tightly regulated (Capdevila, Wang et al. 2016) and the majority of bacterial zinc is expected to reside as cofactors or structural components of proteins (Blaby-Haas, Furman et al. 2011). Therefore, I predict that the range of zinc quantity that living bacteria are able to tolerate is likely somewhat narrow, and may therefore be difficult to detect. Differences in zinc uptake ability might, therefore, be better assessed by pulse-chase experiments using Zn70 isotope labeled zinc (Zackular, Knippel et al. 2020). Zn70 is a nonradioactive isotope of zinc that is rare (0.6% of zinc) in nature (Cloquet, Carignan et al. 2008). It can be detected via mass spectrometry, with a signal distinct from the most naturally abundant (48.6%) zinc isotope, Zn64. The labeled zinc would be spiked into the system as a soluble salt, and its uptake by adherent vs. suspension-grown bacteria could be assessed after a short time interval by ICP-MS.

An additional benefit of this method is that the ratio of Zn70 to Zn64 could be measured. This would describe the proportion of zinc that was taken up during the assay time period versus present beforehand, and reduce the need to normalize to mass or sulfur. Performing this pulse-chase zinc uptake assay will expand our understanding of zinc uptake in adherent and suspension-grown Gc, and help us select likely molecular mediators from the RNAseq screen for further study.

As described in **Chapter 2,** many Gram-negative and Gram-positive bacteria have been shown to overcome metal limitation by expressing high-affinity ABC metal transporters such as ZnuABC or MntABC(Liu, Jellbauer et al. 2012, D'Orazio, Mastropasqua et al. 2015, Hesse, Lonergan et al. 2019). This was the rationale for overexpressing the periplasmic zinc shuttle protein ZnuA in suspension-grown Gc in (**figure 2.8.1).** However, other possibilities exist, including synergy or interaction between ZnuA and NGO_1049 to enhance zinc uptake, or the involvement of other transport proteins.

NGO_1049 is a predicted periplasmic protein with a zinc-binding site (unpublished data, Ian Liyayi, Aloke Bera, Nicholas Noinaj and Alison Criss). NGO_1049, like ZnuA, was upregulated in both suspension and adherent Gc by zinc sequestration, but was also more highly expressed in adherent and zinc-sequestered than suspension and zinc-sequestered Gc. It is possible that ZnuA and NGO_1049 synergize to enhance zinc uptake and improve survival in the face of zinc sequestration. In *E. coli*, a second periplasmic protein, in addition to

ZnuA, enhances zinc uptake, ZinT (Gabbianelli, Scotti et al. 2011). NGO_1049 does not exhibit homology with ZinT (IL and AKC, unpublished data) but this provides a paradigm for accessory zinc shuttle proteins.

The periplasmic ferric iron (Fe^{3+}) transporter FbpA was also upregulated in Gc adherent to glass vs. in suspension when zinc was sequestered. One of the reasons zinc is considered toxic in high concentrations is its ability to replace other metals in their binding sites (Auld 2001). Although a role for FbpA in zinc acquisition has not been previously reported, it is possible FbpA is able to moonlight as a zinc transporter and enhance the survival of adherent Gc. This could be tested by insertional deletion of *fbpA* and assessing adherent Gc growth when zinc is sequestered but iron is abundant. If deletion of *fbpA* decreases the survival of adherent Gc experiencing zinc sequestration, then further Zn_{70} studies ICP-MS should be conducted to verify that Gc with intact *fbpA* take up zinc more efficiently than the *fbpA* mutants.

3.9.3 Are internal zinc stores greater or more readily accessed in adherent Gc?

As described in **Chapter 1**, bacteria are predicted to have cytoplasmic free zinc concentrations in the femtomolar range, while calculations based on total zinc content and volume suggest the total zinc concentration is in the hundreds of micromolar (Capdevila, Wang et al. 2016). This means that the majority of zinc in the bacterial cell is incorporated into proteins or otherwise sequestered. Studies in *Bacillus subtilis* and *E. coli* have shown that, under zinc

limitation, these bacteria mobilize zinc bound to zinc-dependent ribosomal proteins, such as RpmE, by expressing alternative non-zinc bound paralogs that are repressed by Zur.

The steady-state L31 protein, which binds zinc, is called RpmE. Names for the Zur-repressed paralog vary, but I will refer to them as “RpmE2” for consistency with the nomenclature in Gc. The literature on L31 paralog cycling in *E. coli and B. subtilis* suggests that in these bacteria, expression of RpmE2 has evolved to increase zinc availability, rather than to maintain ribosome functioning in low-zinc environments.

In *E. coli,* L31 is not essential for survival, but bacteria lacking either type of L31 exhibit reduced growth (Ueta, Wada et al. 2020). Ribosomes lacking L31 exhibited a 40% reduction in activity, but ribosomes containing either RpmE or RpmE2 showed the same degree of functionality (Ueta, Wada et al. 2020). Additionally, when both proteins are expressed simultaneously, 80-90% of ribosomes contain RpmE2 and 5-10% contain RpmE, suggesting that RpmE2 has higher affinity for the ribosome. In *Bacillus subtilis,* Akanuma et al demonstrated that the RpmE2 has a much higher affinity for the ribosome than RpmE. When RpmE2 was overexpressed *in vivo*, rapid degradation of RpmE was observed. When RpmE-containing ribosomes were purified and exposed to RpmE2 *in vitro,* RpmE2 replaced RpmE on the ribosomes (Akanuma, Nanamiya et al. 2006). These data, taken together, imply that RpmE and RpmE2 are functionally equivalent, and that when both are present, RpmE2 outcompetes

RpmE for binding to the ribosome, leading to degradation of RpmE. The two types of ribosomes also functioned equivalently when zinc levels were low, suggesting that RpmE2 paralogs are not an adaptation to maintain ribosome functioning when cytoplasmic zinc concentrations drop. Instead, Akanuma and others hypothesized that RpmE functions as a storage depot for zinc when zinc is abundant. When zinc levels drop such that Zur repression is removed, RpmE2 is transcribed and translated to displace RpmE on the ribosome, causing RpmE's degradation and the liberation of bound zinc (**figure 1.5.3**).

Gabriel and Helmann tested this hypothesis by testing what occurs when bacteria cannot switch from RpmE to RpmE2 upon zinc starvation. They insertionally deleted both *rpmE* and *rpmE2*, and then cloned each back in under a constitutive promoter in separate strains. They found that when bacteria constitutively expressed either RpmE or RpmE2 and were moved from rich media into conditions of zinc starvation, they had a growth defect compared to bacteria that could switch between the two (Gabriel and Helmann 2009).

The role of RpmJ and RpmJ2 has been less clearly defined. When both RpmJ and RpmJ2 were expressed in *E. coli*, only 20% of ribosomes were occupied with RpmJ2 compared to 60% occupied with RpmJ (Ueta, Wada et al. 2020). This suggests that RpmJ2 is not as adept at displacing RpmJ as RpmE2 is at displacing its paralog. One possibility is that this provides a slower-release zinc pool compared to early release from RpmE. Overall, these zinc-binding and non-zinc binding ribosomal subunits have been suggested to act as a "ribosomal

switchboard" to fine-tune the zinc available in the bacterium in response to zinc levels (Cheng-Guang and Gualerzi 2020). In this study, one of the genes found to be upregulated by zinc sequestration as well as adherence (under zinc sequestration) was *rpmE2* (NGO_0930), the non-zinc binding paralog of L31 ribosomal protein RpmE. Interestingly, *rpmE* (NGO_2126) was upregulated by adherence whether or not zinc was limited. This opens the door for a new model of enhanced survival under zinc limitation by adherent Gc: adherent Gc increase their expression of *rpmE* during the 2 h adherence phase of the experiment, and accumulate zinc on their ribosomes. When the media is changed and zinc is now sequestered, adherent Gc would possess more zinc on their ribosomes than the suspension-grown Gc that have been inoculated directly into experimental media from TPEN plates. Additionally, *rpmE2* is upregulated in adherent Gc once they sense zinc limitation, and RpmE2 replaces RpmE to make zinc accessible. This hypothesized model is testable, and I propose to do so using the following methods:

The first step would be to generate an *rpmE2* knockout strain, which could be achieved by cloning an Ω spectinomycin resistance cassette into *rpmE2* to disrupt the gene. This may disrupt *rpmJ2* as well, because the two genes appear to be in an operon together, with the stop codon of *rpmE2* overlapping by one nucleotide with the start codon of *rpmJ2*.

If the *rpmE2* knockout is still able to survive in the presence of murine calprotectin while adherent to glass coverslips, then ribosomal zinc store

mobilization is unlikely to explain the phenotype. If, however, the mutant is no longer protected, additional studies should be undertaken.

For instance, complementing the mutant with *rpmE2* on a plasmid with a lac promoter would not be predicted to restore protection, because constitutive RpmE2 expression would prevent zinc from accumulating on the ribosomes in association with RpmE. By contrast, complementation with *rpmE2* with its native (zur-repressed) promoter should restore protection.

These studies would be facilitated by creation of anti-RpmE2 and anti-RpmJ2 antibodies so that protein expression may be verified by Western blot and we can determine whether *rpmJ2* expression is also disrupted.

3.10 Final conclusions:

In this body of research, I showed that adherent *Neisseria gonorrhoeae* are less susceptible to growth restriction due to zinc sequestration by nutritional immunity proteins. To our knowledge, this is the first study in which a pathogen has been shown to gain protection from zinc sequestration through adherence. My results expand the field's understanding of zinc dynamics during infection, expression changes in adherent bacteria, and the role of zinc uptake proteins at mucosal surfaces. Additionally, these studies have generated numerous new questions ranging from zinc stores and metal homeostasis in Gc, sensing of adherence and its effect on Gc biology, transcriptional regulation of zinc uptake proteins, and the potential role of thus far uncharacterized proteins in zinc uptake.

References

Abergel, R. J., M. K. Wilson, J. E. Arceneaux, T. M. Hoette, R. K. Strong, B. R. Byers and K. N. Raymond (2006). "Anthrax pathogen evades the mammalian immune system through stealth siderophore production." Proc Natl Acad Sci U S A **103**(49): 18499-18503.

Abtin, A., L. Eckhart, M. Mildner, F. Gruber, J. M. Schroder and E. Tschachler (2008). "Flagellin is the principal inducer of the antimicrobial peptide S100A7c (psoriasin) in human epidermal keratinocytes exposed to Escherichia coli." FASEB J **22**(7): 2168-2176.

Aisen, P., A. Leibman and J. Zweier (1978). "Stoichiometric and site characteristics of the binding of iron to human transferrin." J Biol Chem **253**(6): 1930-1937.

Akanuma, G., H. Nanamiya, Y. Natori, N. Nomura and F. Kawamura (2006). "Liberation of zinc-containing L31 (RpmE) from ribosomes by its paralogous gene product, YtiA, in Bacillus subtilis." J Bacteriol **188**(7): 2715-2720.

Alker, W. and H. Haase (2018). "Zinc and Sepsis." Nutrients **10**(8).

Anderson, J. E., P. A. Leone, W. C. Miller, C. Chen, M. M. Hobbs and P. F. Sparling (2001). "Selection for expression of the gonococcal hemoglobin receptor during menses." J Infect Dis **184**(12): 1621-1623.

Anderson, M. T., L. Byerly, M. A. Apicella and H. S. Seifert (2016). "Seminal Plasma Promotes Neisseria gonorrhoeae Aggregation and Biofilm Formation." J Bacteriol **198**(16): 2228-2235.

Anderson, M. T., L. Dewenter, B. Maier and H. S. Seifert (2014). "Seminal plasma initiates a Neisseria gonorrhoeae transmission state." mBio **5**(2): e01004-01013.

Andrade, W. A., S. Agarwal, S. Mo, S. A. Shaffer, J. P. Dillard, T. Schmidt, V. Hornung, K. A. Fitzgerald, E. A. Kurt-Jones and D. T. Golenbock (2016). "Type I Interferon Induction by Neisseria gonorrhoeae: Dual Requirement of Cyclic GMP-AMP Synthase and Toll-like Receptor 4." Cell Rep **15**(11): 2438-2448.

Andreini, C., L. Banci, I. Bertini, S. Elmi and A. Rosato (2007). "Non-heme iron through the three domains of life." Proteins **67**(2): 317-324.

Andreini, C., L. Banci, I. Bertini and A. Rosato (2006). "Counting the zinc-proteins encoded in the human genome." J Proteome Res **5**(1): 196-201.

Andreini, C., L. Banci, I. Bertini and A. Rosato (2008). "Occurrence of copper proteins through the three domains of life: a bioinformatic approach." J Proteome Res **7**(1): 209-216.

Andreini, C., I. Bertini, G. Cavallaro, G. L. Holliday and J. M. Thornton (2008). "Metal ions in biological catalysis: from enzyme databases to general principles." J Biol Inorg Chem **13**(8): 1205-1218.

Andreini, C. B., L.; Bertini, I.; Rosato, A. (2006). "Zinc through the Three Domains of Life." Journal of Proteome Research **5**: 3173-3178.

Andrews, S. (2010). "FastQC: A Quality Control Tool for High Throughput Sequence Data." Retrieved July 2021, from **https://www.bioinformatics.babraham.ac.uk/projects/fastqc/**.

Anjem, A., S. Varghese and J. A. Imlay (2009). "Manganese import is a key element of the OxyR response to hydrogen peroxide in Escherichia coli." Mol Microbiol **72**(4): 844-858.

Apicella, M. A., M. Ketterer, F. K. Lee, D. Zhou, P. A. Rice and M. S. Blake (1996). "The pathogenesis of gonococcal urethritis in men: confocal and immunoelectron microscopic analysis of urethral exudates from men infected with Neisseria gonorrhoeae." J Infect Dis **173**(3): 636-646.

Atack, J. M., I. Ibranovic, C. L. Ong, K. Y. Djoko, N. H. Chen, R. Vanden Hoven, M. P. Jennings, J. L. Edwards and A. G. McEwan (2014). "A role for lactate dehydrogenases in the survival of Neisseria gonorrhoeae in human polymorphonuclear leukocytes and cervical epithelial cells." J Infect Dis **210**(8): 1311-1318.

Auld, D. S. (2001). "Zinc coordination sphere in biochemical zinc sites." Biometals **14**(3-4): 271-313.

Aydemir, T. B., S. M. Chang, G. J. Guthrie, A. B. Maki, M. S. Ryu, A. Karabiyik and R. J. Cousins (2012). "Zinc transporter ZIP14 functions in hepatic zinc, iron and glucose homeostasis during the innate immune response (endotoxemia)." PLoS One **7**(10): e48679.

Baarda, B. I., R. A. Zielke, A. K. Holm and A. E. Sikora (2021). "Comprehensive Bioinformatic Assessments of the Variability of Neisseria gonorrhoeae Vaccine Candidates." mSphere **6**(1).

Ball, L. M. and A. K. Criss (2013). "Constitutively Opa-expressing and Opa-deficient neisseria gonorrhoeae strains differentially stimulate and survive exposure to human neutrophils." J Bacteriol **195**(13): 2982-2990.

Bando, M., Y. Hiroshima, M. Kataoka, M. C. Herzberg, K. F. Ross, Y. Shinohara, T. Yamamoto, T. Nagata and J. Kido (2010). "Modulation of calprotectin in human keratinocytes by keratinocyte growth factor and interleukin-1alpha." Immunol Cell Biol **88**(3): 328-333.

Bastian, F. B., J. Roux, A. Niknejad, A. Comte, S. S. Fonseca Costa, T. M. de Farias, S. Moretti, G. Parmentier, V. R. de Laval, M. Rosikiewicz, J. Wollbrett, A. Echchiki, A. Escoriza, W. H. Gharib, M. Gonzales-Porta, Y. Jarosz, B. Laurenczy, P. Moret, E. Person, P. Roelli, K. Sanjeev, M. Seppey and M. Robinson-Rechavi (2021). "The Bgee suite: integrated curated expression atlas and comparative transcriptomics in animals." Nucleic Acids Res **49**(D1): D831-D847.

Becker, K. W. and E. P. Skaar (2014). "Metal limitation and toxicity at the interface between host and pathogen." FEMS Microbiol Rev **38**(6): 1235-1249.

Behnsen, J. and M. Raffatellu (2016). "Siderophores: More than Stealing Iron." mBio **7**(6).

Bennett, D. R., C. J. Baird, K. M. Chan, P. F. Crookes, C. G. Bremner, M. M. Gottlieb and W. Y. Naritoku (1997). "Zinc toxicity following massive coin ingestion." Am J Forensic Med Pathol **18**(2): 148-153.

Bera, A. K., R. Wu, S. Harrison, C. N. Cornelissen, W. J. Chazin and N. Noinaj (2022). "TdfH selectively binds metal-loaded tetrameric calprotectin for zinc import." Commun Biol **5**(1): 103.

Besold, A. N., B. A. Gilston, J. N. Radin, C. Ramsoomair, E. M. Culbertson, C. X. Li, B. P. Cormack, W. J. Chazin, T. E. Kehl-Fie and V. C. Culotta (2018). "Role of Calprotectin in Withholding Zinc and Copper from Candida albicans." Infect Immun **86**(2).

Bettelli, E., Y. Carrier, W. Gao, T. Korn, T. B. Strom, M. Oukka, H. L. Weiner and V. K. Kuchroo (2006). "Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells." Nature **441**(7090): 235-238.

Bhattacharyya, M. N., A. E. Jephcott and R. S. Morton (1973). "Diagnosis of gonorrhoea in women: comparison of sampling sites." Br Med J **2**(5869): 748-750.

Birse, K. M., A. Burgener, G. R. Westmacott, S. McCorrister, R. M. Novak and T. B. Ball (2013). "Unbiased proteomics analysis demonstrates significant variability in mucosal immune factor expression depending on the site and method of collection." PLoS One **8**(11): e79505.

Blaby-Haas, C. E., R. Furman, D. A. Rodionov, I. Artsimovitch and V. de Crecy-Lagard (2011). "Role of a Zn-independent DksA in Zn homeostasis and stringent response." Mol Microbiol **79**(3): 700-715.

Blaskewicz, C. D., J. Pudney and D. J. Anderson (2011). "Structure and function of intercellular junctions in human cervical and vaginal mucosal epithelia." Biol Reprod **85**(1): 97-104.

Bobrov, A. G., O. Kirillina, J. D. Fetherston, M. C. Miller, J. A. Burlison and R. D. Perry (2014). "The Yersinia pestis siderophore, yersiniabactin, and the ZnuABC system both contribute to zinc acquisition and the development of lethal septicaemic plague in mice." Mol Microbiol **93**(4): 759-775.

Bobrov, A. G., O. Kirillina, M. Y. Fosso, J. D. Fetherston, M. C. Miller, T. T. VanCleave, J. A. Burlison, W. K. Arnold, M. B. Lawrenz, S. Garneau-Tsodikova and R. D. Perry (2017). "Zinc transporters YbtX and ZnuABC are required for the virulence of Yersinia pestis in bubonic and pneumonic plague in mice." Metallomics **9**(6): 757-772.

Borgdorff, H., R. Gautam, S. D. Armstrong, D. Xia, G. F. Ndayisaba, N. H. van Teijlingen, T. B. Geijtenbeek, J. M. Wastling and J. H. van de Wijgert (2016). "Cervicovaginal microbiome dysbiosis is associated with proteome changes related to alterations of the cervicovaginal mucosal barrier." Mucosal Immunol **9**(3): 621-633.

Brodersen, D. E., J. Nyborg and M. Kjeldgaard (1999). "Zinc-binding site of an S100 protein revealed. Two crystal structures of Ca2+-bound human

psoriasin (S100A7) in the Zn2+-loaded and Zn2+-free states." Biochemistry **38**(6): 1695-1704.

Brophy, M. B., J. A. Hayden and E. M. Nolan (2012). "Calcium ion gradients modulate the zinc affinity and antibacterial activity of human calprotectin." J Am Chem Soc **134**(43): 18089-18100.

Brophy, M. B., T. G. Nakashige, A. Gaillard and E. M. Nolan (2013). "Contributions of the S100A9 C-terminal tail to high-affinity Mn(II) chelation by the host-defense protein human calprotectin." J Am Chem Soc **135**(47): 17804-17817.

Brunham, R. C., S. L. Gottlieb and J. Paavonen (2015). "Pelvic inflammatory disease." N Engl J Med **372**(21): 2039-2048.

Bryborn, M., M. Adner and L. O. Cardell (2005). "Psoriasin, one of several new proteins identified in nasal lavage fluid from allergic and non-allergic individuals using 2-dimensional gel electrophoresis and mass spectrometry." Respir Res **6**: 118.

Bushnell, B. (2014). "BBMap: A Fast, Accurate, Splice-Aware Aligner." Retrieved July 2021, from **https://www.osti.gov/servlets/purl/1241166**.

Cahoon, L. A. and H. S. Seifert (2009). "An alternative DNA structure is necessary for pilin antigenic variation in Neisseria gonorrhoeae." Science **325**(5941): 764-767.

Calmettes, C., C. Ing, C. M. Buckwalter, M. El Bakkouri, C. Chieh-Lin Lai, A. Pogoutse, S. D. Gray-Owen, R. Pomes and T. F. Moraes (2015). "The molecular mechanism of Zinc acquisition by the neisserial outer-membrane transporter ZnuD." Nat Commun **6**: 7996.

Capdevila, D. A., J. Wang and D. P. Giedroc (2016). "Bacterial Strategies to Maintain Zinc Metallostasis at the Host-Pathogen Interface." J Biol Chem **291**(40): 20858-20868.

Capel, E., A. L. Zomer, T. Nussbaumer, C. Bole, B. Izac, E. Frapy, J. Meyer, H. Bouzinba-Segard, E. Bille, A. Jamet, A. Cavau, F. Letourneur, S. Bourdoulous, T. Rattei, X. Nassif and M. Coureuil (2016). "Comprehensive Identification of Meningococcal Genes and Small Noncoding RNAs Required for Host Cell Colonization." mBio **7**(4).

Carson, S. D., P. E. Klebba, S. M. Newton and P. F. Sparling (1999). "Ferric enterobactin binding and utilization by Neisseria gonorrhoeae." J Bacteriol **181**(9): 2895-2901.

Cassat, J. E. and E. P. Skaar (2013). "Iron in infection and immunity." Cell Host Microbe **13**(5): 509-519.

Centers for Disease, C. and Prevention (2013). "CDC Grand Rounds: the growing threat of multidrug-resistant gonorrhea." MMWR Morb Mortal Wkly Rep **62**(6): 103-106.

Chai, F., A. Q. Truong-Tran, A. Evdokiou, G. P. Young and P. D. Zalewski (2000). "Intracellular zinc depletion induces caspase activation and p21 Waf1/Cip1 cleavage in human epithelial cell lines." J Infect Dis **182 Suppl 1**: S85-92.

Chaturvedi, K. S., C. S. Hung, J. R. Crowley, A. E. Stapleton and J. P. Henderson (2012). "The siderophore yersiniabactin binds copper to protect pathogens during infection." Nat Chem Biol **8**(8): 731-736.

Chen, C., X. Song, W. Wei, H. Zhong, J. Dai, Z. Lan, F. Li, X. Yu, Q. Feng, Z. Wang, H. Xie, X. Chen, C. Zeng, B. Wen, L. Zeng, H. Du, H. Tang, C. Xu, Y. Xia, H. Xia, H. Yang, J. Wang, J. Wang, L. Madsen, S. Brix, K. Kristiansen, X. Xu, J. Li, R. Wu and H. Jia (2017). "The microbiota continuum along the female reproductive tract and its relation to uterine-related diseases." Nat Commun **8**(1): 875.

Chen, C. Y. and S. A. Morse (2001). "Identification and characterization of a high-affinity zinc uptake system in Neisseria gonorrhoeae." FEMS Microbiol Lett **202**(1): 67-71.

Cheng-Guang, H. and C. O. Gualerzi (2020). "The Ribosome as a Switchboard for Bacterial Stress Response." Front Microbiol **11**: 619038.

Chimalapati, S., J. M. Cohen, E. Camberlein, N. MacDonald, C. Durmort, T. Vernet, P. W. Hermans, T. Mitchell and J. S. Brown (2012). "Effects of deletion of the Streptococcus pneumoniae lipoprotein diacylglyceryl transferase gene lgt on ABC transporter function and on growth in vivo." PLoS One **7**(7): e41393.

Cho, Y. E., R. A. Lomeda, S. H. Ryu, J. H. Lee, J. H. Beattie and I. S. Kwun (2007). "Cellular Zn depletion by metal ion chelators (TPEN, DTPA and chelex resin) and its application to osteoblastic MC3T3-E1 cells." Nutr Res Pract **1**(1): 29-35.

Chuang, I. C., P. N. Lee, T. H. Lin and G. S. Chen (2002). "Determination of some elements in the cervical mucus of healthy Taiwanese women, by GF-AAS." Biol Trace Elem Res **86**(2): 137-143.

Clapham, D. E. (2007). "Calcium signaling." Cell **131**(6): 1047-1058.

Cloquet, C., J. Carignan, M. F. Lehmann and F. Vanhaecke (2008). "Variation in the isotopic composition of zinc in the natural environment and the use of zinc isotopes in biogeosciences: a review." Anal Bioanal Chem **390**(2): 451-463.

Coleman, N. and M. A. Stanley (1994). "Expression of the myelomonocytic antigens CD36 and L1 by keratinocytes in squamous intraepithelial lesions of the cervix." Hum Pathol **25**(1): 73-79.

Colvin, R. A., W. R. Holmes, C. P. Fontaine and W. Maret (2010). "Cytosolic zinc buffering and muffling: their role in intracellular zinc homeostasis." Metallomics **2**(5): 306-317.

Corbin, B. D., E. H. Seeley, A. Raab, J. Feldmann, M. R. Miller, V. J. Torres, K. L. Anderson, B. M. Dattilo, P. M. Dunman, R. Gerads, R. M. Caprioli, W. Nacken, W. J. Chazin and E. P. Skaar (2008). "Metal chelation and inhibition of bacterial growth in tissue abscesses." Science **319**(5865): 962-965.

Cornelissen, C. N. (2018). "Subversion of nutritional immunity by the pathogenic Neisseriae." Pathog Dis **76**(1).

Cornelissen, C. N., G. D. Biswas, J. Tsai, D. K. Paruchuri, S. A. Thompson and P. F. Sparling (1992). "Gonococcal transferrin-binding protein 1 is required for transferrin utilization and is homologous to TonB-dependent outer membrane receptors." J Bacteriol **174**(18): 5788-5797.

Cornelissen, C. N. and A. Hollander (2011). "TonB-Dependent Transporters Expressed by Neisseria gonorrhoeae." Front Microbiol **2**: 117.

Cornelissen, C. N., M. Kelley, M. M. Hobbs, J. E. Anderson, J. G. Cannon, M. S. Cohen and P. F. Sparling (1998). "The transferrin receptor expressed by gonococcal strain FA1090 is required for the experimental infection of human male volunteers." Mol Microbiol **27**(3): 611-616.

Costello, L. C. and R. B. Franklin (2016). "A comprehensive review of the role of zinc in normal prostate function and metabolism; and its implications in prostate cancer." Arch Biochem Biophys **611**: 100-112.

Criss, A. K. and H. S. Seifert (2012). "A bacterial siren song: intimate interactions between Neisseria and neutrophils." Nat Rev Microbiol **10**(3): 178-190.

Crouch, M. L., M. Castor, J. E. Karlinsey, T. Kalhorn and F. C. Fang (2008). "Biosynthesis and IroC-dependent export of the siderophore salmochelin are essential for virulence of Salmonella enterica serovar Typhimurium." Mol Microbiol **67**(5): 971-983.

Cunden, L. S., M. B. Brophy, G. E. Rodriguez, H. A. Flaxman and E. M. Nolan (2017). "Biochemical and Functional Evaluation of the Intramolecular Disulfide Bonds in the Zinc-Chelating Antimicrobial Protein Human S100A7 (Psoriasin)." Biochemistry **56**(43): 5726-5738.

D'Orazio, M., M. C. Mastropasqua, M. Cerasi, F. Pacello, A. Consalvo, B. Chirullo, B. Mortensen, E. P. Skaar, D. Ciavardelli, P. Pasquali and A. Battistoni (2015). "The capability of Pseudomonas aeruginosa to recruit zinc under conditions of limited metal availability is affected by inactivation of the ZnuABC transporter." Metallomics **7**(6): 1023-1035.

Damjanovich, P., A. G. Sipos, K. Larson, T. D. Cunningham, P. Takacs and B. Kozma (2020). "Cervicovaginal lavage fluid zinc level as a marker of vaginal atrophy." Menopause **27**(7): 776-779.

Damo, S. M., T. E. Kehl-Fie, N. Sugitani, M. E. Holt, S. Rathi, W. J. Murphy, Y. Zhang, C. Betz, L. Hench, G. Fritz, E. P. Skaar and W. J. Chazin (2013). "Molecular basis for manganese sequestration by calprotectin and roles in the innate immune response to invading bacterial pathogens." Proc Natl Acad Sci U S A **110**(10): 3841-3846.

de Jonge, M. I., H. J. Hamstra, L. van Alphen, J. Dankert and P. van der Ley (2003). "Mapping the binding domains on meningococcal Opa proteins for CEACAM1 and CEA receptors." Mol Microbiol **50**(3): 1005-1015.

DeJong, A. (2018). "Genome2D Webserver." 2.0. Retrieved 10-29-21, from **http://genome2d.molgenrug.nl/index.html**.

Detels, R., A. M. Green, J. D. Klausner, D. Katzenstein, C. Gaydos, H. Handsfield, W. Pequegnat, K. Mayer, T. D. Hartwell and T. C. Quinn (2011). "The incidence and correlates of symptomatic and asymptomatic

Chlamydia trachomatis and Neisseria gonorrhoeae infections in selected populations in five countries." Sex Transm Dis **38**(6): 503-509.

Diaz-Ochoa, V. E., S. Jellbauer, S. Klaus and M. Raffatellu (2014). "Transition metal ions at the crossroads of mucosal immunity and microbial pathogenesis." Front Cell Infect Microbiol **4**: 2.

Diaz-Ochoa, V. E., D. Lam, C. S. Lee, S. Klaus, J. Behnsen, J. Z. Liu, N. Chim, S. P. Nuccio, S. G. Rathi, J. R. Mastroianni, R. A. Edwards, C. M. Jacobo, M. Cerasi, A. Battistoni, A. J. Ouellette, C. W. Goulding, W. J. Chazin, E. P. Skaar and M. Raffatellu (2016). "Salmonella Mitigates Oxidative Stress and Thrives in the Inflamed Gut by Evading Calprotectin-Mediated Manganese Sequestration." Cell Host Microbe **19**(6): 814-825.

Dillard, J. P. (2011). "Genetic Manipulation of Neisseria gonorrhoeae." Curr Protoc Microbiol **Chapter 4**: Unit4A 2.

DiPaolo, J. A., N. C. Popescu, L. Alvarez and C. D. Woodworth (1993). "Cellular and molecular alterations in human epithelial cells transformed by recombinant human papillomavirus DNA." Crit Rev Oncog **4**(4): 337-360.

Dobin, A., C. A. Davis, F. Schlesinger, J. Drenkow, C. Zaleski, S. Jha, P. Batut, M. Chaisson and T. R. Gingeras (2013). "STAR: ultrafast universal RNA-seq aligner." Bioinformatics **29**(1): 15-21.

Du, Y., J. Lenz 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**(8): 4834-4845.

Edgeworth, J., M. Gorman, R. Bennett, P. Freemont and N. Hogg (1991). "Identification of p8,14 as a highly abundant heterodimeric calcium binding protein complex of myeloid cells." J Biol Chem **266**(12): 7706-7713.

Edwards, J. B., E.J.; Uk-Nham, S.; Cannon, J.G.; Blake, M.S.; Apicella, M.A. (2002). "A co-operative interaction between Neisseria gonorrhoeae and complement receptor 3 mediates infection of primary cervical epithelial cells." Cellular Microbiology **4**(5): 571-584.

Edwards, J. L. and M. A. Apicella (2004). "The molecular mechanisms used by Neisseria gonorrhoeae to initiate infection differ between men and women." Clin Microbiol Rev **17**(4): 965-981, table of contents.

Edwards, J. L. and M. A. Apicella (2005). "I-domain-containing integrins serve as pilus receptors for Neisseria gonorrhoeae adherence to human epithelial cells." Cell Microbiol **7**(8): 1197-1211.

Edwards, J. L., E. J. Brown, K. A. Ault and M. A. Apicella (2001). "The role of complement receptor 3 (CR3) in Neisseria gonorrhoeae infection of human cervical epithelia." Cell Microbiol **3**(9): 611-622.

Edwards, J. L. and E. K. Butler (2011). "The Pathobiology of Neisseria gonorrhoeae Lower Female Genital Tract Infection." Front Microbiol **2**: 102.

Edwards, J. L., J. Q. Shao, K. A. Ault and M. A. Apicella (2000). "Neisseria gonorrhoeae elicits membrane ruffling and cytoskeletal rearrangements

upon infection of primary human endocervical and ectocervical cells." Infect Immun **68**(9): 5354-5363.
Evans, B. A. (1977). "Ultrastructural study of cervical gonorrhea." J Infect Dis **136**(2): 248-255.
Falsetta, M. L., T. B. Bair, S. C. Ku, R. N. Vanden Hoven, C. T. Steichen, A. G. McEwan, M. P. Jennings and M. A. Apicella (2009). "Transcriptional profiling identifies the metabolic phenotype of gonococcal biofilms." Infect Immun **77**(9): 3522-3532.
Feinen, B., A. E. Jerse, S. L. Gaffen and M. W. Russell (2010). "Critical role of Th17 responses in a murine model of Neisseria gonorrhoeae genital infection." Mucosal Immunol **3**(3): 312-321.
Fichorova, R. N., P. J. Desai, F. C. Gibson, 3rd and C. A. Genco (2001). "Distinct proinflammatory host responses to Neisseria gonorrhoeae infection in immortalized human cervical and vaginal epithelial cells." Infect Immun **69**(9): 5840-5848.
Fichorova, R. N., J. G. Rheinwald and D. J. Anderson (1997). "Generation of papillomavirus-immortalized cell lines from normal human ectocervical, endocervical, and vaginal epithelium that maintain expression of tissue-specific differentiation proteins." Biol Reprod **57**(4): 847-855.
Flo, T. H., K. D. Smith, S. Sato, D. J. Rodriguez, M. A. Holmes, R. K. Strong, S. Akira and A. Aderem (2004). "Lipocalin 2 mediates an innate immune response to bacterial infection by sequestrating iron." Nature **432**(7019): 917-921.
Forng, R. Y., C. R. Ekechukwu, S. Subbarao, S. A. Morse and C. A. Genco (1997). "Promoter mapping and transcriptional regulation of the iron-regulated Neisseria gonorrhoeae fbpA gene." J Bacteriol **179**(9): 3047-3052.
Frandoloso, R., S. Martinez-Martinez, C. Calmettes, J. Fegan, E. Costa, D. Curran, R. H. Yu, C. B. Gutierrez-Martin, E. F. Rodriguez-Ferri, T. F. Moraes and A. B. Schryvers (2015). "Nonbinding site-directed mutants of transferrin binding protein B exhibit enhanced immunogenicity and protective capabilities." Infect Immun **83**(3): 1030-1038.
Friedrich, A., C. G. Arvidson, W. M. Shafer, E. H. Lee and M. So (2007). "Two ABC transporter operons and the antimicrobial resistance gene mtrF are pilT responsive in Neisseria gonorrhoeae." J Bacteriol **189**(14): 5399-5402.
Gaballa, A. and J. D. Helmann (1998). "Identification of a zinc-specific metalloregulatory protein, Zur, controlling zinc transport operons in Bacillus subtilis." J Bacteriol **180**(22): 5815-5821.
Gabbianelli, R., R. Scotti, S. Ammendola, P. Petrarca, L. Nicolini and A. Battistoni (2011). "Role of ZnuABC and ZinT in Escherichia coli O157:H7 zinc acquisition and interaction with epithelial cells." BMC Microbiol **11**: 36.

Gabriel, S. E. and J. D. Helmann (2009). "Contributions of Zur-controlled ribosomal proteins to growth under zinc starvation conditions." J Bacteriol **191**(19): 6116-6122.

Gaetke, L. M., C. J. McClain, R. T. Talwalkar and S. I. Shedlofsky (1997). "Effects of endotoxin on zinc metabolism in human volunteers." Am J Physiol **272**(6 Pt 1): E952-956.

Gammoh, N. Z. and L. Rink (2017). "Zinc in Infection and Inflammation." Nutrients **9**(6).

Ganz, T. (2006). "Hepcidin and its role in regulating systemic iron metabolism." Hematology Am Soc Hematol Educ Program: 29-35, 507.

Garreis, F., M. Gottschalt, T. Schlorf, R. Glaser, J. Harder, D. Worlitzsch and F. P. Paulsen (2011). "Expression and regulation of antimicrobial peptide psoriasin (S100A7) at the ocular surface and in the lacrimal apparatus." Invest Ophthalmol Vis Sci **52**(7): 4914-4922.

Gilston, B. A., E. P. Skaar and W. J. Chazin (2016). "Binding of transition metals to S100 proteins." Sci China Life Sci **59**(8): 792-801.

Glaser, R., U. Meyer-Hoffert, J. Harder, J. Cordes, M. Wittersheim, J. Kobliakova, R. Folster-Holst, E. Proksch, J. M. Schroder and T. Schwarz (2009). "The antimicrobial protein psoriasin (S100A7) is upregulated in atopic dermatitis and after experimental skin barrier disruption." J Invest Dermatol **129**(3): 641-649.

Goire, N., M. M. Lahra, M. Chen, B. Donovan, C. K. Fairley, R. Guy, J. Kaldor, D. Regan, J. Ward, M. D. Nissen, T. P. Sloots and D. M. Whiley (2014). "Molecular approaches to enhance surveillance of gonococcal antimicrobial resistance." Nat Rev Microbiol **12**(3): 223-229.

Gray-Owen, S. D., C. Dehio, A. Haude, F. Grunert and T. F. Meyer (1997). "CD66 carcinoembryonic antigens mediate interactions between Opa-expressing Neisseria gonorrhoeae and human polymorphonuclear phagocytes." EMBO J **16**(12): 3435-3445.

Gray-Owen, S. D., D. R. Lorenzen, A. Haude, T. F. Meyer and C. Dehio (1997). "Differential Opa specificities for CD66 receptors influence tissue interactions and cellular response to Neisseria gonorrhoeae." Mol Microbiol **26**(5): 971-980.

Greiner, L. L., J. L. Edwards, J. Shao, C. Rabinak, D. Entz and M. A. Apicella (2005). "Biofilm Formation by Neisseria gonorrhoeae." Infect Immun **73**(4): 1964-1970.

Grimley, D. M., L. Annang, I. Lewis, R. W. Smith, I. Aban, T. Hooks, S. Williams, E. W. Hook, 3rd and J. St Lawrence (2006). "Sexually transmitted infections among urban shelter clients." Sex Transm Dis **33**(11): 666-669.

Gunesekere, I. C., C. M. Kahler, D. R. Powell, L. A. Snyder, N. J. Saunders, J. I. Rood and J. K. Davies (2006). "Comparison of the RpoH-dependent regulon and general stress response in Neisseria gonorrhoeae." J Bacteriol **188**(13): 4769-4776.

Gunesekere, I. C., C. M. Kahler, C. S. Ryan, L. A. Snyder, N. J. Saunders, J. I. Rood and J. K. Davies (2006). "Ecf, an alternative sigma factor from Neisseria gonorrhoeae, controls expression of msrAB, which encodes methionine sulfoxide reductase." J Bacteriol **188**(10): 3463-3469.

Harkness, A. H. (1948). "The pathology of gonorrhoea." Br J Vener Dis **24**(4): 137-147.

Harvey, H. A., M. P. Jennings, C. A. Campbell, R. Williams and M. A. Apicella (2001). "Receptor-mediated endocytosis of Neisseria gonorrhoeae into primary human urethral epithelial cells: the role of the asialoglycoprotein receptor." Mol Microbiol **42**(3): 659-672.

Hayden, J. A., M. B. Brophy, L. S. Cunden and E. M. Nolan (2013). "High-affinity manganese coordination by human calprotectin is calcium-dependent and requires the histidine-rich site formed at the dimer interface." J Am Chem Soc **135**(2): 775-787.

Hegyi, Z., S. Zwicker, D. Bureik, M. Peric, S. Koglin, A. Batycka-Baran, J. C. Prinz, T. Ruzicka, J. Schauber and R. Wolf (2012). "Vitamin D analog calcipotriol suppresses the Th17 cytokine-induced proinflammatory S100 "alarmins" psoriasin (S100A7) and koebnerisin (S100A15) in psoriasis." J Invest Dermatol **132**(5): 1416-1424.

Hein, K. Z., H. Takahashi, T. Tsumori, Y. Yasui, Y. Nanjoh, T. Toga, Z. Wu, J. Grotzinger, S. Jung, J. Wehkamp, B. O. Schroeder, J. M. Schroeder and E. Morita (2015). "Disulphide-reduced psoriasin is a human apoptosis-inducing broad-spectrum fungicide." Proc Natl Acad Sci U S A **112**(42): 13039-13044.

Hesse, L. E., Z. R. Lonergan, W. N. Beavers and E. P. Skaar (2019). "The Acinetobacter baumannii Znu System Overcomes Host-Imposed Nutrient Zinc Limitation." Infect Immun **87**(12).

Higashi, D. L., S. W. Lee, A. Snyder, N. J. Weyand, A. Bakke and M. So (2007). "Dynamics of Neisseria gonorrhoeae attachment: microcolony development, cortical plaque formation, and cytoprotection." Infect Immun **75**(10): 4743-4753.

Hood, M. I., B. L. Mortensen, J. L. Moore, Y. Zhang, T. E. Kehl-Fie, N. Sugitani, W. J. Chazin, R. M. Caprioli and E. P. Skaar (2012). "Identification of an Acinetobacter baumannii zinc acquisition system that facilitates resistance to calprotectin-mediated zinc sequestration." PLoS Pathog **8**(12): e1003068.

Hu, L. I., S. Yin, E. A. Ozer, L. Sewell, S. Rehman, J. A. Garnett and H. S. Seifert (2020). "Discovery of a New Neisseria gonorrhoeae Type IV Pilus Assembly Factor, TfpC." mBio **11**(5).

Huang, J., Q. Zhang, J. Chen, T. Zhang, Z. Chen, Z. Chen, J. Yang, Y. Wang, Z. Min, M. Huang and X. Min (2020). "Neisseria gonorrhoeae NGO2105 Is an Autotransporter Protein Involved in Adhesion to Human Cervical Epithelial Cells and in vivo Colonization." Front Microbiol **11**: 1395.

Huber, W., V. J. Carey, R. Gentleman, S. Anders, M. Carlson, B. S. Carvalho, H. C. Bravo, S. Davis, L. Gatto, T. Girke, R. Gottardo, F. Hahne, K. D. Hansen, R. A. Irizarry, M. Lawrence, M. I. Love, J. MacDonald, V. Obenchain, A. K. Oles, H. Pages, A. Reyes, P. Shannon, G. K. Smyth, D. Tenenbaum, L. Waldron and M. Morgan (2015). "Orchestrating high-throughput genomic analysis with Bioconductor." Nat Methods **12**(2): 115-121.

Hubert, K., N. Devos, I. Mordhorst, C. Tans, G. Baudoux, C. Feron, K. Goraj, J. Tommassen, U. Vogel, J. T. Poolman and V. Weynants (2013). "ZnuD, a potential candidate for a simple and universal Neisseria meningitidis vaccine." Infect Immun **81**(6): 1915-1927.

Hussain, L. A., C. G. Kelly, A. Rodin, M. Jourdan and T. Lehner (1995). "Investigation of the complement receptor 3 (CD11b/CD18) in human rectal epithelium." Clin Exp Immunol **102**(2): 384-388.

Hyun, H. J., J. H. Sohn, D. W. Ha, Y. H. Ahn, J. Y. Koh and Y. H. Yoon (2001). "Depletion of intracellular zinc and copper with TPEN results in apoptosis of cultured human retinal pigment epithelial cells." Invest Ophthalmol Vis Sci **42**(2): 460-465.

Isabella, V. M. and V. L. Clark (2011). "Deep sequencing-based analysis of the anaerobic stimulon in Neisseria gonorrhoeae." BMC Genomics **12**: 51.

Jackson, E., S. Little, D. S. Franklin, J. A. Gaddy and S. M. Damo (2017). "Expression, Purification, and Antimicrobial Activity of S100A12." J Vis Exp(123).

Jackson, L. A., T. F. Ducey, M. W. Day, J. B. Zaitshik, J. Orvis and D. W. Dyer (2010). "Transcriptional and functional analysis of the Neisseria gonorrhoeae Fur regulon." J Bacteriol **192**(1): 77-85.

Jean, S., R. A. Juneau, A. K. Criss and C. N. Cornelissen (2016). "Neisseria gonorrhoeae Evades Calprotectin-Mediated Nutritional Immunity and Survives Neutrophil Extracellular Traps by Production of TdfH." Infect Immun **84**(10): 2982-2994.

Jerse, A. E., H. Wu, M. Packiam, R. A. Vonck, A. A. Begum and L. E. Garvin (2011). "Estradiol-Treated Female Mice as Surrogate Hosts for Neisseria gonorrhoeae Genital Tract Infections." Front Microbiol **2**: 107.

Johne, B., M. K. Fagerhol, T. Lyberg, H. Prydz, P. Brandtzaeg, C. F. Naess-Andresen and I. Dale (1997). "Functional and clinical aspects of the myelomonocyte protein calprotectin." Mol Pathol **50**(3): 113-123.

Johnson, M. B., L. M. Ball, K. P. Daily, J. N. Martin, L. Columbus and A. K. Criss (2015). "Opa+ Neisseria gonorrhoeae exhibits reduced survival in human neutrophils via Src family kinase-mediated bacterial trafficking into mature phagolysosomes." Cell Microbiol **17**(5): 648-665.

Johnson, M. B. and A. K. Criss (2011). "Resistance of Neisseria gonorrhoeae to neutrophils." Front Microbiol **2**: 77.

Kallstrom, H., M. K. Liszewski, J. P. Atkinson and A. B. Jonsson (1997). "Membrane cofactor protein (MCP or CD46) is a cellular pilus receptor for pathogenic Neisseria." Mol Microbiol **25**(4): 639-647.

Kammerman, M. T., A. Bera, R. Wu, S. A. Harrison, C. N. Maxwell, K. Lundquist, N. Noinaj, W. J. Chazin and C. N. Cornelissen (2020). "Molecular Insight into TdfH-Mediated Zinc Piracy from Human Calprotectin by Neisseria gonorrhoeae." mBio **11**(3).

Kattner, C., J. Zaucha, F. Jaenecke, U. Zachariae and M. Tanabe (2013). "Identification of a cation transport pathway in Neisseria meningitidis PorB." Proteins **81**(5): 830-840.

Kehl-Fie, T. E., S. Chitayat, M. I. Hood, S. Damo, N. Restrepo, C. Garcia, K. A. Munro, W. J. Chazin and E. P. Skaar (2011). "Nutrient metal sequestration by calprotectin inhibits bacterial superoxide defense, enhancing neutrophil killing of Staphylococcus aureus." Cell Host Microbe **10**(2): 158-164.

Kehl-Fie, T. E. and E. P. Skaar (2010). "Nutritional immunity beyond iron: a role for manganese and zinc." Curr Opin Chem Biol **14**(2): 218-224.

Kimura, T. and T. Kambe (2016). "The Functions of Metallothionein and ZIP and ZnT Transporters: An Overview and Perspective." Int J Mol Sci **17**(3): 336.

Klein, E. J., L. S. Fisher, A. W. Chow and L. B. Guze (1977). "Anorectal gonococcal infection." Ann Intern Med **86**(3): 340-346.

Komaroff, A. L., M. D. Aronson, T. M. Pass and C. T. Ervin (1980). "Prevalence of pharyngeal gonorrhea in general medical patients with sore throats." Sex Transm Dis **7**(3): 116-119.

Kothari, R. P. and A. R. Chaudhari (2016). "Zinc Levels in Seminal Fluid in Infertile Males and its Relation with Serum Free Testosterone." J Clin Diagn Res **10**(5): CC05-08.

Kumar, P., S. Sannigrahi and Y. L. Tzeng (2012). "The Neisseria meningitidis ZnuD zinc receptor contributes to interactions with epithelial cells and supports heme utilization when expressed in Escherichia coli." Infect Immun **80**(2): 657-667.

Kunimi, K., M. Maegawa, M. Kamada, S. Yamamoto, T. Yasui, T. Matsuzaki, A. Kuwahara, H. Furumoto, Y. Ohmoto, H. Kido and M. Irahara (2006). "Myeloid-related protein-8/14 is associated with proinflammatory cytokines in cervical mucus." J Reprod Immunol **71**(1): 3-11.

Lenz, J. D. and J. P. Dillard (2018). "Pathogenesis of Neisseria gonorrhoeae and the Host Defense in Ascending Infections of Human Fallopian Tube." Front Immunol **9**: 2710.

Leukert, N., T. Vogl, K. Strupat, R. Reichelt, C. Sorg and J. Roth (2006). "Calcium-dependent tetramer formation of S100A8 and S100A9 is essential for biological activity." J Mol Biol **359**(4): 961-972.

Li, B. and C. N. Dewey (2011). "RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome." BMC Bioinformatics **12**: 323.

Liang, S. C., X. Y. Tan, D. P. Luxenberg, R. Karim, K. Dunussi-Joannopoulos, M. Collins and L. A. Fouser (2006). "Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides." J Exp Med **203**(10): 2271-2279.

Liu, J. Z., S. Jellbauer, A. J. Poe, V. Ton, M. Pesciaroli, T. E. Kehl-Fie, N. A. Restrepo, M. P. Hosking, R. A. Edwards, A. Battistoni, P. Pasquali, T. E. Lane, W. J. Chazin, T. Vogl, J. Roth, E. P. Skaar and M. Raffatellu (2012). "Zinc sequestration by the neutrophil protein calprotectin enhances Salmonella growth in the inflamed gut." Cell Host Microbe **11**(3): 227-239.

Liu, Y., E. A. Islam, G. A. Jarvis, S. D. Gray-Owen and M. W. Russell (2012). "Neisseria gonorrhoeae selectively suppresses the development of Th1 and Th2 cells, and enhances Th17 cell responses, through TGF-beta-dependent mechanisms." Mucosal Immunol **5**(3): 320-331.

Liuzzi, J. P., L. A. Lichten, S. Rivera, R. K. Blanchard, T. B. Aydemir, M. D. Knutson, T. Ganz and R. J. Cousins (2005). "Interleukin-6 regulates the zinc transporter Zip14 in liver and contributes to the hypozincemia of the acute-phase response." Proc Natl Acad Sci U S A **102**(19): 6843-6848.

Lonergan, Z. R. and E. P. Skaar (2019). "Nutrient Zinc at the Host-Pathogen Interface." Trends Biochem Sci **44**(12): 1041-1056.

Lopez, A., P. Cacoub, I. C. Macdougall and L. Peyrin-Biroulet (2016). "Iron deficiency anaemia." Lancet **387**(10021): 907-916.

Love, M. I., W. Huber and S. Anders (2014). "Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2." Genome Biol **15**(12): 550.

Ma, Z., S. E. Gabriel and J. D. Helmann (2011). "Sequential binding and sensing of Zn(II) by Bacillus subtilis Zur." Nucleic Acids Res **39**(21): 9130-9138.

Madu, A. J. and M. D. Ughasoro (2017). "Anaemia of Chronic Disease: An In-Depth Review." Med Princ Pract **26**(1): 1-9.

Maurakis, S., K. Keller, C. N. Maxwell, K. Pereira, W. J. Chazin, A. K. Criss and C. N. Cornelissen (2019). "The novel interaction between Neisseria gonorrhoeae TdfJ and human S100A7 allows gonococci to subvert host zinc restriction." PLoS Pathog **15**(8): e1007937.

Mayer, L. W. (1982). "Rates in vitro changes of gonococcal colony opacity phenotypes." Infect Immun **37**(2): 481-485.

McClure, R., K. Nudel, P. Massari, B. Tjaden, X. Su, P. A. Rice and C. A. Genco (2015). "The Gonococcal Transcriptome during Infection of the Lower Genital Tract in Women." PLoS One **10**(8): e0133982.

McConaghy, J. R. and B. Panchal (2016). "Epididymitis: An Overview." Am Fam Physician **94**(9): 723-726.

Mendivil-Perez, M., C. Velez-Pardo and M. Jimenez-Del-Rio (2012). "TPEN induces apoptosis independently of zinc chelator activity in a model of acute lymphoblastic leukemia and ex vivo acute leukemia cells through oxidative stress and mitochondria caspase-3- and AIF-dependent pathways." Oxid Med Cell Longev **2012**: 313275.

Merz, A. J., M. So and M. P. Sheetz (2000). "Pilus retraction powers bacterial twitching motility." Nature **407**(6800): 98-102.

Meyer, J. E., J. Harder, B. Sipos, S. Maune, G. Kloppel, J. Bartels, J. M. Schroder and R. Glaser (2008). "Psoriasin (S100A7) is a principal antimicrobial peptide of the human tongue." Mucosal Immunol **1**(3): 239-243.

Michalek, M., C. Gelhaus, O. Hecht, R. Podschun, J. M. Schroder, M. Leippe and J. Grotzinger (2009). "The human antimicrobial protein psoriasin acts by permeabilization of bacterial membranes." Dev Comp Immunol **33**(6): 740-746.

Mikhaylina, A., A. Z. Ksibe, D. J. Scanlan and C. A. Blindauer (2018). "Bacterial zinc uptake regulator proteins and their regulons." Biochem Soc Trans **46**(4): 983-1001.

Mildner, M., M. Stichenwirth, A. Abtin, L. Eckhart, C. Sam, R. Glaser, J. M. Schroder, R. Gmeiner, V. Mlitz, J. Pammer, A. Geusau and E. Tschachler (2010). "Psoriasin (S100A7) is a major Escherichia coli-cidal factor of the female genital tract." Mucosal Immunol **3**(6): 602-609.

Moroz, O. V., A. A. Antson, S. J. Grist, N. J. Maitland, G. G. Dodson, K. S. Wilson, E. Lukanidin and I. B. Bronstein (2003). "Structure of the human S100A12-copper complex: implications for host-parasite defence." Acta Crystallogr D Biol Crystallogr **59**(Pt 5): 859-867.

Moroz, O. V., W. Burkitt, H. Wittkowski, W. He, A. Ianoul, V. Novitskaya, J. Xie, O. Polyakova, I. K. Lednev, A. Shekhtman, P. J. Derrick, P. Bjoerk, D. Foell and I. B. Bronstein (2009). "Both Ca2+ and Zn2+ are essential for S100A12 protein oligomerization and function." BMC Biochem **10**: 11.

Murphy, G. L., T. D. Connell, D. S. Barritt, M. Koomey and J. G. Cannon (1989). "Phase variation of gonococcal protein II: regulation of gene expression by slipped-strand mispairing of a repetitive DNA sequence." Cell **56**(4): 539-547.

Murray, J. I., M. L. Tonkin, A. L. Whiting, F. Peng, B. Farnell, J. T. Cullen, F. Hof and M. J. Boulanger (2012). "Structural characterization of S100A15 reveals a novel zinc coordination site among S100 proteins and altered surface chemistry with functional implications for receptor binding." BMC Struct Biol **12**: 16.

Nakashige, T. G., J. R. Stephan, L. S. Cunden, M. B. Brophy, A. J. Wommack, B. C. Keegan, J. M. Shearer and E. M. Nolan (2016). "The Hexahistidine Motif of Host-Defense Protein Human Calprotectin Contributes to Zinc Withholding and Its Functional Versatility." J Am Chem Soc **138**(37): 12243-12251.

Nanamiya, H., G. Akanuma, Y. Natori, R. Murayama, S. Kosono, T. Kudo, K. Kobayashi, N. Ogasawara, S. M. Park, K. Ochi and F. Kawamura (2004). "Zinc is a key factor in controlling alternation of two types of L31 protein in the Bacillus subtilis ribosome." Mol Microbiol **52**(1): 273-283.

Neumann, W., R. C. Hadley and E. M. Nolan (2017). "Transition metals at the host-pathogen interface: how Neisseria exploit human metalloproteins for acquiring iron and zinc." Essays Biochem **61**(2): 211-223.

Noinaj, N., M. Guillier, T. J. Barnard and S. K. Buchanan (2010). "TonB-dependent transporters: regulation, structure, and function." Annu Rev Microbiol **64**: 43-60.

Nudel, K., R. McClure, M. Moreau, E. Briars, A. J. Abrams, B. Tjaden, X. H. Su, D. Trees, P. A. Rice, P. Massari and C. A. Genco (2018). "Transcriptome Analysis of Neisseria gonorrhoeae during Natural Infection Reveals Differential Expression of Antibiotic Resistance Determinants between Men and Women." mSphere **3**(3).

O'Brien, J. P., D. L. Goldenberg and P. A. Rice (1983). "Disseminated gonococcal infection: a prospective analysis of 49 patients and a review of pathophysiology and immune mechanisms." Medicine (Baltimore) **62**(6): 395-406.

Ong, C. L., C. M. Gillen, T. C. Barnett, M. J. Walker and A. G. McEwan (2014). "An antimicrobial role for zinc in innate immune defense against group A streptococcus." J Infect Dis **209**(10): 1500-1508.

Palmer, A. and A. K. Criss (2018). "Gonococcal Defenses against Antimicrobial Activities of Neutrophils." Trends Microbiol **26**(12): 1022-1034.

Panicker, G., Y. Ye, D. Wang and E. R. Unger (2010). "Characterization of the Human Cervical Mucous Proteome." Clin Proteomics **6**(1-2): 18-28.

Park, H. S., M. Wolfgang and M. Koomey (2002). "Modification of type IV pilus-associated epithelial cell adherence and multicellular behavior by the PilU protein of Neisseria gonorrhoeae." Infect Immun **70**(7): 3891-3903.

Parrow, N. L., R. E. Fleming and M. F. Minnick (2013). "Sequestration and scavenging of iron in infection." Infect Immun **81**(10): 3503-3514.

Pawlik, M. C., K. Hubert, B. Joseph, H. Claus, C. Schoen and U. Vogel (2012). "The zinc-responsive regulon of Neisseria meningitidis comprises 17 genes under control of a Zur element." J Bacteriol **194**(23): 6594-6603.

Phillips, N. J., C. T. Steichen, B. Schilling, D. M. Post, R. K. Niles, T. B. Bair, M. L. Falsetta, M. A. Apicella and B. W. Gibson (2012). "Proteomic analysis of Neisseria gonorrhoeae biofilms shows shift to anaerobic respiration and changes in nutrient transport and outermembrane proteins." PLoS One **7**(6): e38303.

Postle, K. (2007). "TonB system, in vivo assays and characterization." Methods Enzymol **422**: 245-269.

Quillin, S. J. and H. S. Seifert (2018). "Neisseria gonorrhoeae host adaptation and pathogenesis." Nat Rev Microbiol **16**(4): 226-240.

R_Core_Team. (2020). "A language and environment for statistical computing." Retrieved July 2021, from **https://www.R-project.org/**.

Raffatellu, M., M. D. George, Y. Akiyama, M. J. Hornsby, S. P. Nuccio, T. A. Paixao, B. P. Butler, H. Chu, R. L. Santos, T. Berger, T. W. Mak, R. M. Tsolis, C. L. Bevins, J. V. Solnick, S. Dandekar and A. J. Baumler (2009).

"Lipocalin-2 resistance confers an advantage to Salmonella enterica serotype Typhimurium for growth and survival in the inflamed intestine." Cell Host Microbe **5**(5): 476-486.

Ray, J. C., A. Smirnov, S. A. Maurakis, S. A. Harrison, E. Ke, W. J. Chazin, C. N. Cornelissen and A. K. Criss (2022). "Adherence enables Neisseria gonorrhoeae to overcome zinc limitation imposed by nutritional immunity proteins." Infect Immun: iai0000922.

Reich, O., S. Regauer, W. G. McCluggage, C. Bergeron and C. Redman (2017). "Defining the Cervical Transformation Zone and Squamocolumnar Junction: Can We Reach a Common Colposcopic and Histologic Definition?" Int J Gynecol Pathol **36**(6): 517-522.

Reyes-Caballero, H., G. C. Campanello and D. P. Giedroc (2011). "Metalloregulatory proteins: metal selectivity and allosteric switching." Biophys Chem **156**(2-3): 103-114.

Rice, P. A. (2005). "Gonococcal arthritis (disseminated gonococcal infection)." Infect Dis Clin North Am **19**(4): 853-861.

Ross, K. F. and M. C. Herzberg (2001). "Calprotectin expression by gingival epithelial cells." Infect Immun **69**(5): 3248-3254.

Rowley, J., S. Vander Hoorn, E. Korenromp, N. Low, M. Unemo, L. J. Abu-Raddad, R. M. Chico, A. Smolak, L. Newman, S. Gottlieb, S. S. Thwin, N. Broutet and M. M. Taylor (2019). "Chlamydia, gonorrhoea, trichomoniasis and syphilis: global prevalence and incidence estimates, 2016." Bull World Health Organ **97**(8): 548-562P.

Rudel, T., J. P. van Putten, C. P. Gibbs, R. Haas and T. F. Meyer (1992). "Interaction of two variable proteins (PilE and PilC) required for pilus-mediated adherence of Neisseria gonorrhoeae to human epithelial cells." Mol Microbiol **6**(22): 3439-3450.

Sarantis, H. and S. D. Gray-Owen (2012). "Defining the roles of human carcinoembryonic antigen-related cellular adhesion molecules during neutrophil responses to Neisseria gonorrhoeae." Infect Immun **80**(1): 345-358.

Schroeder, J. J. and R. J. Cousins (1990). "Interleukin 6 regulates metallothionein gene expression and zinc metabolism in hepatocyte monolayer cultures." Proc Natl Acad Sci U S A **87**(8): 3137-3141.

Seifert, H. S. (1996). "Questions about gonococcal pilus phase- and antigenic variation." Mol Microbiol **21**(3): 433-440.

Seifert, H. S. (2019). "Location, Location, Location-Commensalism, Damage and Evolution of the Pathogenic Neisseria." J Mol Biol **431**(16): 3010-3014.

Seifert, H. S., C. J. Wright, A. E. Jerse, M. S. Cohen and J. G. Cannon (1994). "Multiple gonococcal pilin antigenic variants are produced during experimental human infections." J Clin Invest **93**(6): 2744-2749.

Semchenko, E. A., A. V. Everest-Dass, F. E. Jen, T. D. Mubaiwa, C. J. Day and K. L. Seib (2019). "Glycointeractome of Neisseria gonorrhoeae:

Identification of Host Glycans Targeted by the Gonococcus To Facilitate Adherence to Cervical and Urethral Epithelial Cells." mBio **10**(4).

Shin, J. H. and J. D. Helmann (2016). "Molecular logic of the Zur-regulated zinc deprivation response in Bacillus subtilis." Nat Commun **7**: 12612.

Sigdel, T. K., J. A. Easton and M. W. Crowder (2006). "Transcriptional response of Escherichia coli to TPEN." J Bacteriol **188**(18): 6709-6713.

Sintsova, A., H. Sarantis, E. A. Islam, C. X. Sun, M. Amin, C. H. Chan, C. P. Stanners, M. Glogauer and S. D. Gray-Owen (2014). "Global analysis of neutrophil responses to Neisseria gonorrhoeae reveals a self-propagating inflammatory program." PLoS Pathog **10**(9): e1004341.

Sirisaengtaksin, N., M. A. Odem, R. E. Bosserman, E. M. Flores and A. M. Krachler (2020). "The E. coli transcription factor GrlA is regulated by subcellular compartmentalization and activated in response to mechanical stimuli." Proc Natl Acad Sci U S A **117**(17): 9519-9528.

Snyder, L. A. and N. J. Saunders (2006). "The majority of genes in the pathogenic Neisseria species are present in non-pathogenic Neisseria lactamica, including those designated as 'virulence genes'." BMC Genomics **7**: 128.

Soneson, C., M. I. Love and M. D. Robinson (2015). "Differential analyses for RNA-seq: transcript-level estimates improve gene-level inferences." F1000Res **4**: 1521.

Song, W., Q. Yu, L. C. Wang and D. C. Stein (2020). "Adaptation of Neisseria gonorrhoeae to the Female Reproductive Tract." Microbiol Insights **13**: 1178636120947077.

St Cyr, S., L. Barbee, K. A. Workowski, L. H. Bachmann, C. Pham, K. Schlanger, E. Torrone, H. Weinstock, E. N. Kersh and P. Thorpe (2020). "Update to CDC's Treatment Guidelines for Gonococcal Infection, 2020." MMWR Morb Mortal Wkly Rep **69**(50): 1911-1916.

StdGen. (2003). "STD Sequence Databases." 2021, from **http://stdgen.northwestern.edu/**.

Stern, A., M. Brown, P. Nickel and T. F. Meyer (1986). "Opacity genes in Neisseria gonorrhoeae: control of phase and antigenic variation." Cell **47**(1): 61-71.

Stevens, J. S. and A. K. Criss (2018). "Pathogenesis of Neisseria gonorrhoeae in the female reproductive tract: neutrophilic host response, sustained infection, and clinical sequelae." Curr Opin Hematol **25**(1): 13-21.

Stevens, J. S., M. C. Gray, C. Morisseau and A. K. Criss (2018). "Endocervical and Neutrophil Lipoxygenases Coordinate Neutrophil Transepithelial Migration to Neisseria gonorrhoeae." J Infect Dis **218**(10): 1663-1674.

Stohl, E. A., A. K. Criss and H. S. Seifert (2005). "The transcriptome response of Neisseria gonorrhoeae to hydrogen peroxide reveals genes with previously uncharacterized roles in oxidative damage protection." Mol Microbiol **58**(2): 520-532.

Stork, M., M. P. Bos, I. Jongerius, N. de Kok, I. Schilders, V. E. Weynants, J. T. Poolman and J. Tommassen (2010). "An outer membrane receptor of Neisseria meningitidis involved in zinc acquisition with vaccine potential." PLoS Pathog **6**: e1000969.

Stork, M., J. Grijpstra, M. P. Bos, C. Manas Torres, N. Devos, J. T. Poolman, W. J. Chazin and J. Tommassen (2013). "Zinc piracy as a mechanism of Neisseria meningitidis for evasion of nutritional immunity." PLoS Pathog **9**(10): e1003733.

Strange, H. R., T. A. Zola and C. N. Cornelissen (2011). "The fbpABC operon is required for Ton-independent utilization of xenosiderophores by Neisseria gonorrhoeae strain FA19." Infect Immun **79**(1): 267-278.

Stroncek, D. F., R. A. Shankar and K. M. Skubitz (2005). "The subcellular distribution of myeloid-related protein 8 (MRP8) and MRP14 in human neutrophils." J Transl Med **3**: 36.

Takacs, P., P. Damjanovich, A. G. Sipos and B. Kozma (2020). "The effect of oral zinc supplementation on cervicovaginal lavage fluid zinc level." Eur J Obstet Gynecol Reprod Biol **248**: 106-109.

Tanaka, T., M. Narazaki and T. Kishimoto (2014). "IL-6 in inflammation, immunity, and disease." Cold Spring Harb Perspect Biol **6**(10): a016295.

Thees, A. V., K. M. Pietrosimone, C. K. Melchiorre, J. N. Marden, J. Graf, M. A. Lynes and M. Maltz-Matyschsyk (2021). "PmtA Regulates Pyocyanin Expression and Biofilm Formation in Pseudomonas aeruginosa." Front Microbiol **12**: 789765.

Trebach, J. D., C. P. Chaulk, K. R. Page, S. Tuddenham and K. G. Ghanem (2015). "Neisseria gonorrhoeae and Chlamydia trachomatis among women reporting extragenital exposures." Sex Transm Dis **42**(5): 233-239.

Tuddenham, S., M. M. Hamill and K. G. Ghanem (2022). "Diagnosis and Treatment of Sexually Transmitted Infections: A Review." JAMA **327**(2): 161-172.

Ueta, M., C. Wada and A. Wada (2020). "YkgM and YkgO maintain translation by replacing their paralogs, zinc-binding ribosomal proteins L31 and L36, with identical activities." Genes Cells **25**(8): 562-581.

Uhlen, M., L. Fagerberg, B. M. Hallstrom, C. Lindskog, P. Oksvold, A. Mardinoglu, A. Sivertsson, C. Kampf, E. Sjostedt, A. Asplund, I. Olsson, K. Edlund, E. Lundberg, S. Navani, C. A. Szigyarto, J. Odeberg, D. Djureinovic, J. O. Takanen, S. Hober, T. Alm, P. H. Edqvist, H. Berling, H. Tegel, J. Mulder, J. Rockberg, P. Nilsson, J. M. Schwenk, M. Hamsten, K. von Feilitzen, M. Forsberg, L. Persson, F. Johansson, M. Zwahlen, G. von Heijne, J. Nielsen and F. Ponten (2015). "Proteomics. Tissue-based map of the human proteome." Science **347**(6220): 1260419.

Unemo, M., H. S. Seifert, E. W. Hook, 3rd, S. Hawkes, F. Ndowa and J. R. Dillon (2019). "Gonorrhoea." Nat Rev Dis Primers **5**(1): 79.

Urban, C. F., D. Ermert, M. Schmid, U. Abu-Abed, C. Goosmann, W. Nacken, V. Brinkmann, P. R. Jungblut and A. Zychlinsky (2009). "Neutrophil

extracellular traps contain calprotectin, a cytosolic protein complex involved in host defense against Candida albicans." PLoS Pathog **5**(10): e1000639.

Vasak, M. and G. Meloni (2011). "Chemistry and biology of mammalian metallothioneins." J Biol Inorg Chem **16**(7): 1067-1078.

Virji, M., D. Evans, A. Hadfield, F. Grunert, A. M. Teixeira and S. M. Watt (1999). "Critical determinants of host receptor targeting by Neisseria meningitidis and Neisseria gonorrhoeae: identification of Opa adhesiotopes on the N-domain of CD66 molecules." Mol Microbiol **34**(3): 538-551.

Wainwright, L. A., J. V. Frangipane and H. S. Seifert (1997). "Analysis of protein binding to the Sma/Cla DNA repeat in pathogenic Neisseriae." Nucleic Acids Res **25**(7): 1362-1368.

Waldron, K. J., J. C. Rutherford, D. Ford and N. J. Robinson (2009). "Metalloproteins and metal sensing." Nature **460**(7257): 823-830.

Wan, C., J. L. Latter, A. Amirshahi, I. Symonds, J. Finnie, N. Bowden, R. J. Scott, K. A. Cunningham, P. Timms and K. W. Beagley (2014). "Progesterone activates multiple innate immune pathways in Chlamydia trachomatis-infected endocervical cells." Am J Reprod Immunol **71**(2): 165-177.

Wang, J., S. D. Gray-Owen, A. Knorre, T. F. Meyer and C. Dehio (1998). "Opa binding to cellular CD66 receptors mediates the transcellular traversal of Neisseria gonorrhoeae across polarized T84 epithelial cell monolayers." Mol Microbiol **30**(3): 657-671.

Werner, L. M., A. Palmer, A. Smirnov, M. Belcher Dufrisne, L. Columbus and A. K. Criss (2020). "Imaging Flow Cytometry Analysis of CEACAM Binding to Opa-Expressing Neisseria gonorrhoeae." Cytometry A **97**(10): 1081-1089.

Wierzbicki, I. H., R. A. Zielke, K. V. Korotkov and A. E. Sikora (2017). "Functional and structural studies on the Neisseria gonorrhoeae GmhA, the first enzyme in the glycero-manno-heptose biosynthesis pathways, demonstrate a critical role in lipooligosaccharide synthesis and gonococcal viability." Microbiologyopen **6**(2).

Wilkinson, M. M., A. Busuttil, C. Hayward, D. J. Brock, J. R. Dorin and V. Van Heyningen (1988). "Expression pattern of two related cystic fibrosis-associated calcium-binding proteins in normal and abnormal tissues." J Cell Sci **91 (Pt 2)**: 221-230.

Wolfgang, M., P. Lauer, H. S. Park, L. Brossay, J. Hebert and M. Koomey (1998). "PilT mutations lead to simultaneous defects in competence for natural transformation and twitching motility in piliated Neisseria gonorrhoeae." Mol Microbiol **29**(1): 321-330.

Wong, W. Y., G. Flik, P. M. Groenen, D. W. Swinkels, C. M. Thomas, J. H. Copius-Peereboom, H. M. Merkus and R. P. Steegers-Theunissen (2001). "The impact of calcium, magnesium, zinc, and copper in blood and seminal plasma on semen parameters in men." Reprod Toxicol **15**(2): 131-136.

Workowski, K. A., L. H. Bachmann, P. A. Chan, C. M. Johnston, C. A. Muzny, I. Park, H. Reno, J. M. Zenilman and G. A. Bolan (2021). "Sexually Transmitted Infections Treatment Guidelines, 2021." MMWR Recomm Rep **70**(4): 1-187.

Wu, H. J., K. L. Seib, Y. N. Srikhanta, S. P. Kidd, J. L. Edwards, T. L. Maguire, S. M. Grimmond, M. A. Apicella, A. G. McEwan and M. P. Jennings (2006). "PerR controls Mn-dependent resistance to oxidative stress in Neisseria gonorrhoeae." Mol Microbiol **60**(2): 401-416.

Yadav, R., N. Noinaj, N. Ostan, T. Moraes, J. Stoudenmire, S. Maurakis and C. N. Cornelissen (2019). "Structural Basis for Evasion of Nutritional Immunity by the Pathogenic Neisseriae." Front Microbiol **10**: 2981.

Yu, Q., L. C. Wang, S. Di Benigno, S. D. Gray-Owen, D. C. Stein and W. Song (2019). "Neisseria gonorrhoeae infects the heterogeneous epithelia of the human cervix using distinct mechanisms." PLoS Pathog **15**(12): e1008136.

Zackular, J. P., R. J. Knippel, C. A. Lopez, W. N. Beavers, C. N. Maxwell, W. J. Chazin and E. P. Skaar (2020). "ZupT Facilitates Clostridioides difficile Resistance to Host-Mediated Nutritional Immunity." mSphere **5**(2).

Zegels, G., G. A. Van Raemdonck, E. P. Coen, W. A. Tjalma and X. W. Van Ostade (2009). "Comprehensive proteomic analysis of human cervical-vaginal fluid using colposcopy samples." Proteome Sci **7**: 17.

Zouboulis, C. C., C. Beutler, H. F. Merk and J. M. Baron (2017). "RIS-1/psoriasin expression in epithelial skin cells indicates their selective role in innate immunity and in inflammatory skin diseases including acne." Dermatoendocrinol **9**(1): e1338993.

Zygiel, E. M. and E. M. Nolan (2019). "Exploring Iron Withholding by the Innate Immune Protein Human Calprotectin." Acc Chem Res **52**(8): 2301-2308.