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Sulfur metabolism in Neisseria gonorrhoeae

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Abstract

Elucidating essential pathways for the pathogenicity of *Neisseria gonorrhoeae* is crucial for the discovery of novel therapeutics required to combat the threat of extensively drug-resistant gonorrhoeae. Sulfur metabolic pathways have been proposed as attractive targets as they are absent in mammals, and cysteine, the central node in sulfur metabolism, is critical to a broad range of cellular functions. Accordingly, the acquisition of sulfur sources to fulfil cysteine requirements is a crucial determinant of bacterial survival. Compared to our understanding of how *N. gonorrhoeae* acquires essential nutrients such as carbon, zinc, and iron, sulfur acquisition and metabolism remain poorly defined.

Here, we investigate the metabolic flexibility of *N. gonorrhoeae* with respect to sulfur acquisition and examine how the gonococcus adapts to conditions of sulfur limitation. By integrating bioinformatic analyses with experimental growth data and enzyme kinetic assays, we mapped the sulfur metabolic network in *N. gonorrhoeae*. We confirm that direct acquisition of cystine (and cysteine) supports growth and further demonstrate that cystine uptake is essential for colonization of epithelial cells under aerobic conditions. In the absence of cysteine, *N. gonorrhoeae* exhibits a limited capacity to utilize alternative sulfur sources, with only glutathione supporting proliferation, albeit inefficiently, via an unidentified mechanism. Growth under these conditions was restored by further supplementation with thiosulfate or methionine, indicating that glutathione utilization is constrained by downstream metabolic limitations, likely associated with inefficient cysteine liberation and restricted hydrogen sulfide availability required for methionine biosynthesis.

This limited capacity to utilize alternative sulfur acquisition strategies is notable given that cysteine availability within the host is tightly regulated and maintained at low concentrations. Transcriptomic analyses across cysteine-independent growth conditions revealed the sulfur landscape influences transcriptional responses in a metabolite specific manner. More specifically, in response to sulfur starvation, *N. gonorrhoeae* does not appear to overcome this limitation through diverse acquisition strategies but instead adjusts cellular processes to compensate for inefficient sulfur acquisition via resource reallocation, where possible.

By defining the sulfur metabolic network and uncovering the transcriptional responses, we conclude that *N. gonorrhoeae* exhibits metabolic inflexibility when its preferred substrate, cysteine, is unavailable, with proliferation supported by condition-specific adaptive responses rather than broad metabolic versatility. These findings provide valuable insights into how the gonococcus adapts to nutrient scarcity and advances our understanding of the fundamental physiology of this host-adapted pathogen.

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1 | Chapter One

Introduction

1.1 Preface

Neisseria gonorrhoeae is the causative agent of the sexually transmitted infection, gonorrhoea. Gonorrhoea remains a major public health, social, and economic burden, with an estimated ~82 million new infections globally each year (World Health Organization, WHO, n.d.), disproportionately affecting marginalized populations (Kirkcaldy et al., 2019). In Aotearoa New Zealand, the incidence of gonorrhoea has almost made a full recovery to pre-pandemic levels, following the post-pandemic decline, reporting annual laboratory-confirmed cases fluctuating around seven and a half thousand since 2023 (New Zealand Institute for Public Health and Forensic Science, n.d.). However, reported gonorrhoea incidences underestimate the full extent of gonococcal infection globally, given the reliance on testing-dependent surveillance systems, paired with the frequently asymptomatic nature of gonorrhoea (Walker & Sweet, 2011; Quillin & Seifert, 2018).

Compounding this burden of gonococcal disease, *N. gonorrhoeae* has demonstrated an extraordinary capability to develop resistance to every class of antimicrobial used for its treatment since the introduction of antibiotics in the 1930s (Unemo & Shafer, 2011). In recent years, multidrug-resistant strains have emerged, including resistance to ceftriaxone, the last remaining option for monotherapy, raising concerns regarding the efficacy of utilizing current therapies to treat gonorrhoea infections in the future (Fletcher-Lartey et al., 2019; Unemo et al., 2021; Adamson et al., 2024; World Health Organization, n.d.). Thus, the emerging threat of extensively drug-resistant gonococci underscores an urgent need for development of novel therapeutics.

Pathways related to sulfur metabolism have been proposed as promising targets for therapeutic intervention due to the central role of cysteine in core cellular metabolism, in addition to the involvement of sulfur and sulfur-containing molecules in bacterial redox homeostasis, stress resistance, and virulence (Jochim et al., 2019; Das et al., 2021; Shatalin et al., 2021; Varatnitskaya et al., 2021; Tikhomirova et al., 2023; Fatima et al., 2025; Fuchs et al., 2025). In addition to being vital for pathogenesis, several of these sulfur metabolic pathways are

compelling candidates as they are absent in mammals, reducing the likelihood of off-target effects. Indeed, inhibitors of several key enzymes involved sulfur acquisition pathways, namely *de novo* cysteine biosynthesis and sulfate assimilation pathways, have been developed over the past decade (Campanini, Pieroni, et al., 2015; Palde et al., 2016; Hicks et al., 2022; Tao et al., 2024). As a host-restricted pathogen, *N. gonorrhoeae* has an absolute reliance on host-derived nutrients, such as sulfur, for survival and proliferation during infection. Consequently, nutrient acquisition strategies are expected to be central to gonococcal infection outcomes and represent potential vulnerabilities that may be exploited.

1.2 Summary of *N. gonorrhoeae* pathogenesis

N. gonorrhoeae predominately infects the mucosal epithelium of the urogenital tract where infection typically presents as urethritis in men and mild cervicitis in women (Colón Pérez et al., 2024). However, gonococcal infection is frequently asymptomatic, particularly in females, which contributes to prolonged undetected transmission and an increased risk of ascending infection in women, resulting in complications such as pelvic inflammatory disease, ectopic pregnancy, and infertility. In addition to the urogenital tract, *N. gonorrhoeae* can colonize distinct anatomical sites, including the rectum, pharynx, and conjunctiva. Although infections at these sites are less common, they contribute to the burden of disease, transmission, and antimicrobial resistance (Adamson & Klausner, 2021; Mahapure & Singh, 2023; Colón Pérez et al., 2024).

Gonococcal infection proceeds through several stages of pathogenesis encompassing transmission, adherence, colonization, invasion, and immune evasion. As an organism highly adapted to mucosal epithelia, *N. gonorrhoeae* is armed with several surface-exposed structures, including, type IV pili (T4P), opacity (Opa) proteins, lipopolysaccharide (LOS), and porins, in addition to secreted outer membrane vesicles (OMV) that promotes the establishment of infection while evading immune detection and maintaining minimal invasiveness (Quillin & Seifert, 2018).

Adherence of *N. gonorrhoeae* to host cells is the essential first step in establishing infection following transmission. Initial attachment is facilitated by the T4P, subsequently enabling the interaction of Opa proteins and host cell-receptors to

facilitate tighter binding, among various other roles (Merz & So, 2000; Hauck & Meyer, 2003). These interactions trigger host signaling pathways that both enhance bacterial association with epithelial cells and promote T4P-mediated microcolony formation (Quillin & Seifert, 2018; Walker et al., 2023). Microcolony formation precedes biofilm formation and colonization, although the precise role of biofilms in gonococcal pathogenesis is uncertain, it has been proposed to contribute to asymptomatic infections and persistence (Greiner et al., 2005; Steichen et al., 2008).

Successful colonization of the epithelium is contingent upon the ability to overcome nutritional immunity to acquire essential nutrients for proliferation and is further shaped by dynamic pathogen-host interactions. During early colonization events, host-interactions mediated by LOS and OMV activate host signaling cascades that drives cytokine and chemokine production ultimately leading to the recruitment of large numbers of neutrophils to the infection site, a hallmark of gonococcal disease (Kaparakis et al., 2010; Mavrogiorgos et al., 2014; Zhou et al., 2014). Despite the hostile conditions usually generated by such infiltration, the gonococcus is well adapted to survive and replicate both extracellularly and within neutrophils under these conditions (Palmer & Criss, 2018). As *N. gonorrhoeae* is not a producer of potent exotoxins, tissue damage associated with activated innate immunity is thought to contribute to the pathology of symptomatic gonococcal infection (Quillin & Seifert, 2018).

Although gonococcal colonization occurs primarily at the epithelial surface, *N. gonorrhoeae* can occupy and replicate within intracellular niches, including epithelial cells, neutrophils, and macrophages, which contributes to persistence during infection. Intracellular survival within neutrophils is further thought to facilitate transmission by enabling viable bacteria to be transferred within purulent exudates upon sexual contact (Criss & Seifert, 2012). In addition, a subset of gonococci can undergo transcytosis across the epithelial barrier, enabling access to subepithelial tissues and contributing to disseminated infection in rare cases (Merz et al., 1996).

Owing to its long-standing co-evolution with the human host, *N. gonorrhoeae* employs multiple immune evasion and host manipulation strategies that enable persistent infection within both extracellular and intracellular environments (Quillin & Seifert, 2018; Walker et al., 2023). Central to immune evasion are

sophisticated mechanisms of antigenic and phase variation of virulence factors including T4P, Opa proteins, and LOS. These processes effectively limit recognition by the adaptive immune system, posing a significant challenge for vaccine development (Belcher et al., 2023). Beyond immune evasion, the gonococcus demonstrates diverse mechanisms of host manipulation that modulate both innate and adaptive immune responses ultimately dampening effect clearance by the immune system. These include, but are not limited to, the suppression of neutrophils oxidative burst, interference with phagosomal maturation, modulation of apoptosis, serum resistance, and alterations of epithelial cell turnover (Walker et al., 2023). However, in some instances whether the consequences of these gonococcal-host interactions ultimately serve to help or hinder gonococcal pathogenesis is debated (Walker et al., 2023). Nevertheless, strategies of immune evasion and manipulation are crucial aspects of gonococcal pathogenesis, enabling the bacterium to evade detection, persist at mucosal surfaces, and, ultimately, facilitate ongoing transmission.

1.3 Sulfur metabolism in *N. gonorrhoeae*

Acquisition of host-derived nutrients from the host-pathogen interface is essential for the survival and proliferation of *N. gonorrhoeae* throughout infection. While the nutrient compositions of gonococcal niches are largely unknown, factors that influence nutrient availability, including oxygen tension, resident microbiota, and immune pressures, are heterogenic across anatomical sites, between intracellular and extracellular environments, and among individuals (Edwards & Butler, 2011; Quillin & Seifert, 2018). This diversity of host environments encountered by *N. gonorrhoeae* would be expected to favor metabolic flexibility to support adaptation to dynamic, and often challenging, conditions (Chen et al., 2021). However, whether *N. gonorrhoeae* possesses such metabolic flexibility with respect to sulfur is unclear.

It is known that *N. gonorrhoeae* possesses high affinity transporters for the acquisition of exogenous cysteine and cystine (the oxidized form of cysteine), which represents the most efficient strategy to fulfil sulfur requirements (Bulut et al., 2012). However, free cysteine is present at low concentrations within the human host and is tightly regulated (Stipanuk, 2004), rendering the use of alternative sulfur acquisition strategies, such as *de novo* cysteine biosynthesis, highly relevant for

N. gonorrhoeae. At present, multiple lines of evidence support the importance of these sulfur acquisition pathways in *N. gonorrhoeae*. The gene encoding the first enzyme in cysteine biosynthesis, *cysE*, is predicted to be essential for gonococcal viability (Remmele et al., 2014). In addition, the second gene in the cysteine biosynthesis pathway, *cysK*, alongside a gene involved in cystine acquisition (NGO0372) are upregulated upon adhesion to epithelial cells, suggesting both cystine acquisition and *de novo* cysteine biosynthesis are important for gonococcal colonization (Du et al., 2005). Moreover, genetic deletion of *cysK* reduces adherence and invasion of epithelial cells *ex vivo*, providing functional support for the role of sulfur acquisition in the pathogenesis of *N. gonorrhoeae* (Du & Arvidson, 2006). However, beyond these examples, the relative importance of different sulfur acquisition strategies during various aspects of gonococcal pathogenesis has not been investigated.

Currently, the full repertoire of sulfur sources utilized by *N. gonorrhoeae* and the pathways used to acquire and assimilate them remain poorly defined. Moreover, previous efforts carried out by the Hicks lab to characterize the *de novo* cysteine biosynthesis pathway have raised intriguing questions regarding how the gonococcus acquires sulfur required for the synthesis of cysteine (Hicks & Mullholland, 2018; McGarvie et al., 2025). Given that the gonococcus is likely exposed to diverse sulfur landscapes during infection, understanding these pathways is essential for interpreting their contribution to pathogenesis, and consequently, the feasibility of targeting such pathways.

Sulfur metabolism in bacterial pathogens does not appear highly conserved between related species or different species that colonize similar host niches. Thus, how sulfur metabolism in *N. gonorrhoeae* compares to that observed in other pathogens is unknown. In the next section we examine the literature on sulfur metabolism in bacterial pathogens, building the foundation to answer questions regarding sulfur metabolism in *N. gonorrhoeae*.

1.4 Sulfur metabolism in bacterial pathogens

Sulfur is an essential element that contributes to a plethora of fundamental cellular processes including redox homeostasis, synthesis of sulfur containing amino acids and cofactors, and protein architecture (Kies & Hammer, 2022; Lensmire & Hammer, 2019). In bacteria, sulfur requirements are met through the acquisition of

exogenous inorganic and organic sulfur compounds, which are processed through assimilatory and salvaging pathways that converge at cysteine. Cysteine lies at the center of bacterial sulfur metabolism, representing the bioavailable and metabolically versatile form of sulfur. Through cysteine, sulfur is mobilized for the synthesis of numerous sulfur-containing biomolecules vital for fundamental metabolic processes, including methionine, iron-sulfur (Fe–S) clusters, molybdopterin, biotin, thiamine, lipoic acid, and thiolated tRNA nucleosides (Das et al., 2021). Moreover, cysteine is an essential precursor for the synthesis of low molecular weight thiols, glutathione, mycothiol or bacillithiol. These thiols are crucially involved in mitigating oxidative stress, buffering fluctuations in redox state, and repairing damaged proteins, highlighting a pivotal role for sulfur in the survival of bacterial pathogens encountering the hostile environment of the human host (Rawat & Maupin-Furlow, 2020). Accordingly, sulfur acquisition has widespread implications for pathogenesis including colonization, stress-resistance, and virulence.

Despite these essential roles, cysteine is maintained as a tightly regulated intermediate and does not accumulate as elevated levels are cytotoxic. This toxicity arises from the redox activity thiol group (-SH), which reduces intracellular iron and promotes reactive oxygen species (ROS) generation via Fenton chemistry, leading to oxidative stress, DNA and protein damage, inhibition of metabolic enzymes, and impaired bacterial growth (Park & Imlay, 2003; Smirnova et al., 2024). As such, mechanisms to manage cysteine homeostasis are crucial and contribute to various aspects of bacterial pathogenesis (Soutourina et al., 2009; Oguri et al., 2012; Takumi & Nonaka, 2016; Díaz-Yáñez et al., 2020; Kies & Hammer, 2022; Farman et al., 2023; Gebhardt et al., 2020). Sulfur metabolism, therefore, encompasses coordinated processes of sulfur acquisition, assimilation, mobilization, degradation, and export.

This review examines and integrates the current knowledge of sulfur metabolism in human bacterial pathogens, with a specific focus on sulfur acquisition and cysteine degradation pathways and their contribution to bacterial survival and pathogenesis. In doing so, it provides an integrated perspective on sulfur metabolic networks across pathogens, highlighting both shared sulfur metabolic pathways and interspecies variability that reflect environmental constraints, metabolic requirements, and pathogen-specific pathoadaptive strategies.

1.4.1 Sulfur acquisition strategies

The acquisition of host-derived nutrients is an essential step for colonization. During infection, pathogens must navigate complex and often hostile host microenvironments that impose nutrient restrictions (Abu Kwaik & Bumann, 2013). Direct acquisition of cysteine or cystine, the oxidized dimer of cysteine, constitutes the most direct means to fulfil sulfur requirements, involving minimal processing. However, free cysteine concentrations are low within the host and tightly regulated (Stipanuk, 2004). Furthermore, during infection and inflammation, the host demand for cysteine increases (Takeda et al., 2023), presumably further restricting the pool of free accessible cysteine. As such, access to cysteine within the host is generally considered restricted, requiring pathogens to scavenge alternative, and often more abundant, sulfur compounds. Accordingly, pathogens often employ diverse strategies to fulfil sulfur requirements, including synthesizing cysteine *de novo* which is a fundamental mechanism by which inorganic sulfur is incorporated into organic compounds, and/or recycling of organosulfur compounds, including glutathione, methionine, and taurine.

1.4.1.1 Sulfur landscape within the human host

The sulfur landscape encountered by pathogens during infection of different niches is largely unknown, owing to the complexity of several interacting and dynamic factors that collectively govern the availability of sulfur compounds, including the influence of host cells, resident microbes, and local physiochemical conditions (Carbonero et al., 2012; Poole, 2015; Chikina & Vignjevic, 2021; Even et al., 2025; Hansen & Venkatachalam, 2023). While the anatomical infection site, encompassing both host and resident microbial factors influences which sulfur metabolites are accessible, the local physiochemical environment dictates the speciation of redox-sensitive sulfur metabolites. Unlike most nutritional elements, sulfur exists across a wide range of oxidation states (-2 to +6). This unique property is why sulfur is integral to redox biology; however, it presents unique challenges for the acquisition of redox sensitive sulfur sources, in particular cysteine and inorganic sulfur.

Under oxidizing conditions, cysteine rapidly forms cystine while hydrogen sulfide (H_2S), the most reduced species of inorganic sulfur, is progressively oxidized through intermediate species thiosulfate ($S_2O_3^{2-}$) or sulfite (SO_3^{2-}), to the fully

oxidized sulfate (SO_4^{2-}) (Anderson et al., 2007; Hartle & Pluth, 2016). At physiologically relevant conditions (pH 7.4), hydrogen sulfide mainly exists as hydrosulfide anions (HS^-), with the remaining ~20% existing in the form of H_2S gas (dissolved and uncharged) (Wang, 2012). This equilibrium is strongly pH-dependent, with acidic conditions (pH <5), such as those encountered in lysosomes, favoring H_2S speciation (~99%) (Hansen & Venkatachalam, 2023). Henceforth we use the abbreviation H_2S, but acknowledge this reflects a collective, dynamic pool of reduced sulfide species.

This chemical speciation of inorganic sulfur and cysteine directly impacts acquisition and assimilation pathways as oxidized and reduced species are metabolically distinct (Lensmire & Hammer, 2019). As such, different species often require separate import systems and influence the energetic cost associated with such pathways, as oxidized sulfur species require enzymatic reduction to usable reduced forms. A further challenge in the acquisition of these redox-sensitive compounds is that the interplay between physiochemical factors that contribute to the overall redox state of an environment, including oxygen tension, pH, and levels of host-derived ROS/RNS, is dynamic within the host and generates microenvironments where sulfur speciation may differ sharply, even within the same niche (Chikina & Vignjevic, 2021). Consequently, as pathogens navigate a variety of host niches during infection, they encounter diverse (micro)environments of constantly shifting mixtures of reduced and oxidized sulfur species, which, in addition to fluctuations in alternative organosulfur compounds, is likely to necessitate flexible acquisition strategies to exploit the sulfur sources locally available. However, despite the importance of environmental factors in determining the sulfur metabolite pool accessible to pathogens, availability of sulfur compounds is not the sole contributor of sulfur acquisition strategy employed, which is highlighted throughout this section.

1.4.1.2 Transport systems for the acquisition of sulfur metabolites

Acquisition of most exogenous sulfur metabolites proceeds via active transport, facilitated by ATP-Binding Cassette (ABC) transporter systems. Bacterial ABC transport systems are comprised of a periplasmic substrate binding protein (SBP), two transmembrane domains, and two ATP binding domains (Akhtar & Turner, 2022). The SBP is the principal determinant of substrate specificity of the conjugate transport system and binds the ligand, often with high affinity. Bound ligand is

subsequently translocated through the transmembrane permease, powered via ATP-hydrolysis. Alternative, ATP-independent systems, such as permeases and ion symporters, may also be involved in the uptake of sulfur compounds (Chonoles Imlay et al., 2015; Lensmire et al., 2020; Man et al., 2020). Compared to ABC transporters, these alternative systems typically exhibit broader substrate specificity and lower affinity at a reduced energetic cost.

1.4.1.3 Acquisition of exogenous cystine and cysteine

Extracellular environments are predominately oxidizing, favoring cystine, and as such, cystine is often the preferred sulfur source (Ottaviano et al., 2008). Advantageously, the acquisition of cystine has been shown to satisfy intracellular sulfur demands more effectively than cysteine, despite requiring NADPH-dependent reduction upon internalization (Lensmire et al., 2020). Cystine uptake is mediated by either ABC transporters and/or H^+/Na^+ symporters. Biochemical characterization of several ABC transporters and homologs of the H^+/Na^+ symporter, TcyP, from different pathogens reveal these transporters may additionally bind cysteine-related metabolites, although with lower affinity and efficiency (Kim et al., 2012; Chonoles Imlay et al., 2015; Lensmire et al., 2020; Brenner et al., 2022). Whether this substrate versatility is conserved across all homologs or if such versatility is advantageous for sulfur acquisition of during infection remains unclear, but it suggests the potential for substrate flexibility under cystine limitation.

Numerous pathogens encode at least two distinct cystine transporters, including *Listeria monocytogenes*, *Campylobacter jejuni*, *Staphylococcus aureus*, *Streptococcus mutans*, *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium), and *Escherichia coli* (Baptist & Kredich, 1977; Kim et al., 2012; Chonoles Imlay et al., 2015; Man et al., 2020; Lensmire et al., 2020; Brenner et al., 2022), highlighting the prioritization of cystine as a sulfur source for these pathogens. The specific combination of cystine transporters varies between species, with some pathogens carrying two ABC-type systems while others encode both an ABC transporter and TcyP. In other cases, the primary and secondary cystine transporters remain unidentified, suggesting additional cystine transport systems are yet to be elucidated. Further species-dependent nuances occur in the implementation of these systems even among pathogens that employ the same transporter types. For example, *E. coli* and *S. aureus* both encode a TcyP symporter

and an ABC transporter system (Chonoles Imlay et al., 2015; Lensmire et al., 2020). In *E. coli*, the ABC transporter, TcyJLN, represents the high-affinity uptake system, while the symporter, TcyP, provides a lower-affinity, energetically favorable alternative. Whereas in *S. aureus*, TcyP functions as the primary high-affinity importer, and the ABC transporter, TcyABC, operates as the low-affinity transporter. Despite this, general concepts regarding how these transport systems are implemented in response to sulfur availability are consistent. Both pathogens employ the low-affinity transporter (TcyP in *E. coli* and TcyABC in *S. aureus*), when exogenous cystine is abundant while expression of both systems is transcriptionally induced to maximize cystine scavenging under sulfur-limitation.

Further supporting the critical role of cystine acquisition during infection, loss of function studies demonstrate that the inability to effectively import cystine impairs various aspects of pathogenesis *in vitro*, including biofilm formation, stress response, survival under sulfur limitation, and activation of virulence-associated traits (Brenner et al., 2022; Man et al., 2020). The physiological significance of cystine acquisition for fitness of *S. aureus* during infection is further highlighted in an *in vivo* model of competitive infection (Lensmire et al., 2020). Mutants impaired in cystine uptake via deletion of *tcyA* and/or *tcyP* were less recoverable than WT in a strain and tissue specific manner, underscoring intraspecies variability and niche-dependent sulfur acquisition strategies during infection. Importantly, double mutants were still recoverable from the mouse infection model, indicating that metabolic flexibility provides a survival advantage *in vivo*.

In contrast to the prevalent cystine uptake systems, cysteine-specific transporters appear rare among pathogens and the physiological relevance of cysteine acquisition during pathogenesis is not understood. The latter is, in part, due to practical limitations of laboratory environments being aerobic, contributing to difficulties in differentiating cysteine from cystine uptake systems. Moreover, existing cystine transporters may facilitate cysteine acquisition at reduced affinity, making distinguishing between cysteine versus cystine uptake challenging. Generic transporters, such as those involved in import of branched-chain amino acid (BCAA), facilitate cysteine import in binding assays (Zhou & Imlay, 2020). However, cysteine uptake is outcompeted when native substrates are available in laboratory conditions, leaving it uncertain that the observed cysteine uptake via such transporters *in vitro* translates *in vivo*. Other permease-type transporters, such

as CyuP in *E. coli* and *Salmonella enterica*, which facilitates the import of cysteine does so for cysteine catabolism, enabling the utilization of cysteine as a source of carbon rather than sulfur and are not thought to contribute to sulfur acquisition (Loddeke et al., 2017; Zhou & Imlay, 2022).

First discovered and characterized in *C. jejuni*, the ABC substrate binding protein, CjaA, remains the only high-specificity cysteine transporter biochemically characterized to date (Müller et al., 2005). A bioinformatic analysis identified CjaA homologs in only few bacterial pathogens, including *Helicobacter pylori*, *Streptococcus pneumoniae*, *N. gonorrhoeae, Bacillus cereus*, and *Fusobacterium nucleatum* (Müller et al., 2005). The CjaA homologs in *H. pylori* and *N. gonorrhoeae* were originally suspected to be glutamine transporters based on sequence homology (Friedrich et al., 2007; Merrell et al., 2003). However, subsequent kinetic characterization of the *N. gonorrhoeae* CjaA homolog confirms specificity for cysteine and does not recognize glutamine or cystine (Bulut et al., 2012). Later evidence also rejects the glutamine transport annotation in *H. pylori* based on the finding that this bacterium cannot import extracellular glutamine (Shibayama et al., 2007).

Despite the identification of these transporters in clinically relevant, non-related pathogens, conditions under which CjaA supports or contributes to pathogenesis are unresolved. In *C. jejuni*, CjaA (*Cj*CjaA) is dispensable during growth in liquid media *in vitro.* Furthermore, the acquisition of cysteine does not compensate for defects in cystine acquisition, despite cysteine being readily accessible and a sulfur starvation response being triggered. This suggests that cysteine does not contribute to growth under sulfur limitation *in vitro* (Man et al., 2020). In *S. pneumoniae*, transcription of *cjaA* is induced during cysteine limitation suggesting a possible role for cysteine acquisition under sulfur limitation, however, skeptically, the resulting protein is truncated relative to *Cj*CjaA, and the functional consequences of this are unknown (Afzal et al., 2016). In *H. pylori*, *cjaA* is upregulated under acidic conditions, which the bacterium encounters during infection of gastric mucosa, however as the stomach is a strongly oxidized environment, it remains unclear and unexplored how cysteine acquisition might facilitate *H. pylori* infection in this environment (Merrell et al., 2003; Even et al., 2025).

The limited distribution of high-affinity cysteine transporters among bacterial pathogens is striking and raises evolutionary questions regarding the involvement of cysteine acquisition during infection. While this disparity may reflect the inherent instability of cysteine within host extracellular environments, several infection relevant niches are reducing environments which would favor cysteine speciation, including intracellular compartments such as the cytosol (Ottaviano et al., 2008). Moreover, given that cysteine is limited within the host, these conditions would, in principle, be expected to favor high-affinity cysteine uptake, yet such transport systems appear rare among bacterial pathogens. Notably, the occurrence of CjaA homologs appears among un-related gastrointestinal tract, nasopharynx, and urogenital tract colonizers, suggesting a common anatomical infection niche is not responsible for driving selection. However, in common, these pathogens represent anaerobic, microaerobic, or facultative anaerobic pathogens, supporting a possible environmental adaptation to hypoxic conditions (Müller et al., 2005). As such, it has previously been proposed that perhaps a strategy for pathogens residing in predominantly oxygen-rich environments dominated in cystine is to employ high-affinity cystine transporters, as these can import cysteine at a reduced efficacy should cysteine become available. On the other hand, pathogens that thrive in oxygen-limited environments may benefit from maintaining functionally distinct cysteine and cystine transporters (Müller et al., 2005). However, many pathogens survive and replicate in oxygen-limited environments and, of these, only a handful of pathogens encode for high-affinity cysteine transporters.

An alternative, but complementary, explanation for the selection against high-affinity cysteine transporters is a strategic shift toward *de novo* cysteine biosynthesis under microaerobic or anaerobic environments (Zhou & Imlay, 2020). As reduced inorganic sulfur species, such as H_2S, are more stable under these circumstances, the energic burden associated with inorganic sulfur assimilation required for cysteine biosynthesis is significantly minimized. However, these hypotheses collectively fail to explain the implementation of sulfur acquisitions strategies across all pathogens. For example, despite being an obligate aerobe, *Legionella pneumophila* cannot acquire cystine, demonstrating an exclusive preference for cysteine (Ewann & Hoffman, 2006). Outside of its intracellular replicative niche within macrophages or amoeba, *L. pneumophila* persists in the environmental reservoir in a transmissive, non-replicative state (Oliva et al., 2018).

This restriction for cysteine acquisition ensures that replication is initiated after entry into host cells, where cysteine, rather than cystine, is encountered. Cysteine acquisition therefore acts as a metabolic switch out of the transmissive state into the replicative stage of its lifecycle, consequently ensuring replication does not occur outside of favorable environments (Ewann & Hoffman, 2006). *N. gonorrhoeae* provides a further example in which oxygen availability does not explain the implementation of sulfur acquisition strategies as genes involved in *de novo* cysteine biosynthesis, cysteine transport, and cystine transport are all downregulated in response to anaerobic conditions (Isabella & Clark, 2011). Similar incongruencies are observed in the reported responses of *de novo* cysteine biosynthesis to oxygen availability in other pathogens (Salmon et al., 2005; Fuchs et al., 2007).

Collectively, this indicates that sulfur acquisition strategies cannot be reliably predicted by oxygen availability alone. Rather, sulfur acquisition strategies sit at the intersection of environmental conditions, shaped by the complex interplay of various physiochemical factors which govern redox state, alongside pathogen-specific pathoadaptive strategies and metabolic constraints.

1.4.1.4 *De novo* cysteine biosynthesis

The capacity to synthesize cysteine *de novo* is prevalent among pathogens and is crucial for survival, particularly under conditions of sulfur limitation, oxidative stress, and upon interaction with host cells, underscoring the importance of cysteine during colonization events and defense against host-induced stress (Lithgow et al., 2004; Du & Arvidson, 2006; Turnbull & Surette, 2008; Bachman et al., 2015; Gebhardt et al., 2015; Khan et al., 2024).

Cysteine biosynthesis proceeds via a two-step pathway (Figure 1.1, red) that begins with the activation of serine to generate *O*-acetylserine (OAS) catalyzed by serine acetyltransferase (SAT encoded for by *cysE*) (Guédon & Martin-Verstraete, 2007; Kredich, 2008). In the terminal step, OAS is converted to cysteine, which requires the incorporation of an inorganic sulfur source, H_2S or thiosulfate. Two *O*-acetylserine sulfhydrylase (OASS) isoforms mediate this terminal reaction; OASS-A (commonly referred to as CysK) accepts H_2S and OASS-B (commonly referred to as CysM) which utilizes thiosulfate in addition to accepting H_2S. CysM condenses thiosulfate with OAS to form *S*-sulfocysteine (SSC), which is

subsequently reduced to cysteine through an NADPH-dependent reaction that is likely mediated by a glutaredoxin or glutaredoxin-like proteins (Nakatani et al., 2012). In parallel, sulfite is also produced from the reduction of SSC and may be further reduced to H_2S and used as a substrate for CysK or CysM. The OASS isoform carried by different bacterial species is variable, with some species encoding both *cysK* and *cysM*, while others rely on a single isoform.

Non-canonical routes for cysteine biosynthesis unique to *Actinomycetes* are seen in *Mycobacterium tuberculosis*. In addition to carrying the canonical CysK (designated CysK1), *M. tuberculosis* synthesizes cysteine from *O*-phosphoserine, facilitated by two additional OASS variants, confusingly termed CysK2 and CysM (Schnell et al., 2015). CysK2 utilizes H_2S as the sulfur donor, while CysM uses CysO, a small sulfur carrier protein thereby bypassing the requirement of inorganic sulfur (Figure 1.1, blue). This extensive repertoire for *de novo* cysteine biosynthesis in *M. tuberculosis* reflects the non-redundant roles of CysK1, CysK2, and CysM which contribute uniquely to survival under conditions of redox imbalance and oxidative stress (Khan et al., 2024).

Although *de novo* cysteine biosynthesis is prevalent among pathogens, it is not ubiquitously conserved. Cysteine auxotrophy, defined as the absence of functional *de novo* cysteine biosynthetic pathway, has been documented in a limited number of pathogens, including *H. pylori, Francisella tularensis,* and *L. pneumophila* (Ewann & Hoffman, 2006; Alkhuder et al., 2009; Devi et al., 2017). Despite the availability of inorganic sulfur compounds in their respective niches, *H. pylori* and *F. tularensis* instead exploit organosulfur compounds as alternative means to fulfil sulfur requirements. In particular, the utilization of glutathione as a source of sulfur is closely intertwined with the virulence in these species (discussed in Section 1.4.1.6), suggesting that these strategies confer additional functional advantages alongside nutrient acquisition offering a potential explanation for selection pressure against *de novo* cysteine biosynthesis (McGovern et al., 2001; Shibayama et al., 2007; Alkhuder et al., 2009). Similarly, *L. pneumophilia* has been shown to utilize glutathione as a source of sulfur *in vitro,* however the consequences of this are not understood (Pine et al., 1979). Instead of a reliance on alternative sulfur sources, cysteine auxotrophy in *L. pneumophila* is proposed to be necessary to ensure replication does not occur outside of favorable environments by preventing

interference with the cysteine metabolic signaling previously described (Ewann & Hoffman, 2006).

1.4.1.5 Inorganic sulfur assimilation

The acquisition of inorganic sulfur sources is a prerequisite for cysteine biosynthesis. Thiosulfate requires active transport, while H_2S passively diffuses through the membrane (Kumar Pal et al., 2018). However, the unstable nature of H_2S, particularly under oxidizing conditions, means that H_2S mostly exists as more oxidized species, such as sulfate. Accordingly, the H_2S required for cysteine biosynthesis is typically obtained through the acquisition and subsequent reduction of sulfate through the sulfate assimilation pathway (SAP). The ability of pathogens to utilize different inorganic sulfur species as the sole sulfur source for growth is summarized in Table 1.1.

The acquisition of thiosulfate and sulfate is facilitated via the high-affinity ABC transporter, cysUWA. The genetically unlinked SBP, CysP and the sulfate binding protein (sbp), dictate the preference for thiosulfate and sulfate binding, respectively, although they both demonstrate diminished affinity for the other substrates (Guédon & Martin-Verstraete, 2007; Sirko et al., 1995).

The sulfate assimilation pathway

The sulfate assimilation pathway is an energetically expensive process, collectively consuming two ATP and four NADPH molecules to sequentially reduce sulfate to H_2S (Figure 1.1, blue) (Guédon & Martin-Verstraete, 2007; Kredich, 2008). Sulfate is first adenylated by ATP sulfurylase (encoded by *cysDN*) forming adenosine 5-phosposulfate (APS), which is then converted into 3-phosphoadenosine 5-phosphosulfate (PAPS) catalyzed by APS kinase (encoded by *cysC*). PAPS is subsequently reduced to sulfite by the PAPS reductase (encoded by *cysH*). Alternatively, pathogens such as *Neisseria meningitidis* and *M. tuberculosis* encode an APS reductase (also denoted as *cysH*), which bypasses the PAPS intermediate and directly reduces APS to sulfite (Palde et al., 2016; Rusniok et al., 2009). Finally, sulfite is reduced to H_2S via sulfite reductase (encoded by *cysJI*).

Sulfate has been established as an important sulfur source for numerous pathogens including *M. tuberculosis*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, and *N. meningitidis* (Senaratne et al., 2006; Gebhardt et al., 2015; Robinson et al., 2012;

Le Mouëllic et al., 2025), supporting *de novo* cysteine biosynthesis as an important pathway of sulfur acquisition for these pathogens during infection. The importance of this sulfur acquisition strategy in virulent outcomes is highlighted in aerobic pathogens, *N. meningitidis* and *M. tuberculosis*. In *N. meningitidis*, genes for sulfate acquisition and assimilation are significantly upregulated in disease-associated isolates compared to carriage isolates, associating sulfate assimilation with invasive disease outcomes (Ren et al., 2017). Similarly, in *M. tuberculosis*, the genes of the sulfate assimilation pathway and cysteine biosynthesis pathways are strongly upregulated following infection of human macrophages (Fontán et al., 2008; Pinto et al., 2013) and several studies have investigated the survival of SAP deficient mutants *in vivo*, with conflicting outcomes. Deletion of *cysH*, a central SAP gene, resulted in attenuated virulence in a murine model of persistent infection, while no attenuation is observed in the *Mycobacterium bovis* BCG strain deficient in sulfate uptake via deletion of *cysA* (the ATP-binding protein of the sulfate transporter) compared to wild-type (Wooff et al., 2002; Senaratne et al., 2006). More recently, significant sulfate uptake was demonstrated during infection of macrophages and the survival of a *sbp* mutant, blocking sulfate uptake, was attenuated during murine macrophage and lung infection *in vivo* (Le Mouëllic et al., 2025), providing strong support that sulfate acquisition is important for the pathogenesis of *M. tuberculosis.*

Beyond providing a necessary substrate for *de novo* cysteine biosynthesis, the SAP has been implicated in additional cellular functions, including redox homeostasis and mitigation of antibiotic induced stress, linked to H_2S production which has widespread cytoprotective effects in low abundances (Van De Waterbeemd et al., 2013; Álvarez et al., 2015; Le Mouëllic et al., 2025). In *N. meningitidis*, sulfur limitation results in spontaneous OMV release, with the response being exacerbated when sulfate is the source of sulfur limitation compared to cysteine (Van De Waterbeemd et al., 2013). The authors propose that the heightened OMV response during sulfate depletion reflects reduced flux through the sulfate assimilation pathway and a consequent imbalance in NADPH, linking sulfate assimilation to redox homeostasis in the context of OMV release. For *S.* Typhimurium, growth on sulfate, and not cysteine, was found to confer resistance to ciprofloxacin induced oxidative stress, implicating the SAP as an important pathway of H_2S production for survival mechanisms distinct from nutrient acquisition (Álvarez et al., 2015, 2015).

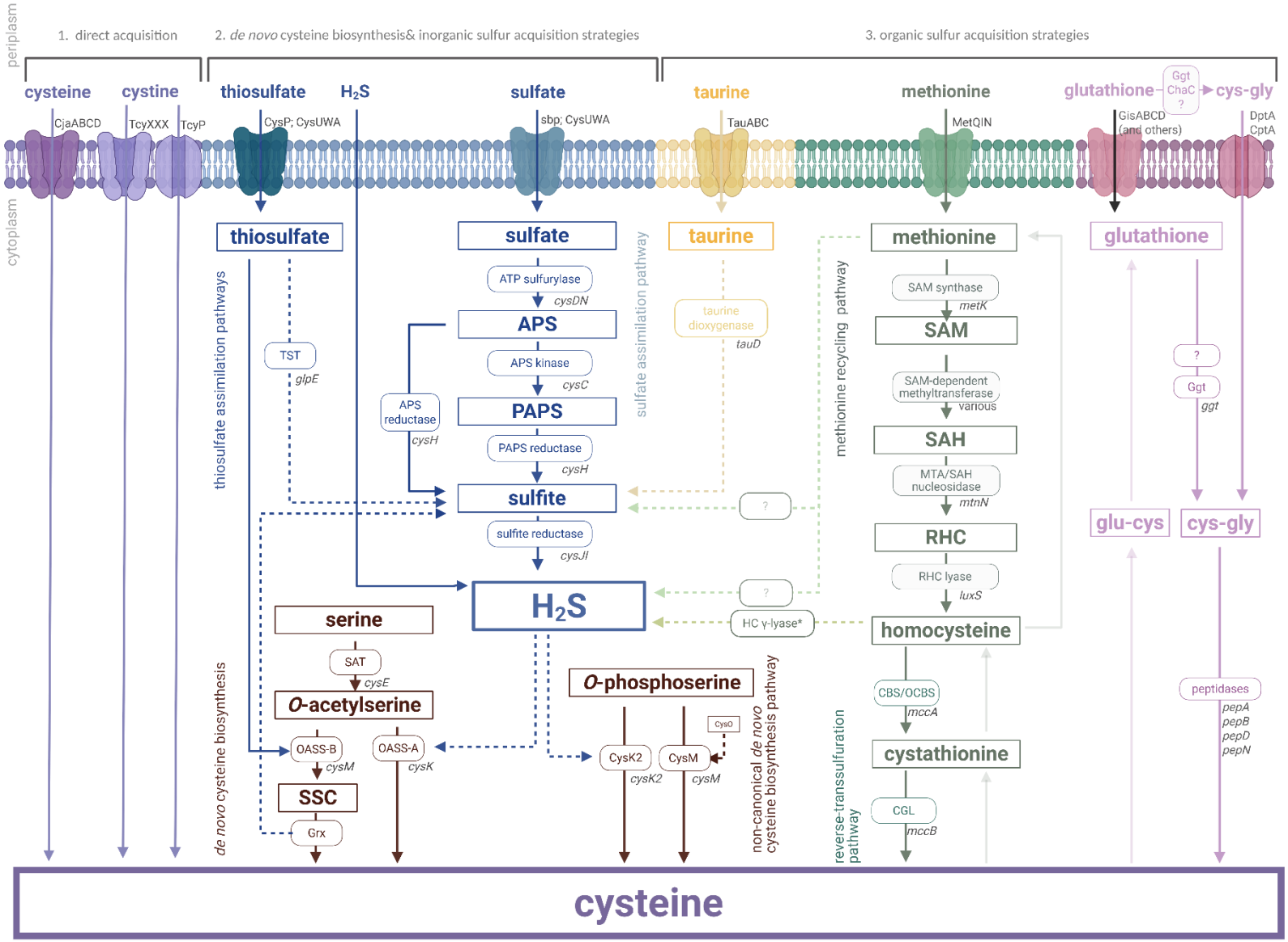


Figure 1.1. Sulfur acquisition in bacterial pathogens. Overview of the pathways of cysteine/cystine acquisition (purple), inorganic sulfur assimilation (blue) and *de novo* cysteine biosynthesis (red), and organosulfur acquisition including taurine (yellow), methionine (green), and glutathione (pink).

Figure 1.1 continued…. Only sulfur containing intermediates are depicted. Enzymes and the genes encoding them are indicated. Dotted lines indicate where the product of one pathway enters another. ? indicates pathways are unidentified and * indicates involvement of the enzyme is hypothesized. Abbreviations: TST; thiosulfate sulfurtransferase, SAT; serine acetyltransferase, OASS; *O*-acetylserine sulfhydrylase, SSC; *S*-sulfocysteine, Grx; glutaredoxin /glutaredoxin-like protein, APS; adenosine 5-phosposulfate, PAPS; 3-phosphoadenosine 5-phosphosulfate, SAM; *S*-adenosylmethionine, SAH; *S*-adenosylhomocysteine, MTA; 5'-methylthioadenosine, RHC; *S*-ribosylhomocysteine, CBS; cystathionine β-synthase, OCBS; *O*-acetylserine cystathionine β-synthase, CGL; cystathionine γ-lyase, Ggt; γ-glutamyl transferase, ChaC; γ-glutamyl cyclotransferase.

Table 1.1. Sulfur source utilization profiles of bacterial pathogens.

Pathogen	Cys	Sulfate	Sulfite	Sulfide	Thiosulfate	Glutathione	Methionine	Taurine	Others	References
Gastrointestinal tract										
H. pylori [a,d]	-	-	-	-	-	**Y**	N	-	HM & CTT	(Baskerville et al., 2023; Doherty et al., 2010)
K. aerogenes [b,f]	Y	Y	Y	Y	Y	-	Y	-	-	(Seiflein & Lawrence, 2001)
E. coli [b,e]	Y	Y	Y	Y	Y	Y	N	Y	-	(Kredich, 2008)
C. difficile [b,e]	Y	N	N	Y	Y	Y	N	-	HM & CTT	(Dubois et al., 2016)
C. perfringens [c,f]	Y	N	Y	Y	Y	Y	N	-	HM & CTT	(André et al., 2010)
C. jejuni [c,d]	Y	N	N	Y	Y	Y	N	-		(Hofreuter et al., 2006; Vorwerk et al., 2014; Man et al., 2020)
L. monocytogenes [c,e,f]	Y	N	-	Y	Y	Y	N	-	GSSG	(Brenner et al., 2022; Berude et al., 2024)
S. Typhimurium [c,e]	Y	Y	Y	Y	Y	Y	N	-		(Baptist & Kredich, 1977; Kredich, 2008)
Respiratory tract										
B. pertussis [a,d]	Y	-	-	-	Y	Y	-	N		(Stenson et al., 2003; Branco Dos Santos et al., 2017)
N. Meningitidis [b,d]	Y	Y	Y	Y	Y	Y	Y	N		(Port et al., 1984; Schoen et al., 2014)
H. influenzae [b,d]	Y	N	-	N	-	Y	-	-	GSSG	(Vergauwen et al., 2003)
S. pneumoniae [b,d]	Y	-	-	-	-	Y	-	-		(Potter et al., 2012; Afzal et al., 2016)
K. pneumoniae [b,f]	Y	Y	-	-	-	-	Y	-		(Seiflein & Lawrence, 2006)
P. aeruginosa [b,f]	Y	Y	Y	Y	Y	-	Y	Y		(Schook & Berk, 1978; Martedi et al., 2025)
F. tularensis [c,e]	Y	-	-	-	-	**Y**	-	-		(Alkhuder et al., 2009; Ramsey et al., 2020)
M. tuberculosis [a,d]	Y	**Y**	-	Y	-	-	Y	-		(Wooff et al., 2002; Wheeler et al., 2005; Le Mouëllic et al., 2025)
L. pneumophila [c,f]	Y	-	-	-	-	Y	-	-		(Pine et al., 1979; Ewann & Hoffman, 2006)
Others										
S. mutans [b,d]	Y	-	-	Y	Y	Y	Y	-		(Sperandio et al., 2010)
S. aureus [b,e]	**Y**	N	N	Y	Y	Y	N	<u>Y</u>	HM &HMC	(Lithgow et al., 2004; Soutourina et al., 2009; Lensmire et al., 2020)
N. gonorrhoeae [a,d]	Y	N	-	-	Y	-	-	-		(Le Faou, 1983, 1984; Bulut et al., 2012)
P. putida [c,f]	Y	Y	-	Y	-	-	Y	Y		(Vermeij & Kertesz, 1999)

Data are derived from in vitro studies, reflecting the capacity of each pathogen to utilize the indicated sulfur source under defined laboratory conditions. Y: growth observed as the sole sulfur source. N; no growth. –; not data available/not tested. Bold entries indicate that utilization of the sulfur source is supported by in vivo data. Entries underlined indicate there is conflicting evidence. a; obligate human pathogen, b; opportunistic pathogen, c; facultative pathogen, d; restricted to human hosts, e; non-human animal hosts, f; associated with environmental reservoirs. Abbreviations: Cys: cystine and or cysteine, CTT: cystathionine, HM: homocysteine, HMC: homocysteine, GSSG: oxidized glutathione.

These examples necessitate careful considerations when correlating defects in sulfate assimilation to sulfur acquisition and, by extension, cysteine biosynthesis.

Thiosulfate assimilation pathways

As a more reduced form of sulfur, the incorporation of thiosulfate into cysteine is advantageous as it requires substantially less energy compared to the reduction of sulfate. Thiosulfate as a sole sulfur source during growth in culture media has been documented for numerous pathogens (Table 1.1). However, the importance of thiosulfate assimilation in pathogenesis has not yet been established, leaving the contribution of thiosulfate acquisition as a nutrient sulfur acquisition strategy during infection unclear.

The canonical pathway of thiosulfate assimilation is the CysM-dependent pathway, which directly incorporates thiosulfate into OAS (Guédon & Martin-Verstraete, 2007; Kredich, 2008). A second, non-canonical, CysM-independent pathway of assimilating thiosulfate for cysteine biosynthesis has been described in *E. coli* (Kawano et al., 2017, 2018). This pathway relies on the reduction of thiosulfate to sulfite and H_2S via a thiosulfate sulfurtransferase (TST), identified as GlpE in *E. coli*. The TST catalyzes the reaction of thiosulfate and glutathione to produce glutathione persulfide (GSSH) and sulfite. Sulfite can then be fed into the sulfate assimilation pathway for further reduction to H_2S, and GSSH spontaneously reacts with an additional glutathione molecule to form glutathione disulfide (GSSG) and H_2S. Additional enzymes may also participate in thiosulfate assimilation as *E. coli cysM* and *glpE* double mutants are capable of minimal growth on thiosulfate, potentially involving an alternative TST, such as PspE (Kawano et al., 2017).

Loss of the sulfur assimilation pathway

Despite the evident importance of sulfate acquisition and reduction for some pathogens, an interesting observation is that numerous pathogens have lost the ability to reduce sulfate, including *Clostridium difficile*, *Clostridium perfringens*, *C. jejuni*, *L. monocytogenes*, *S. aureus*, and *N. gonorrhoeae* (Xayarath et al., 2009; Lithgow et al., 2004; Hicks and Mullholland, 2018). In *C. perfringens*, an incomplete SAP, missing the first enzymes in the pathway, renders *C. perfringens* unable to reduce sulfate to sulfite, but able to reduce sulfite to sulfide, enabling sulfite scavenging (André et al., 2010). In contrast, *C. difficile*, *C. jejuni*, *L. monocytogenes*, *S. aureus* and *N. gonorrhoeae* lack the entire SAP, incapable of

sulfate reduction at any stage, and as such have been described as cysteine auxotrophs (Vorwerk et al., 2014; Dubois et al., 2016; Hicks & Mullholland, 2018; Brenner et al., 2022). It has previously been hypothesized that the loss of the SAP and resulting 'cysteine auxotrophy' in these organisms reflects adaptations to host environments where host reservoirs of organosulfur sources are sufficient to support bacterial requirements, which is favored over *de novo* cysteine biosynthesis due to the comparatively low energy requirement to process organosulfur compounds (Lensmire & Hammer, 2019). However, these pathogens proliferate when thiosulfate is provided as the sole source of sulfur, indicating that the *de novo* cysteine biosynthesis pathway is functional and previously observed 'auxotrophies' more accurately reflects nutrient limitations of experimental conditions (Le Faou, 1984; Lithgow et al., 2004; André et al., 2010; Vorwerk et al., 2014; Lensmire & Hammer, 2019; Berude et al., 2024).

The mechanistic basis for thiosulfate assimilation in these pathogens remains unresolved as, curiously, they lack the CysM isoform required for thiosulfate-dependent cysteine biosynthesis, instead encoding only *cysK*, which has exclusive specificity for H_2S, typically acquired from the reduction of sulfate. Given that TST are ubiquitous enzymes, it is feasible that these pathogens have developed a preference for the TST-mediated thiosulfate reduction pathway to acquire H_2S, in a comparable manner to that described for *E. coli* (Kawano et al., 2018). However, this remains to be demonstrated in pathogens that have lost the capacity for sulfate assimilation.

It is interesting to consider how preferences for sulfate or thiosulfate might reflect adaptations of pathogens to their respective infectious niches. As observed for the acquisition of cysteine or cystine, a correlation between the inorganic sulfur species assimilated by pathogens and their respiratory oxygen requirements is observed. Pathogens that retain the SAP and utilize sulfate include aerobes (*N. meningitidis* and *M. tuberculosis*) and facultative aerobes *(A. baumannii*, *P. aeruginosa,* and *K. aerogenes*) (Table 1.1). Conversely, pathogens that have lost sulfate reduction pathways are a mix of obligate anaerobes (*C. difficile*, *C. perfringens*), microaerobes (*C. jejuni*), and facultative anaerobes (*L. monocytogenes*, *S. aureus*, and *N. gonorrhoeae*) (Table 1.1). This is consistent with the notion that oxygen-limited environments often correlate with more reducing conditions, which may support the stability of reduced inorganic sulfur species, such as H_2S or thiosulfate.

However, as previously discussed, oxygen-levels are only one contributing factor to the sulfur landscape encountered. Local sulfur speciation and sulfur compound accessibility instead reflect the combined influence of host metabolism, microbial activity, and local redox potential (Kies & Hammer, 2022; Hansen & Venkatachalam, 2023). For example, several pathogens that have lost the ability to assimilate sulfate colonize the gastrointestinal tract, an environment rich in dietary sulfate. The gut is host to sulfate-reducing bacteria that produce substantial amounts of H_2S, which is subsequently detoxified to thiosulfate via host epithelial cells (Tomasova et al., 2016). Consequently, multiple inorganic sulfur species are accessible for pathogens to exploit. As regions of the gastrointestinal tract are oxygen-limited (and reducing) (Chikina & Vignjevic, 2021; Even et al., 2025), this may support the persistence of reduced sulfur species, reducing selective pressure to maintain the energetically expensive SAP, while simultaneously reducing competition with resident microbes for sulfate. Thus, the loss of the SAP is not solely dictated by oxygen availability alone but reflects adaptation to niche-specific sulfur landscapes where availability, speciation, and competition collectively shape sulfur acquisition strategy.

1.4.1.6 Recycling of organosulfur compounds

As access to free cysteine is frequently constrained within the host, pathogens have evolved diverse strategies to exploit host-derived organosulfur metabolites, in addition to, or in lieu of, *de novo* cysteine biosynthesis. Glutathione, methionine, and taurine can be metabolically converted into cysteine and support the growth of numerous pathogens as sulfur sources *in vitro* (Table 1.1). However, the contribution of organosulfur compounds to sulfur acquisition during infection remains comparatively underexplored in contrast to cystine/cysteine acquisition and *de novo* cysteine biosynthesis.

Cysteine salvaging from glutathione

Glutathione is a ubiquitous low-molecular weight thiol comprised of glutamate, cysteine, and glycine (Glu-Cys-Gly). Owing to the reactive thiol group provided by cysteine, glutathione is a powerful antioxidant and serves as the major redox buffer in eukaryotes and many prokaryotes, although some Gram-positive bacteria instead rely on thiol functional analogs such as mycothiol or bacillithiol (Rawat & Maupin-Furlow, 2020). Within the human host, glutathione is the most abundant low molecular weight thiol representing a substantial reservoir of cysteine (Forman et

al., 2009). Glutathione is primarily localized to the cytosol where concentrations are within the millimolar range (~ 10 mM), while extracellular concentrations are typically orders of magnitude lower (2 – 20 μM in plasma), with the exception of bile acid where glutathione concentrations can reach 10 mM (Guoyao et al., 2004).

Given the abundance of glutathione within the human host, it is unsurprising that pathogens from diverse niches have evolved mechanisms to degrade glutathione to liberate cysteine as a source of sulfur (Table 1.1). The canonical glutathione degradation pathway is initiated by γ-glutamyl transferase (Ggt), which cleaves the γ-glutamyl bond to form γ-cysteinylglycine (Cys-Gly) and glutamate in the periplasm (Figure 1.1, pink) (Saini et al., 2021). The Cys-Gly dipeptide is subsequently imported into the cytoplasm via proton-dependent dipeptide transporters, such as DptA in *F. tularensis* or CptA in *C. jejuni*, although in most cases the transporters responsible remain unidentified (Vorwerk et al., 2014; Ramsey et al., 2020). Once internalized, Cys-Gly undergoes peptidase-mediated hydrolysis to yield cysteine and glycine (Suzuki et al., 2001). In *E. coli*, at least four such peptidases have been identified, encoded for by *pepA, pepB, pepD* and *pepN*. In parallel, glutamate liberated during the initial cleavage step may be independently acquired and contribute to nitrogen metabolism.

A variation of this canonical pathway is reported in *S. aureus* and *N. meningitidis* where, uncommonly, the Ggt homolog is localized to the cytosol, necessitating glutathione import into the cytosol prior to degradation (Takahashi et al., 2004; Lensmire et al., 2023). In *S. aureus* this is mediated by the high affinity glutathione ABC transporter, GisABCD (Lensmire et al., 2023). Importantly, however, acquisition of host glutathione is not intrinsically linked to the use of glutathione as a sulfur source, as many Gram-positive pathogens lack the capacity to synthesize glutathione, and therefore, rely on exogenous import to establish intracellular glutathione pools required for redox homeostasis and oxidative stress resistance (Brouwer et al., 2022; Potter et al., 2012; Vergauwen et al., 2003). Furthermore, the acquisition of host glutathione has been demonstrated to be involved in the activation of virulence factors, serving as a spatial-temporal cue for virulence, given the substantial difference between extracellular and intracellular glutathione concentrations (Reniere et al., 2015; Berude et al., 2024). Accordingly, the capacity for glutathione acquisition most often reflects roles unrelated to sulfur acquisition.

In addition to the well-characterized Ggt-mediated glutathione degradation pathway, alternative enzymes may also contribute to glutathione catabolism. In *F. tularensis*, γ-glutamyl cyclotransferase (ChaC) functions in parallel with Ggt as a secondary enzyme for initial GSH cleavage in the periplasm, as well as supporting functions distinct from glutathione degradation (Ramsey et al., 2020). However, ChaC homologs appear to be rare among bacteria. Notably, several other pathogens that proliferate when glutathione is provided as the sole sulfur source lack identifiable Ggt and ChaC homologs, including *C. perfringens*, *L. monocytogenes, S. mutans*, and *S. pneumoniae*, supporting the existence of additional, undiscovered mechanisms of glutathione utilization (André et al., 2010; Berude et al., 2024; Potter et al., 2012; Vergauwen et al., 2003). Although alternative peptidases such as PepT, PepM, and PepA, have been proposed to participate in glutathione degradation in the absence of Ggt, there is currently no experimental evidence supporting such a role for these enzymes (André et al., 2010; Dubois et al., 2016). Furthermore, evidence within the literature supports the existence of multiple alternative (or alternative variations) of glutathione utilization pathways, even within the same organism, and suggest these systems may vary in affinity (Potter et al., 2012; Vergauwen et al., 2013; Berude et al., 2024; Vorwerk et al., 2014).

Nevertheless, Ggt-mediated glutathione degradation is recognized as an important contributor to virulence and pathogenesis of several pathogens, underscoring an interesting link between glutathione degradation and disease. In *N. meningitidis*, Ggt contributes to survival in cysteine-deficient environments such as cerebrospinal fluid, a central niche in meningococcal invasive disease (Takahashi et al., 2004). This link between glutathione degradation and virulence is further exemplified in the cysteine auxotrophic pathogens, *F. tularensis* and *H. pylori*. *F. tularensis* leverages abundantly available glutathione to acquire essential cysteine required for replication within the cytosol of macrophages (Alkhuder et al., 2009; Ramsey et al., 2020). Disruption of glutathione degradation pathways severely attenuates replication within macrophages and the ability to cause disease in a mouse model of infection, reinforcing the essential role of nutrient acquisition for virulence *in vivo* (Alkhuder et al., 2009; Ramsey et al., 2020). Accordingly, glutathione degradation has been designated a nutritional virulence mechanism for this bacterium (Abu Kwaik & Bumann, 2013). Similarly, in *H. pylori*, Ggt is classified as a virulence factor that promotes colonization *in vivo* (Chevalier et al., 1999;

McGovern et al., 2001; Fuchs et al., 2025). Intriguingly, *H. pylori* acquires glutathione derived from the host intracellular reservoir following adherence to gastric epithelial cells, through an unidentified mechanism (Baskerville et al., 2023). This exploitation of intracellular glutathione pools for extracellular colonization is curious and is postulated to have additional advantages in the pathogenesis of *H. pylori* beyond sulfur metabolism. Indeed, Ggt mediates the hydrolysis of glutamine to glutamate that can subsequently be assimilated into central carbon metabolism and influences broader metabolic processes including nitrogen metabolism, iron acquisition, and acid resistance that collectively support colonization and persistence of *H. pylori* within the gastric niche (Shibayama et al., 2007; Fuchs et al., 2025). Moreover, given that glutathione is an important antioxidant for maintaining the integrity of gastrointestinal tissue, *H. pylori* induced glutathione depletion is postulated to contribute to epithelial damage and disease progression (Shibayama et al., 2007).

Consistent with the role of Ggt in bacterial pathogenesis, despite not occurring ubiquitously across all strains of *C. jejuni*, Ggt-positive isolates are predominant among human-associated isolates and are found in highly virulent strains, reinforcing a connection between Ggt and adaptation to the human host (Barnes et al., 2007; Hofreuter et al., 2006). In accordance with this, in a highly virulent *C. jejuni* strain, Ggt substantially contributes to colonization in the murine intestine *in vivo* (Hofreuter et al., 2006). However, Ggt is dispensable during invasion and survival within intestinal epithelial cells *ex vivo*, suggesting glutathione degradation may be most relevant during extracellular colonization, as observed for *H. pylori* and in contrast to *F. tularensis*.

Collectively, these examples illustrate that Ggt represents a specialized and context-dependent mechanism of glutathione degradation and utilization as a nutrient source, one that extends beyond merely scavenging behavior and better reflects a niche-tailored virulence strategy for certain pathogens. However, as Ggt-independent mechanisms of glutathione degradation are yet to be fully elucidated, the full extent of glutathione degradation and utilization as sulfur source across pathogens remains a matter of investigation.

Methionine-to-cysteine conversion

Methionine is an indispensable amino acid and the precursor of the universal methyl donor, *S*-adenosylmethionine (SAM). Pathogens can directly acquire exogenous methionine from the host, or synthesize methionine *de novo* using cysteine and/or H_2S as the sulfur donor. Under conditions where cysteine is limited, some pathogens can exploit host derived methionine as a source of sulfur, recycling it back into cysteine. Indeed, growth of numerous pathogens *in vitro* is supported by methionine as the sole sulfur source (Table 1.1). However, evidence detailing methionine as a physiologically relevant sulfur source during infection has not been demonstrated.

Methionine uptake is primarily mediated via the conserved high-affinity ABC transporter, MetINQ (Chen & Yang, 2022). Once internalized, methionine must be processed through two sequential pathways to obtain cysteine, the methionine recycling pathway, required to convert methionine to homocysteine, followed by the reverse-transsulfuration pathway (RTSP). In the methionine recycling pathway (Figure 1.1, green), methionine is first converted to SAM via SAM synthase (encoded by *metK*) (Rodionov et al., 2004). Various SAM-dependent methyltransferases then generate *S*-adenosylhomocysteine (SAH), which is subsequently converted to *S*-ribosylhomocysteine (RHC) via the 5'-methylthioadenosine/*S*-adenosylhomocysteine (MTA/SAH) nucleosidase (encoded by *mtnN)*. Finally, RHC is converted to homocysteine by the RHC lyase (encoded by *luxS*).

The RTSP is then required to convert methionine-derived homocysteine to cysteine (Seiflein & Lawrence, 2001; Wheeler et al., 2005; Doherty et al., 2010; Lee et al., 2019). This involves the condensation of homocysteine with serine or OAS to form cystathionine catalyzed by cystathionine β-synthase (CBS or OCBS, respectively; encoded by *mccA*). Cystathionine is subsequently degraded by cystathionine γ-lyase (CGL; encoded by *mccB***)** to produce cysteine. In addition to facilitating the conversion of methionine to cysteine, CBS/OCBS and CGL further catalyze secondary reactions involved in the degradation of cysteine, and other substrates, to H_2S and thus may be additionally involved in alternative cellular functions (Shatalin et al., 2011; Mothersole & Wolthers, 2019; Pedretti et al., 2024).

Alternative routes for methionine utilization as a sulfur source have also been documented for the opportunistic pathogens *Pseudomonas putida* and *Klebsiella pneumoniae* involving the generation of reduced sulfur species from methionine as substrates for *de novo* cysteine biosynthesis (Seiflein & Lawrence, 2001, 2006; Vermeij & Kertesz, 1999). This is hypothesized to occur through methanethiol-dependent methionine catabolism, although the exact enzymes and intermediates involved remain to be characterized. In *K. pneumoniae* a H_2S intermediate is hypothesized to be produced, whereas in *P. putida*, sulfite is thought to be generated, which can be further reduced by the SAP.

In addition to methionine, RTSP substrates and intermediates, homocysteine and cystathionine, may also be acquired as sulfur sources (Soutourina et al., 2009; André et al., 2010; Doherty et al., 2010; Dubois et al., 2016). Interestingly, several pathogens that proliferate when homocysteine is provided as the sole sulfur source are unable to utilize methionine despite encoding the genes required to convert methionine to homocysteine (Soutourina et al., 2009; André et al., 2010; Dubois et al., 2016). In these organisms, the inability to utilize methionine is not understood but importantly highlights that genomic prediction does not necessarily predict nutrient utilization phenotypes. These observations further suggest that homocysteine and cystathionine can function as sulfur sources independent of methionine recycling. Proliferation with homocysteine and cystathionine is explained by the RTSP in *H. pylori*, *C. perfringens,* and *S. mutans* while *C. difficile* does not encode this pathway. Instead, it is proposed that homocysteine is degraded to H_2S, potentially via homocysteine γ-lyase, and subsequently incorporated into cysteine via the *de novo* synthesis pathway (Dubois et al., 2016).

Investigation into the acquisition of methionine, homocysteine or cystathionine as sulfur sources during infection remains limited. Deletion of *metB* in *M. tuberculosis*, which encodes a bifunctional CGL protein, resulted in minor attenuation of virulence *in vivo*, however it remains to be determined whether this phenotype specifically reflects impaired sulfur acquisition, as has been proposed (Smith et al., 2001; Wheeler et al., 2005). One context where the acquisition of cystathionine may support pathogenesis is within intramacrophage environments. Mammalian cells also encode the RTSP, which is relied upon as the primary pathway to synthesize cysteine (Sbodio et al., 2019). Infection of macrophages with *H. pylori* induces expression of the mammalian CGL homolog, CTH, indicative of an increased flux

through the host RTSP, which is accompanied by increases in intracellular and extracellular cystathionine levels (Gobert et al., 2019). Cystathionine supports *H. pylori* sulfur metabolism and resistance to oxidative and nitrosative stress, indicating that host-derived cystathionine can function as a relevant sulfur source during intracellular infection, which contributes to stress-resistance (Gobert et al., 2019). Subsequent work further demonstrated that *H. pylori* induced CTH drives metabolic remodeling and activation of macrophages, promoting inflammation and contributing to gastritis *in vivo* (Latour et al., 2022). These studies reveal a sophisticated mechanism by which manipulation of host-metabolic pathways can simultaneously influence nutrient availability and immune activation, contributing to the virulence of *H. pylori*.

Taurine assimilation

In mammals, taurine is synthesized from cysteine and is a highly abundant non-proteinogenic amino acid, concentrated in the brain, eyes, heart, muscles, kidneys, and liver (Rais et al., 2023). Bacterial pathogens are not known synthesizers of taurine; however limited reports demonstrate that certain pathogens may acquire taurine as sulfur source (Table 1.1).

The ABC transporter, encoded by *tauABC*, facilitates taurine uptake (Guédon & Martin-Verstraete, 2007). In contrast to other organosulfur compounds that are processed into cysteine, in a 'reverse synthesis' manner, taurine is degraded via α-ketoglutarate-dependent taurine dioxygenase (*tauD*) to produce sulfite, which may then feed into the latter part of the SAP, and subsequently cysteine biosynthesis (Figure 1.1, yellow) (Guédon & Martin-Verstraete, 2007). In *E. coli*, expression of this pathway is induced under sulfur-limiting conditions, suggesting that taurine utilization may function primarily as a conditional sulfur scavenging strategy (van der Ploeg et al., 1997).

Other than *E. coli*, evidence for taurine utilization as a sulfur source is sparse. Early studies demonstrated that *P. aeruginosa* and *P. putida* are capable of growth on taurine as a sole sulfur source in defined media (Shimamoto & Berk, 1979; Vermeij & Kertesz, 1999); however, the genetic basis remains uncharacterized. In *S. aureus*, taurine is efficiently transported into the cell and a putative sulfonate transporter assumed to import taurine demonstrates responsiveness to the primary sulfur regulon transcriptional regulator, CymR (Smiley & Wilkinson, 1983; Soutourina et

al., 2009). However, the absence of the gene required for taurine degradation, *tauD*, and genes required for sulfite reduction, in addition to convincing evidence that taurine supports growth as a sulfur source, taurine is currently not considered a nutritionally relevant sulfur source for *S. aureus* (Soutourina et al., 2009; Lensmire & Hammer, 2019). Instead, postulated functions of taurine acquisition for *S. aureus* include osmoregulation and, in some strains, contribution to capsular polysaccharide composition (Smiley & Wilkinson, 1983; Soutourina et al., 2009,2010).

At present, no studies have directly demonstrated a contribution of taurine assimilation to bacterial fitness or virulence during infection. However, given the presence of resident sulfate-reducing bacteria within the gastrointestinal tract, taurine could represent a less contested sulfur source compared to other abundantly available metabolites such as sulfate.

1.4.1.7 Regulation of sulfur acquisition pathways

Due to the cytotoxic nature of cysteine, tight regulation of sulfur acquisition pathways are important for survival, with dysregulation associated with impaired biofilm formation, increased sensitivity to environmental stress and reduced virulence (Soutourina et al., 2009; Gebhardt et al., 2020). The expression of genes involved in sulfur acquisition, assimilation, and salvaging, collectively referred to as the sulfur regulon, are coordinated through transcriptional regulators. Among them, the LysR-type transcriptional regulator (LTTR), CysB, and the Rrf2 transcriptional regulator, CymR, found in Gram-negative and Gram-positive bacteria, respectively, have been extensively characterized and thoroughly reviewed elsewhere (Guédon & Martin-Verstraete, 2007; Kredich, 2008; Kies & Hammer, 2022). Briefly, cysteine quantities are indirectly sensed through intracellular concentrations of OAS/*N*-acetylserine (NAS; formed spontaneously from OAS). As cysteine directly inhibits CysE activity, the first enzyme in *de novo* cysteine biosynthesis, when intracellular cysteine becomes depleted (resulting from extracellular nutrient limitations), CysE becomes active and intracellular OAS/NAS pools increase and interact with CysB and CymR in different ways. CysB is a positive regulator, which is activated by the binding of OAS or NAS, consequently inducing the transcription of the sulfur regulon. CymR, in contrast, is a negative regulator, inactivated by OAS/NAS. When OAS/NAS levels increase, CymR dissociates from DNA, allowing transcription of the sulfur regulon to occur.

Activation of the sulfur regulon subsequently induces a broad transcriptional response, inducing expression of multiple genes encompassing cystine uptake, *de novo* cysteine biosynthesis, inorganic sulfur assimilation, and recycling of sulfur from organosulfur metabolites.

The sulfur starvation responses as regulated by CysB or CymR in *S. aureus*, *E. coli*, and *Salmonella* equips these pathogens to draw upon diverse sulfur sources when preferred substrates are unavailable. In *E. coli* this response extends to activation of the *cbl* regulator, which controls genes for taurine and sulfonate assimilation (Stec et al., 2006). In other Gram-negative pathogens capable of methionine-to-cysteine conversion, such as *K. aerogenes,* additional signaling mechanisms have been described that allow the bacterium to prioritize sulfur sources, utilizing methionine only in the absence of both cysteine and sulfate (Seiflein & Lawrence, 2001). In this way, APS, an intermediate of the SAP, acts as a signal of sulfate availability, and therefore, active cysteine biosynthesis. It is only when APS levels decrease does the RTSP become induced mediated by CysB.

Although CysB and CymR represent the canonical transcriptional regulators of the sulfur regulon in bacteria, these are not conserved across all pathogens. At least three alternative LTTR regulators have been identified in Gram-positive and Gram-negative pathogens. The sulfur regulon in species belonging to the *Streptococcus* and *Lactococcus* genera are controlled by the LTTR, CysR, with some streptococcal species additionally utilizing a second LTTR, HomR (Sperandio et al., 2010). In *S. mutans*, for example, CysR and HomR likewise respond to intracellular OAS/NAS concentrations. CysR, induces transcription of *cysK*, *tcyABC, gshT*, in addition to inducing transcription of *homR* (Sperandio et al., 2010). In turn, HomR further activates transcription of additional sulfur acquisition genes, *tcyDEFGH*, and as-yet unidentified thiosulfate metabolism genes, as well as genes involved in methionine biosynthesis demonstrating a cascade-like regulatory network. In contrast, the CysR homolog in *S. pneumoniae*, CmbR, broadly regulates the expression of all sulfur acquisition genes, including cysteine acquisition, glutathione acquisition, *de novo* cysteine biosynthesis, and cystine transport without a secondary regulator, emphasizing variation in the mode of regulation of sulfur acquisition genes among related streptococci (Afzal et al., 2016). More recently, in the absence of an obvious CysB homolog, the transcriptional regulator, GigC, was described in *A. baumannii* as an important regulator of genes involved in sulfate

assimilation and *de novo* cysteine biosynthesis required for virulence *in vivo* (Gebhardt et al., 2020).

The regulation of sulfur acquisition is pivotal for adaptations within dynamic host environments, enabling pathogens to balance the necessity and toxicity of cysteine to maintain intracellular homeostasis. Like the pathways they govern, these transcriptional regulatory networks also exhibit interspecies variation, further illustrating the diversity of sulfur metabolism across pathogens.

1.4.2 The mechanisms and function of cysteine degradation

Although cysteine is an essential metabolite, mechanisms of cysteine degradation are equally important for pathogenesis contributing to numerous, often non-exclusive, cellular functions including maintenance of cysteine homeostasis, endogenous H_2S production, the use of cysteine as a carbon or nitrogen source, and the regulation of virulence factors (Bogdan et al., 2001; Loddeke et al., 2017; Gu et al., 2018; Díaz-Yáñez et al., 2020; Kanojiya et al., 2022; Farman et al., 2023; Smirnova et al., 2024; Liu et al., 2025).

Cysteine catabolism is mediated by several distinct enzyme classes, including cysteine desulfhydrases (CDS), cysteine desulfidases (CD), cysteine dioxygenases (CDO), and cysteine desulfurases (CSD). CDS and CD both degrade cysteine to pyruvate, ammonia, and H_2S (Carbonero et al., 2012; Gervason et al., 2025). CDS are PLP-dependent enzymes that are broadly distributed across diverse taxa, whereas CD are Fe-S cluster containing proteins that predominantly occur in bacteria adapted to anaerobic or microaerobic environments. By contrast, CDOs catalyze the oxidation of cysteine, forming cysteine sulfonic acid which can be further metabolized to taurine or to pyruvate and sulfite (Dominy et al., 2006). Although predominantly described in eukaryotes, CDO homologs have also been identified in a subset of prokaryotes across several phyla (Dominy et al., 2006). Finally, the CSDs are highly conserved, ubiquitous PLP-dependent housekeeping enzymes that are functionally and mechanistically distinct from the aforementioned enzyme classes. CSDs function primarily to mobilize sulfur from cysteine for the biosynthesis of downstream sulfur-containing biomolecules by forming an enzyme-linked persulfide and releasing alanine (Das et al., 2021). Several CSD isoforms have been identified as belonging to separate classes of CSD proteins, the

classification and contribution of these enzymes to pathogenesis have been extensively reviewed elsewhere (Das et al., 2021).

Another pathway to degrade cysteine involves the sequential activity of aspartate aminotransferase (AspC) and 3-mercaptopyruvate sulfurtransferase (3-MST) (Shatalin et al., 2011). In this pathway, cysteine is first deaminated to yield 3-mercaptopyruvate via AspC, which is used as a substrate to generate pyruvate and the 3-MST persulfide intermediate, which subsequently reacts with reduced thiols or thioredoxins to release H_2S.

Beyond these dedicated cysteine-degrading enzymes, cysteine desulfhydrase activity has been reported as a secondary function of several PLP-dependent enzymes, although these are less efficient (Awano et al., 2005). These include enzymes involved in cysteine biosynthesis (CysK and CysM), methionine biosynthesis (cystathionine β-lyase, MetC, and related homolog, MalY), and tryptophan degradation (tryptophanase, TnaA). Additionally, enzymes of the RTSP, CBS and CGL, also catalyze several alternative reactions involving the release of H_2S from cysteine and homocysteine (Shatalin et al., 2011; Devi et al., 2017; Pedretti et al., 2024).

Due to this catalytic redundancy, pathogens generally encode multiple enzymes capable of cysteine degradation suggesting a high degree of functional redundancy thus complicating efforts to discern the specific contributions of different enzymes to cysteine degradation and the role of cysteine degradation to bacterial fitness and pathogenesis.

1.4.2.1 Cysteine homeostasis

A fundamental physiological role of cysteine degradation is maintenance of intracellular cysteine as evident by increased sensitivity to cysteine toxicity observed in mutants strains defective in cysteine-degradation *in vitro* (Kunota et al., 2021; Oguri et al., 2012). Although cysteine is generally considered limited within the host, certain anatomical sites, such as the gastrointestinal tract may be subject to increased cysteine exposure necessitating robust mechanisms of cysteine degradation (Díaz-Yáñez et al., 2020). This is exemplified in *S.* Typhimurium where CdsH, a CD that protects against cysteine toxicity, promotes intracellular survival in epithelial cells when extracellular cysteine levels are elevated (Oguri et al., 2012; Díaz-Yáñez et al., 2020). This scenario is proposed to reflect fluctuations

in luminal cysteine concentrations associated with dietary cycles, suggesting that cysteine detoxification represents a pathoadaptive strategy most relevant to the intestinal phase of infection. Consistently, CdsH does not confer a survival advantage for survival within macrophages (Oguri et al., 2012).

In contrast to *S.* Typhimurium, mechanisms of cysteine degradation are important contributors to the adaptation of *B. pertussis* to the intramacrophage environment. Two CDO genes are strongly induced during intramacrophage survival, and deletion of these genes is associated with increased viability of infected macrophages (Farman et al., 2023). Although these enzymes are proposed to function in cysteine homeostasis, additional evidence is required to validate this physiological function for the CDOs in *B. pertussis*.

1.4.2.2 Source of endogenous hydrogen sulfide production

H_2S has emerged as an important gasotransmitter and is increasingly recognized as an important contributor to pathogenesis (Liu et al., 2025). Endogenous H_2S production is implicated in diverse roles, ranging from, but not limited to, modulation of core cellular functions to mechanisms of protection against oxidative and antibiotic-induced stress, as well as direct contributions to virulence (Lee et al., 2006; Großhennig et al., 2016; Rahman et al., 2020; Chen et al., 2022; Liu et al., 2025). As cysteine is a major substrate for endogenous H_2S production, pathways of cysteine degradation play a central role in processes in which H_2S influences pathogenesis.

Endogenous H_2S confers resistance to oxidative- and/or antibiotic-induced stress in multiple pathogens under aerobic conditions, including *E. coli, S. aureus, Bacillus anthracis, P. aeruginosa,* and *Vibrio cholerae* (Shatalin et al., 2011; Ma et al., 2021). In these pathogens, the cysteine degrading enzymes, 3-MST in *E. coli* and CBS/CGL in the remaining pathogens, were implicated in mediating stress-resistance phenotypes. Furthermore, 3-MST-deficient *E. coli* and CBS/CGL deficient *S. aureus* exhibit increased susceptibility to immune-mediated killing and are cleared more efficiently in infection-relevant murine models, supporting a protective role of H_2S against early immune responses during infection (Toliver-Kinsky et al., 2019). However, attribution of these *in vivo* infection phenotypes to H_2S production in *S. aureus* warrants caution as CBS and CGL form the RTSP, the disruption of which may have broader consequences for sulfur metabolism.

Despite the evident contribution of 3-MST in stress resistance, the mechanism by which 3-MST confers cytoprotection is debated (Li et al., 2019; Mironov et al., 2017; Shatalin et al., 2011). It has been proposed that the AspC/3-MST pathway represents the major route for endogenous H_2S production, which confers protection via sequestering free iron thereby mitigating cellular damage arising from Fenton chemistry (Mironov et al., 2017). However, subsequent work argues that 3-MST instead generates reactive sulfone sulfur, which underlies protection against oxidative stress, while H_2S production is predominantly mediated by CDS, primarily TnaA, which is subsequently lost rapidly through diffusion (Li et al., 2019).

In addition to protection against oxidative stress, endogenous H_2S in *E. coli* has been further shown to support growth and energy metabolism under reducing and anaerobic conditions (Wang et al., 2019). Under these conditions, the CSD, IscS, represents the primary contributor to H_2S production, illustrating that pathways responsible for cysteine degradation within the same organism can vary depending on environmental conditions (Wang et al., 2019).

Similar roles for cysteine-derived H_2S have also been demonstrated in *M. tuberculosis* (Kunota et al., 2021). The CDS, Cds1, has been identified as the major H_2S-producing enzyme, contributing to H_2S-mediated modulation of central metabolism, respiration, redox homeostasis, and antibiotic susceptibility. However, despite this central role, *M. tuberculosis cds1* mutants are not attenuated during macrophage infection, presumably due to the existence of multiple H_2S producing enzymes.

Beyond these roles in metabolism and cytoprotection, production of H_2S is notable for its contribution to virulence for some pathogens as H_2S is a volatile sulfur compound which can contribute to host cell damage (Großhennig et al., 2016; Kawahara et al., 2020; Chen et al., 2022). The opportunistic pathogen, *F. nucleatum* for example, is among the most prominent H_2S producers in the oral cavity, contributing to malodor and tissue damage in patients with periodontal disease (Chen et al., 2022). Although multiple cysteine-degrading enzymes contribute to H_2S production, in *F. nucleatum* the methionine γ-lyase, MegL, has been determined to be the major H_2S producing enzyme, contributing substantially to both virulence and antibiotic susceptibility (Chen et al., 2022). Similarly, in

Mycoplasma pneumoniae, cysteine-degradation via the dual-functioning CDS/CSD enzyme, HpaE, drives haemolysis, establishing HapE as a candidate virulence factor and highlighting how cysteine catabolism and H_2S production can mechanistically contribute to host cell damage (Großhennig et al., 2016).

1.4.2.3 Source of carbon and/or nitrogen

Cysteine degradation commonly yields pyruvate and ammonia, alongside H_2S. Accordingly, the exploitation of cysteine as a carbon and/or nitrogen source has been documented for some pathogens. In *E. coli* and *S. enterica*, cysteine degradation under anaerobic conditions is mediated by the CD, CyuA, which is encoded within the *cyuPA* operon, alongside the cysteine permease, CyuP, which facilitates cysteine import (Loddeke et al., 2017; Zhou & Imlay, 2022). Notably, this cysteine uptake system is distinct from transporters involved in acquiring cysteine as a sulfur source. Although *E. coli cyuA* mutants exhibit sensitivity to exogenous cysteine, the orchestration of CyuA and CyuP within the same operon suggests this system predominantly functions to enable cysteine uptake for contributing to metabolism rather than a mechanism of cysteine homeostasis.

The utilization of cysteine as a carbon and/or nitrogen source has been reported in other bacterial pathogens including *N. meningitidis* and *L. pneumophila,* however, in these organisms the enzymes responsible for cysteine degradation have not been identified (Ewann & Hoffman, 2006; Kanojiya et al., 2022). In *N. meningitidis*, cysteine facilitates growth as the sole nitrogen source *in vitro* and leads to niche-specific infection phenotypes, enhancing intramacrophage survival while reducing epithelial cell adhesion and invasion, indicating that nutrient availability, specifically source of nitrogen, can differentially influence meningococcal host-interactions (Kanojiya et al., 2022). Finally, *L. pneumophila* utilizes host-derived cysteine as a source of carbon, which supports the transition of this bacterium into a metabolically active stage of its lifecycle by supporting intracellular proliferation (Ewann & Hoffman, 2006; Price et al., 2014).

1.4.2.4 Cysteine as post-translational regulator of virulence

Bacteria frequently use metabolic cues to modulate metabolism and virulence functions. In this regard, it is notable that cysteine availability contributes to the regulation of virulence factors in *C. difficile* and *B. pertussis*. Cysteine strongly represses production of the *C. difficile* toxins, TcdA and TcdB, the major virulence

factors responsible for disease progression and colon dysfunction (Dubois et al., 2016; Gu et al., 2018). Cysteine is degraded by the CD, CdsB, generating pyruvate and H_2S. The accumulation of pyruvate acts as a metabolic signal that leads to the repression of the toxin genes. Thus, when cysteine is limited and pyruvate quantities diminish, toxin expression is induced.

Likewise, cysteine availability has been implicated in the regulation of virulence factors in *B. pertussis*, with enhanced production of the pertussis toxin (Ptx) under conditions of cysteine limitation (Bogdan et al., 2001). The conversion of cysteine to pyruvate and sulfate (oxidized H_2S) has been proposed as contributing to this regulatory mechanism, with sulfate specifically identified as acting as a negative regulator of Ptx expression (Bogdan et al., 2001). However, the exact pathway by which this occurs has not been identified. Interestingly, *B. pertussis* is the causative agent of whooping cough, a disease prevalent in younger populations. As cysteine concentrations are reportedly lower in infants, it has been postulated that cysteine availability within these host populations may contribute to differences in disease manifestation between infant and adult populations (Bogdan et al., 2001).

1.4.3 Systems of cysteine export

Cysteine export is another potential mechanism used by bacteria to maintain cysteine homeostasis and confer protection against oxidative stress. However, whether this is a conserved mechanism utilized by pathogens is unclear as the extent of our understanding of the existence and functions of cysteine exporter systems is limited to research conducted in *E. coli*.

Several cysteine exporter proteins have been described in *E. coli* including AlaE, EamA, CydDC, and Bcr. AlaE was originally identified as an alanine exporter protein; however more recent transcriptomic and functional evidence identifies AlaE as the primary exporter of excess intracellular cysteine, supporting a role for AlaE in cysteine homeostasis (Korshunov et al., 2020). EamA is a member of the drug/metabolite exporter family of proteins and exports metabolites of the cysteine biosynthesis pathway, cysteine and OAS, in addition to other amino acids such as glutamine and asparagine (Dassler et al., 2000; Franke et al., 2003). EamA has been implicated as a component of the cystine/cysteine shuttle system, that delivers reducing equivalents in the form of cysteine to the periplasm to mitigate oxidative stress (Ohtsu et al., 2010, 2015) Similarly, the ABC transporter, CydDC, was

originally proposed to contribute to redox homeostasis in the periplasm via export of cysteine (Pittman et al., 2002). However, it was later determined that the principle function of CydDC is to maintain cysteine in the reduced form by reducing cystine thus contributing to the cytoplasmic thiol redox balance (Mironov et al., 2020). Finally, Bcr is a multidrug efflux pump belonging to the major facilitator superfamily of membrane transporters, which has also been reported to contribute to cysteine exportation in *E. coli* strains engineered to overproduce cysteine, in addition to its primary role as conferring resistance to several antibiotics (Yamada et al., 2006). The extent to which such systems are conserved or are functionally relevant across bacterial pathogens has not been investigated, emphasizing a notable gap in our understanding of sulfur metabolism in bacteria pathogens.

1.4.4 Conclusions

The multifaceted nature of cysteine in protein structure and function, redox homeostasis, synthesis of sulfur-containing compounds and stress response, situates pathways of sulfur metabolism as important determinants of pathogenic fitness as they navigate the hostile and dynamic environments of the human host. Furthermore, beyond survival, pathways involved in the pursuit and degradation of cysteine have been implicated as determinants of virulence, acting directly as mechanisms of nutritional virulence and indirectly through the regulation of virulence factors.

A common theme emerging from the current literature is the intriguingly divergent nature of sulfur metabolic networks across bacterial pathogens. Sulfur acquisition strategies display variability among closely related species and pathogens occupying similar host niches. This variability suggests that sulfur acquisition strategies extend beyond simple nutrient scavenging behavior and instead sit at the intersection between pathogen-specific metabolic requirements, pathogenesis strategies, and the complex environments encountered within the host. The primary mechanisms and functions of cysteine degradation pathways likewise appear variable across bacterial pathogens. For some pathogens cysteine degradation may function primarily as a detoxification mechanism, whereas in others these pathways appear to contribute to metabolic flexibility or virulence-associated processes. However, limited research in this area presents challenges in understanding the full scope of conserved versus pathogen-specific functions of cysteine degradation.

This is further compounded by the apparent catalytic redundancy of cysteine degradation, complicating efforts to discern the specific physiological roles of individual enzymes and the subsequent contribution of these functions to infection phenotypes. Together, this observed variability in sulfur metabolic networks presents challenges for the use of model organisms to infer sulfur metabolic strategies across pathogenic species in the absence of experimental validation. As highlighted by this review, genetic prediction does not necessarily correspond to nutrient utilization phenotypes, signifying the limited knowledge in this field.

Despite progress in understanding certain aspects of sulfur metabolism, our overall understanding remains limited. In particular, the pathways that enable the utilization of organosulfur compounds remain poorly defined. Furthermore, how different sulfur sources facilitate pathogens as they transition through different host environments *in vivo* is not well understood and inherently challenging given the unknown compositions of sulfur landscapes encountered in most infection-relevant niches. Addressing these gaps is instrumental in advancing our understanding of how pathogens access and prioritize sulfur sources during infection and how these constraints ultimately shape infection outcomes and host-pathogen interactions.

1.5 Research questions and aims

As sulfur metabolic networks are variable between pathogens, manual curation is often necessary to resolve what pathways pathogens have available in their toolbox, and subsequently the conditions under which these pathways become relevant during infection. This work explores how *N. gonorrhoeae* fulfils its sulfur requirements and lays the groundwork for determining which sulfur acquisition strategies are relevant across distinct stages of infection.

1.5.1 Aim one: Mapping the sulfur metabolic network in *N. gonorrhoeae*

One key challenge in discerning sulfur metabolism networks is the largely predictive process of genome annotation. The first aim of this research is to address this issue by comprehensively mapping the sulfur metabolism network in *N. gonorrhoeae* using integrated approaches. This included bioinformatic analysis, informed by the literature review to identify all plausible pathways relevant to sulfur metabolism, integrated with experimental evidence (Chapter Three).

1.5.2 Aim two: Investigate thiosulfate assimilation in *N. gonorrhoeae*

As part of mapping the sulfur metabolic network, we investigated the hypothesis that H_2S required for *de novo* cysteine biosynthesis in *N. gonorrhoeae* is facilitated by the reduction of thiosulfate via thiosulfate sulfurtransferases, in the non-canonical thiosulfate assimilation pathway. This pathway has been previously described in *E. coli* and is theoretically applicable to several clinically relevant pathogens. To this end, *N. gonorrhoeae* is used as model organism to study alternative thiosulfate assimilation pathways that may be relevant to other pathogens.

1.5.3 Aim three: Elucidating the role of sulfur acquisition during epithelial cell infection

Furthermore, while experimental evidence currently supports the importance of enzymes of *de novo* cysteine biosynthesis for infection in *N. gonorrhoeae* (Du & Arvidson, 2006), one major concern of targeting *de novo* biosynthesis pathways is the potential for pathogens to acquire end point metabolites. We begin to address this concern by examining how different sulfur acquisition strategies affect the pathogenesis of *N. gonorrhoeae* including adherence and invasion of endocervical cells.

1.5.4 Aim four: Transcriptomic response to cysteine-limitations

As a continuation of the mapped sulfur metabolic network in *N. gonorrhoeae*, the final aim herein sought to understand in greater depth how the gonococcus responds to the availability of different sulfur sources and adapts to conditions of cysteine depletion. To address this, a multi-omics approach was implemented, integrating transcriptomic and metabolomic techniques to gain a detailed understanding of how *N. gonorrhoeae* responds to various sulfur landscapes. Although both these datasets were generated, inconsistencies in the metabolomics annotation pipeline precluded confident interpretation of the metabolite profiles. Accordingly, the analysis presented in Chapter Four focuses on transcriptomic data.

2 | Chapter Two

Methods

2.1 Bacterial strains and culture conditions

Bacterial strains and plasmids used in the present study are listed in the Supplementary material 6.1. *E. coli* BL21 strains were routinely cultured aerobically on Luria–Bertani (LB) medium agar or in LB liquid culture at 37 °C supplemented, when applicable, with the antibiotics; ampicillin (100 $\mu g.ml^{-1}$), kanamycin (50 $\mu g.ml^{-1}$) or erythromycin (100 $\mu g.ml^{-1}$). *E. coli* strains were stored in LB medium containing 20% glycerol at −80 °C.

The *N. gonorrhoeae* FA19 strain was kindly provided by the So laboratory (Department of Immunobiology, University of Arizona), and all isogenic mutants were derived from this parent strain. *N. gonorrhoeae* strains were routinely cultured from glycerol stocks onto GCB agar (15 $g.L^{-1}$ proteose peptone #3, 4.0 $g.L^{-1}$, K_2HPO_4, 1.0 $g.L^{-1}$, KH_2PO_4, 1.0 $g.L^{-1}$ NaCl) (BD Difco™) supplemented with Kellogg's supplements I and II (22.22 mM glucose, 0.68 mM glutamine, 0.45 mM cocarboxylase, 1.23 mM $Fe(NO_3)_3$) (Kellogg et al., 1963). *N. gonorrhoeae* strains were stored in GCBL medium containing 20% glycerol at −80 °C.

Unless otherwise stated, *N. gonorrhoeae* was cultured at 37 °C in a humidified 5% CO_2 atmosphere. For growth in liquid culture, *N. gonorrhoeae* was cultured from frozen glycerol stocks onto GCB agar and incubated for 16 – 18 hr. Approximately 20 piliated (P^+) and opacity protein negative (Opa^-) colonies were identified using a stereomicroscope and passaged for heavy growth (7 – 9 hr). Cells were resuspended in pre-warmed Graver-Wade complete medium (GW) (Wade & Graver, 2007) or GCBL and adjusted to OD_{550} 0.07 – 0.08. Cultures were incubated at 30 °C (50 rpm) for 15 – 18 hr, diluted to OD_{550} 0.2, and grown for ~1.5 hr at (250 rpm). For use in growth curve, metabolomic, and transcriptomic experiments, cultures were then diluted to an OD_{550} of 0.07 – 0.08 and grown at 37 °C and 250 rpm as required. When necessary, liquid cultures were further supplemented with 0.042% (w/v) sodium bicarbonate.

2.2 Media for *N. gonorrhoeae* liquid culturing and composition of chemically defined media.

Three liquid media were routinely used for gonococcal culturing and subsequent experiments: GCBL supplemented with Kellogg's supplements I and II, GW, and the sulfur-free GW medium. The GCBL medium was used predominantly for culturing and gonococcal preparation for experimentation. For experiments involving the manipulation of sulfur sources, the GW medium was used as the control condition and comprised of the following sulfur sources and quantities: sulfate (803.38 μM), cystine (83.0 μM), cysteine (0.626 μM), methionine (100 μM), and reduced glutathione (0.162 μM). For the sulfur-free media and manipulation of sulfur sources, the base of the GW media, M199 cell culture media was specifically manufactured to omit all sulfur containing compounds. Sulfur sources were manually added to at the following concentrations: sulfate (1 mM), sulfite (1 mM), thiosulfate (2mM), glutathione (1 - 10 mM), methionine (2 mM), and cysteine (0.25 – 6 mM). For growth conditions containing cysteine or glutathione, stocks were freshly prepared and added to cultures immediately prior to incubation. The full recipes for GW media are provided in the Supplementary material (6.2).

2.3 Genetic manipulation of *N. gonorrhoeae*

N. gonorrhoeae isogenic mutant strains were generated via homologous recombination following the spot or liquid transformation protocol as described in Dillard (2011). Gene disruptions were achieved by insertion – deletion mutagenesis, in which the gene of interest was replaced or interrupted with an antibiotic resistance cassette (kanamycin or erythromycin). The erythromycin gene was codon optimized for *N. gonorrhoeae*. Each construct contained the resistance cassette flanked by ~100-200 base pair (bp) homologous regions upstream and downstream of the target locus, along with the corresponding promoter and terminator. With the exception of the *pspE* construct, where the construct was designed to interrupt and partially replace the *pspE* gene. The terminator of the erythromycin cassette in this instance was also omitted as to not disrupt transcription of the remaining genes in the operon. DNA constructs were ordered as gene fragments from Twist Biosciences, amplified via PCR, purified using the High Pure PCR Clean-Up Micro Kit (Roche Applied Science) as per manufacturer's instructions, and visualized by agarose gel electrophoresis. Primer

sequences, PCR cycling conditions, and deletion strain genetic constructs are provided in the Supplementary material 6.3. Following transformation, mutant strains were selected on GCB agar containing kanamycin (50 μg.mL^{-1}) or erythromycin (10 ug.mL^{-1}), where appropriate.

For confirmation of correct integration and assessment of off-target mutations following transformation, the genomes of the isogenic mutants were sequenced via Nanopore Sequencing. Briefly, genomic DNA (gDNA) was extracted from *N. gonorrhoeae* strains using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific) and accessed for quality and size via LabChip (PerkinElmer). gDNA was then prepared according to the SQK-NBD114.24 Oxford Nanopore Technologies (ONT, UK) ligation sequencing amplicons protocol and kit (200 fmol input) and sequenced using a R10.4.1 flow cell (ONT). Base-calling and demultiplexing of raw read data was performed using Dorado (v0.4.0, ONT). Genome *de novo* assembly from these reads were performed using a standard Trycycler pipeline. The mutant genomes were aligned and compared to the FA19 parent strain (GenBank accession: NZ_CP012026.1) using Geneious Prime and Mauve alignment.

2.3.1 Growth curve experiments

N. gonorrhoeae was prepared in liquid media as previously described (Section 2.2). For the testing of various sulfur sources, cultures were pelleted by centrifugation for 10 min at 2000 × g to remove all traces of sulfur sources from culturing. The pellet was then resuspended in 5 mL of sulfur free media and diluted to an OD_{550} of 0.07 – 0.08. Sulfur sources of interest were added to individual cultures as required, at the following concentrations: 0.25 – 6 mM cysteine, 1 mM sulfate, 1 mM sulfite, 2 mM methionine, 2 mM thiosulfate, and 1 – 10 mM glutathione. Cultures were incubated for 5 - 6 hr at 37 °C, 250 rpm. Samples for OD_{550} readings and CFU plating (where applicable) were collected hourly. Samples for CFU plating were serial diluted 1:10 until required dilutions were obtained (between 10^{-4} – 10^{-6}) and 100 μl were plated, spread with sterile glass beads and incubated for 24 – 48 hr. Biological replicates were collected as technical duplicates or triplicates.

For each biological replicate in each condition, OD_{550} readings were blank-corrected, normalized to a starting OD_{550} of 0.075, transformed to natural log (ln) values and plotted against time to identify the steady exponential window

(Supplementary material 6.4). This was determined to be between 0 – 3 hr for all growth curves except for those performed in section 3.1.1 corresponding to the cysteine concentration curves. For these growth curves, the exponential phase used for growth rate calculations was 0 – 2 hr as, due to the substantial growth differences, this window was relevant for all concentrations. The exponential (Malthusian) growth equation in Graph Pad Prism software (version 10) was fitted to determine doubling times, calculated as ln(2)/k, where *k* is the growth-rate constant returned by the fit. All exponential growth equations fit had an $R^2 > 0.9$. Growth yields are defined as the maximum OD_{550} of the culture within the window of growth. Values were obtained independently for each biological replicate, and summary statistics are reported as mean ± standard error of the mean (SEM).

Statistically significant differences ($p < 0.05$) for growth curve experiments were determined by One-Way analysis of variance (ANOVA) with Dunnett's multiple comparison t-test, assuming normal distribution in Graph Pad Prism software (version 10).

2.4 *N. gonorrhoeae* infection of human epithelial cells

The human cervical carcinoma cell line ME-180 (HTB-33) were grown in a at 37 °C in a humidified 5% CO_2 atmosphere. ME-180 cells were maintained in McCoys 5A medium (phenol red, eagles salts, glutamine) (Gibco), supplemented with 10% heat-inactivated FBS as per the ATCC guidelines. Cells were grown to confluency in T25 or T75 tissue culture treated flasks (Falcon) before passaging. For infection experiments, ME-180 cells were harvested via centrifugation (500 × g, 5 min), and variable cells were quantified using an Invitrogen Countess 3 Automated Cell Counter. Cells were seeded at 9×10^4 cells per well in a 12-well tissue culture treated plate (Corning) 2 days prior to infection.

To determine the adhesion and invasion indices of *N. gonorrhoeae* WT and mutant strains, ME-180 cells were infected with an *N. gonorrhoeae* at an MOI of 20 (WT, Δ*tcyA*, and Δ*cjaA*) or 50 (WT, Δ*str*, Δ*pspE*, and Δ*str:pspE*). Gonococcal infection inoculums were prepared from a ~17 hr lawn plate on GCB agar and resuspended in 5 mL of GCBL. Bacterial density was determined spectrophotometrically (OD_{550}), and the required volume of the resuspension was added to McCoys 5A cell culture medium supplemented with 10% FBS and 48 μM $Fe(NO_3)_3$. To confirm

gonococcal challenge, inoculums were serially diluted and plated to enumerate CFU.

ME-180 cell in the 12-well plate were infected with 1 mL WT or mutant strains for 4 hr, at which point data was collected to quantify intracellular, cell-associated, and non-cell-associated bacterial populations. To retrieve the intracellular population, infected wells were aspirated and replaced with McCoy's 5A containing 10% FBS and 50 $\mu g.mL^{-1}$ gentamicin and incubated for 1 hr to remove extracellular bacteria. Following this, infected monolayers were washed three times and lysed by incubation with 0.5% saponin for 20 m. Once lysed, the contents of the wells were collected, vortexed for 2 m, and diluted to plate for CFU. The non-cell associated population was retrieved by collecting the infection medium and three subsequent 1 mL washes. The collected suspensions were vortexed, serially diluted, and plated for CFU determination. Following the removal of the non-cell associated fraction, the cell-associated population, which includes both the adherent and intracellular bacteria, was determined by lysing the cells by incubating with 0.5% saponin for 20 m. Once lysed, the contents of the wells were collected, vortexed for 2 m, and diluted to plate for CFU counts. The adhesion and intracellular indices were expressed as a percentage of the infection inoculum. Population growth was quantified as the $\log_{10}$ fold-change ($\log_{10}$FC) in total bacterial counts at 4 hours post-infection relative to the inoculum. Biological replicates were collected as technical duplicates or triplicates. Values were obtained independently for each biological replicate, and summary statistics are reported as mean ± SEM. Statistically significant differences ($p < 0.05$) were determined by One-Way analysis of variance (ANOVA) with Dunnett's multiple comparison t-test, assuming normal distribution in Graph Pad Prism software (version 10).

2.5 *In vitro* sulfurtransferase activity assays

2.5.1 Cloning, expression, and purification of Str and PspE in *E. coli*

The genes encoding Str (NGO0020) and PspE (NGO0369), minus the signal peptide (ΔSP-PspE), were ordered from Twist Bioscience in the pET28α plasmid between the NdeI and XhoI restriction sites for expression with an N-terminal His-tag. Str and PspE protein sequences are provided in the Supplementary material 6.5. For protein expression, the *str*-pET28α and *pspE*-pET28α plasmids were transformed into chemically competent *E. coli* BL21 (DE3) via the heat shock

method. Expression of the recombinant proteins was induced by growing the *E. coli* transformants harbouring the expression plasmids in a 10 mL seeder culture in LB broth with appropriate antibiotic overnight at 37°C with shaking (180 rpm). The seeder culture was used to inoculate large scale expression cultures (1L) which was incubated in a baffled flask with shaking (180 rpm) at 37 ℃ until mid-log growth (OD_{600} 0.4 – 0.6) was reached. At this point, 0.75 min isopropyl β-D-1-thiogalactopyranoside was added to induce protein expression, and the culture was further incubated overnight at 22 ℃ with shaking (180 rpm). Cells were subsequently harvested via centrifugation at 4000 × g at 4 °C for 20 min and resuspended in Lysis buffer (50 mM KPO_4 pH 8, 200 mM NaCl, 20 mM imidazole). Cells were lysed via sonication (Q700 sonicator) on ice with the addition of an EDTA-free protease inhibitor tablet (Roche Applied Science). To separate the soluble and insoluble fractions, the sonicate was then centrifuged at 9 000 × g, 4 °C for 20 min.

Recombinant proteins were purified from the soluble fraction using immobilized metal affinity chromatography (IMAC) with a nickel affinity column (HisTrap, Cytiva), followed by size exclusion chromatography (SEC) using a Superdex 75 16/60 column (Cytiva). All purification steps were performed on a Bio-Rad NGC FPLC System. Briefly, the supernatant was loaded onto a nickel column pre-equilibrated with Lysis buffer (50 mM KPO_4 pH 8, 200 mM NaCl, 20 mM imidazole). The column washed with 2 column volumes of 96 % Lysis buffer, and 4% Elution buffer (50mM KPO_4 pH 8, 200mM NaCl, 1M imidazole) to remove any unbound proteins. Bound proteins were eluted with a gradient of 4 - 50% Elution buffer at a flow rate of 2 $ml.min^{-1}$. Eluted fractions containing proteins of interest were subsequently collated and loaded through a 0.2 μm filter onto the SEC column pre-equilibrated with Sec buffer (50mM Tris pH 8, 200mM NaCl). Protein separation was performed at a flow rate of 0.5 $ml.min^{-1}$ and fractions containing the target protein were collected and visualized by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis to assess purity and quantified by NanoDrop™ absorbance readings. The molar absorption coefficient of the Str protein and PspE were determined to be, 1.4 $M^{-1}.cm^{-1}$ and 0.883 $M^{-1}.cm^{-1}$ respectively, by ProtParam (Gasteiger et al., 2005). Purified protein was snap frozen in 50 mM Tris, 200 mM NaCl, pH 8 containing 50% (v/v) glycerol and stored at −80 ℃.

2.5.2 Thiosulfate sulfurtransferase activity assay

To assess the ability of Str and PspE to reduce thiosulfate to hydrogen sulfide in the presence of various thiol acceptor substrates, including glutathione, cysteine, and dithiothreitol, the lead acetate activity assay was as described by Chiku et al (2009). Briefly, the reaction mixture (0.25 mL) contained lead acetate (0.4 mM), thiosulfate (1 - 50 mM) and glutathione, cysteine, or DTT (1 - 50 mM) in 200 mM sodium phosphate buffer, pH 7.4. The reaction mix was pre-warmed to 37 ℃ for 10 m. To initiate the reaction, 0.125 µg of either Str or PspE was added to the reaction mix. Reactions were carried out in a 96-well plate in the SpectraMax® M4 with the chamber set to 37 ℃ and a brief shake (1 s) before the first reading. The production of H_2S was measured continuously as lead sulfide at 390 nm. The molar extinction coefficient of 5 500 $M^{-1}.cm^{-1}$ for lead sulfide was used to calculate the concentration of hydrogen sulfide produced. At least three parallel experiments were performed for each unique thiosulfate and thiol acceptor substrate combination, for each enzyme.

The steady-state rate at each substrate concentration was extracted from the SpectraMax M4 SoftMax® Pro 7.2 GxP Software and plotted as rate of H_2S production ($\mu mol.min^{-1}.mg^{-1}$) as a function of substrate concentration (0.25 - 50 mM). Enzyme kinetics parameters (V_{max} and K_M) were determined by fitting the Michaelis-Menten equation or substrate inhibition equation using GraphPad Prism (version 10). The catalytic efficiency of Str and PspE was determined by calculating the catalytic constant and specificity constant, *k*cat and K_M/*k*cat, respectively.

2.6 Metabolomic and transcriptomic experiments

2.6.1 Culture conditions and extraction protocols

N. gonorrhoeae WT was prepared in liquid culture as previously described (Section 2.1). To obtain samples for transcriptomic and metabolomic experiments, *N. gonorrhoeae* was grown in 15 mL cultures to obtain enough culture yield for RNA seq and extra-and intra-cellular metabolite analysis. Cultures were supplemented with either (i) 1 mM cyst(e)ine, (ii) 2 mM thiosulfate and 5 mM glutathione, (iii) 2 mM methionine and 5 mM glutathione, or (iv) 5 mM glutathione and incubated for 2.5 hr with shaking (250 rpm). Non-inoculated media control samples of each condition were included as media only samples for metabolomic experiments and incubated for the same length of time.

At the time of extraction, 5 mL of culture was removed. Of this, 500 µL was recovered for total RNA extraction and centrifuged for 2 minutes at maximum speed. The pellet was resuspended in 800 µL of Tri Reagent®, snap-frozen and stored at -80 ºC. Total RNA was extracted using the Direct-zol RNA Miniprep Kit (Zymo Research). RNA quality was assessed using the Agilent TapeStation (Agilent Technologies) with the High Sensitivity RNA ScreenTape Assay. Samples were dried under vacuum in GenTegraRNA tubes and sent to Macrogen (Macrogen Inc.) for total RNA sequencing.

The remaining 4.5 mL of culture and media blank controls were filtered through a 0.2 µM cellulose-acetate filter. Of the filtrate, 1 mL was then added to 0.2 µM of d4-alanine (internal standard) and immediately stored at -80 °C. The remaining 10 mL of the culture was used to extract intracellular metabolites as described in Smart et al., (2010). Briefly, samples were immediately quenched by adding pre-chilled (-20 °C) 50:50 glycerol–saline solution, homogenized, and centrifuged (26,900 × g for 20 min at −20 °C). The supernatant was discarded and the pelleted was resuspended in 1:1 (vol/vol) glycerol:saline solution, and centrifuged (26,900 × g for 20 min at −20 °C). Following removal of the supernatant, the pellet was resuspended in 2.5 mL of the extraction solution (50% (v/v) methanol–water) and 0.2 µM d4-alanine (internal standard) was added. Samples were then subjected to three freeze–thaw cycles consisting of 4 min on ice, 1 min vortex, and 30 min at −80 °C. Extracted metabolites were recovered by centrifugation (20,800 × g for 15 min at −20 °C,) and collecting the supernatant. An additional 2.5 mL methanol-water (50% vol/vol) solution was added to the pellet, resuspended, centrifuged and recovered as before. Supernatants were combined and stored at −80 °C until further analysis. All intra- and extracellular metabolite samples were freeze-dried for 48 hr prior to derivation and detection by gas chromatography-mass spectrometry (GC-MS).

All metabolomic and RNA-sequencing samples were collected as biological triplicates. For extracellular and media blank samples, the biological replicates were collected in technical duplicates.

2.6.2 Methyl chloroformate (MCF)-derivatization and GC-MS

Metabolites were derivatized using the methyl chloroformate (MCF) method as described in Smart et al., (2010). Briefly, 400 µL of 1 min sodium hydroxide, 334

µL methanol, and 68 µL pyridine were added to metabolite extracts, derivatization blanks, and an amino acid standard mix. Samples were then derivatized by the addition of 40 µL MCF, followed by a 30 sec vortex, which was repeated once. Subsequently, 400 µL chloroform was added and samples were vortexed for 10 s, followed by the addition of 800 µL 50 mM sodium bicarbonate and a further 10 sec vortex. Samples were centrifuged at 2,500 rpm for 5 min at 6 °C. The upper aqueous phase was aspirated to recover the lower organic phase. Residual water was removed by the addition of sodium sulfate.

After derivatizations, samples were analyzed with a GC (GC7890B, Agilent Technologies, USA) - MS (MSD5977A, Agilent Technologies, USA) operated under electron ionization at 70 eV. For each sample, 1 µL was injected for analysis. The injector temperature set to 290 °C under pulsed split-less mode (1.8 bars for 1 min, 20 ml min^{-1} split flow after 1.01 min), carried by helium gas with a constant flow of 1 mL min^{-1} through a ZB-1701 capillary column (30 min × 250 µm internal diameter × 0.15 µm film thickness; 14% cyanopropyl phenyl, 86% dimethyl polysiloxane) fitted with a 5 min guard column (Phenomenex). The GC oven temperature was initially held at 45 °C for 2 min. Following this, the temperature is raised with a gradient of 9 °C min^{-1} until it reaches 180 °C, which is held for 5 min. The temperature is then raised with a gradient of 40 °C min^{-1} until 220 °C and held for 5 min, after which the temperature is raised to 240 °C and held for 11.5 min. Finally, the temperature is raised with a gradient of 40 °C min^{-1} until it reaches 280 °C, where it is held for 10 min. The ion source and quadrupole temperature was 250 °C and 150 °C, respectively. The MS device is operated in scan mode, starting after 4.7 min with a mass range of 38–550 m/z at four scans per second (A/D sampling = 4), and a detection threshold set to 50 ion counts. AMU at 1.47 scans per second.

Quality control samples were included and consisted of pure chloroform solvent, blank samples, and an amino acid standard mix, which were co-derivatized with 20 µL of 10 mM d4-alanine as an internal standard. QC samples were injected at the beginning of the GC-MS run and all experimental samples were injected in a randomized order.

2.7 Bioinformatics

2.7.1 Construction of the sulfur metabolic network in *N. gonorrhoeae*

Genes associated with characterized sulfur-related metabolic pathways were identified based on the literature review (Section 1.4) and searched for in *N. gonorrhoeae* FA1090 (reference strain) and FA19 (strain used in this study). Homologs sequences were identified using both the BioCyc database and protein BLAST (pBLAST) of *E. coli* K-12 proteins as query sequences (unless otherwise indicated in Table 3.1). Proteins were considered homologous if they met the following criteria, in the order listed: 1) E value < 1 $x10^{-20}$, 2) coverage > 85%, and 3) sequence similarity >25%.

2.7.2 Transcriptomics (RNA preparation, sequencing, and sequencing data)

Total RNA libraries were prepared using the NEBNext rRNA Depletion Kit (Bacteria) followed by the TruSeq Stranded Total RNA Library Prep Gold protocol to generate stranded libraries. Sequencing was carried out on an Illumina platform using paired-end reads of 151 bp, with an average yield of ~30 million paired-end reads per sample. Sequencing quality was uniformly high, with Q30 values exceeding 94% for all libraries.

Adapter sequences and low-quality bases were removed from raw paired-end FASTQ files using fastp. Trimmed reads were aligned to the *N. gonorrhoeae* FA19 genome (NZ_CP012026.1) using bwa-mem. Alignments were converted to BAM format, sorted, and indexed using SAMtools. Gene-level read counts were quantified from sorted BAM files using featureCounts. Count matrices were analyzed in R (version 4.5.2) using DESeq2 to identify differentially expressed genes (log_2 fold-change >1 and an adjusted p-value (p_{adj}) < 0.05). Differentially expressed genes were subjected to manual literature surveys for assignment into functional categories.

2.7.3 Metabolomics peak identification

Chromatographic deconvolution and peak detection were conducted in Automated Mass Spectral Deconvolution and Identification System (AMDIS) based on comparison of mass spectra and retention times. For peak annotation, an in-house MCF spectral library, which is the intellectual property of the University of

Auckland, was used. Metabolite peak intensities were subsequently integrated into a summary report using R MassOmics software package developed by the University of Auckland Mass Spectrometry (MaSH) facility.

3 | Chapter Three

Mapping the sulfur metabolic network in *Neisseria gonorrhoeae*

Sulfur metabolism networks present as variable between different pathogens and are governed both by sulfur landscapes encountered during infection and unique pathogenic requirements. To determine ideal candidates for targeting sulfur metabolism pathways, is it necessary to establish which pathways occur in *N. gonorrhoeae* and consequently their relative importance for pathogenesis within the host.

To obtain a comprehensive understanding of sulfur metabolism in *N. gonorrhoeae*, bioinformatic and literature-based evidence was integrated with data from growth experiments to construct sulfur-related metabolic pathways. Given the central role of cysteine in cellular sulfur metabolism, pathways involving cysteine acquisition, recycling, and degradation were considered as part of the sulfur network construction (Table 3.1). To confirm gonococcal sulfur acquisition strategies, examination of sulfur source utilization by *N. gonorrhoeae* FA19 WT during growth was assessed using the Graver-Wade chemically defined medium (Wade & Graver, 2007), which consists of the M199 cell culture medium as a base and an additional 13 components to specifically support gonococcal growth (Supplementary Material 6.2). Here, the M199 base component was specifically manufactured to omit all sulfur-containing compounds present and was subsequently supplemented with one or a combination of the following organic and inorganic sulfur sources known to facilitate pathogenic growth; sulfite, sulfate, thiosulfate, glutathione, cysteine, and/or methionine. We confirmed that in the absence of sulfur supplementation *N. gonorrhoeae* did not proliferate when using this modified version of M199 medium (Figure 3.1). The complete unmodified medium (GW) served as the control condition and contained the following sulfur compounds in the M199 base: sulfate, cystine, cysteine, methionine, and reduced glutathione.

In this chapter, we individually consider each major sulfur source that contributes to sulfur acquisition strategies in bacterial pathogens as outlined in the literature review undertaken in Chapter 1, including an investigation of the genetic basis of

these, integrated with experimental observations. We conclude this section by discussing the subsequent implications of these pathways in the context of *N. gonorrhoeae* infection.

Table 3.1. Genes involved in sulfur metabolism considered when constructing the *N. gonorrhoeae* sulfur metabolism network map.

Gene	Protein	FA1090 locus
Cysteine/Cystine transport		
tcyP	Cystine Na^+/H^+ symporter	—
tcyABC[a]	ABC cystine transporter	NGO0372 – 0374
tcyDEFGH[b]	ABC cystine transporter	—
tcyKLMN[c]	ABC cystine transporter	—
tcyJLN	ABC cystine transporter	—
cjaA[d]	ABC cysteine transporter	NGO2011 – 2014
Cysteine export		
alaE	Alanine exporter	—
eamA	Cysteine/O-acetylserine exporter (DMT)	NGO1512/NGO00320*
bcr	Major facilitator superfamily (MFS) class	NGO0903
Cysteine degradation		
cdsH[e]	Cysteine desulfhydrase	—
cyuA	Cysteine desulfidase	—
cdo[d]	Cysteine dioxygenase	—
hapE[f]	Desulfurase/desulfhydrase	—
mstA	Mercaptopyruvate sulfurtransferase	—
csdA	Cysteine desulfurase	—
iscS	Cysteine desulfurase	NGO0636
nifS	Cysteine desulfurase	
sufS	Cysteine desulfurase	
tnaA	cysteine desulfhydrase (secondary)	—
malY	cysteine desulfhydrase (secondary)	—
Glutathione and dipeptide transport		
gshT[b]	Glutathione substrate binding (unlinked)	—
gsiABCD	ABC glutathione transporter	—
oppABCDF	ABC peptide transporter	—
dptA[g]	Proton-dependent oligopeptide transporter	NGO1954
cptA[d]	OPT-family transporter	NGO1900
ctaP[c]	ABC oligopeptide binding protein	—
Glutathione synthesis		
gshA	γ-glutamyl-cysteine ligase	NGO0680
gshB	Glutathione synthetase	NGO1217
gshAB[b]	Bifunctional GSH synthetase	—
Glutathione catabolism		
ggt	γ-glutamyl transpeptidase	NGO04550
chaC[g]	γ-glutamyl cyclotransferase	—

pepT[h]	Peptidase T	—
pepA	Aminopeptidase A	NGO1227
pepB	Aminopeptidase B	—
pepD	Peptidase D	—
pepN	Aminopeptidase N	NGO0158
Methionine transport		
metINQ	ABC methionine transporter	NGO2137 – NGO2139
Methionine synthesis		
metA	Homoserine *O*-succinyltransferase	—
metB	Cystathionine γ-synthase	NGO0386
metC	Cystathionine β-lyase	—
metW[i]	Unknown	NGO0934
metX[i]	Homoserine *O*-succinyltransferase/*O*-acetyltransferase	NGO0933
metY[j]	*O*-acetylhomoserine sulfhydrylase	—
metZ[i]	*O*-succinylhomoserine sulfhydrylase	NGO1149
metI[k]	Cystathionine γ-synthase variant	—
metE	Methionine synthase (cobalamin-independent)	NGO0928
metH	Methionine synthase (cobalamin-dependent)	—
Methionine recycling		
luxS	*S*-ribosylhomocysteine lyase	NGO2102
metK	*S*-adenosylmethionine synthetase	NGO0106
mtnN	5'-methylthioadenosine/*S*-adenosylhomocysteine nucleosidase	NGO0345
mccA[c]	Cystathionine β-synthase	—
mccB[c]	Cystathionine γ-lyase	—
Thiosulfate/sulfate acquisition		
cysP	Thiosulfate binding protein	—
sbp	Sulfate binding protein	NGO02385*
cysUWA	Thiosulfate/sulfate ABC transporter	NGO00445*– 0446*
Sulfate assimilation		
cysDN	ATP sulfurylase	—
cysC	APS kinase/APS reductase	—
cysH	PAPS reductase	—
cysJI	Sulfite reductase	NGO04265* – 04270*
Cysteine biosynthesis		
cysE	Serine acetyltransferase	NGO1423
cysK	*O*-acetylserine sulfhydrylase-A/cysteine desulfhydrase	NGO0340
cysM	*O*-acetylserine sulfhydrylase-B/cysteine desulfhydrase	—
Thiosulfate assimilation		
glpE	Thiosulfate sulfurtransferase	NGO0020
pspE	Thiosulfate sulfurtransferase	NGO0369
Taurine assimilation		

tauABC	ABC transporter	—
tauD	Taurine dioxygenase	—

Locus tags for identified protein homologs in N. gonorrhoeae FA1090 are reported. Protein sequences from E. coli K-12 were used as query sequences unless otherwise indicated. For proteins absent in E. coli, homologs from the following organisms were used: [a]S. aureus, [b]S. mutans [c]L. monocytogenes, [d]C. jejuni, [e]S. Typhimurium*, [f]M. pneumoniae, [g]F. tularensis, [h]C. difficile, [i]P. putida, [j]Corynebacterium glutamicum [k]Bacillus subtilis, [h]H. pylori. Grey text indicates that the involvement of the corresponding protein in sulfur metabolism is hypothesized — indicated protein homolog was not identified in N. gonorrhoeae strains FA19 and FA 1090. *Locus tags are predicted pseudogenes.*

3.1 Cysteine and cystine acquisition and catabolism

N. gonorrhoeae encodes two ABC transporter systems related to sulfur acquisition, NGO2011 – NGO2014 and NGO0372 – NGO0374. These transporters have been kinetically characterized and demonstrate substrate specificity for cysteine and cystine, respectively (Bulut et al., 2012). Herein we propose the nomenclature, CjaABCD, for the cysteine transporter system, where the substrate binding protein (NGO2014) is homologous to the only high-affinity cysteine receptor discovered to date, CjaA, and TcyABC for the cystine transporter system as the substrate binding protein (NGO0372) shares the highest sequence similarity to the *S. aureus* TcyA protein. It has not yet been reported whether *N. gonorrhoeae* encodes additional cystine transport systems, such as the TcyP symporter found in *E. coli* and *S. aureus*, or additional ABC-type transporters as reported for *S. mutans* (Kim et al., 2012; Chonoles Imlay et al., 2015; Lensmire et al., 2020). Homologs of these transporters, however, were not identified within the gonococcal genome (Table 3.1) suggesting that *N. gonorrhoeae* lacks the redundant cystine uptake mechanisms observed in many other pathogens, although this currently lacks experimental support.

Consistent with the presence of cysteine and cystine ABC transporters, *N. gonorrhoeae* displayed robust growth when provided with 1 mM cyst(e)ine as the sole sulfur source, indicating that sulfur requirements can be met solely by the acquisition of exogenous cysteine (Figure 3.1). Given the inherent instability of cysteine under the applied growth conditions (aerobic growth, shaking, 37 °C, pH 6.8) we recognize that the culture medium contains a dynamic mixture of cysteine and its oxidized dimer, cystine (Ewann & Hoffman, 2006). Accordingly, we acknowledge this by referring to this condition as “cyst(e)ine” throughout.

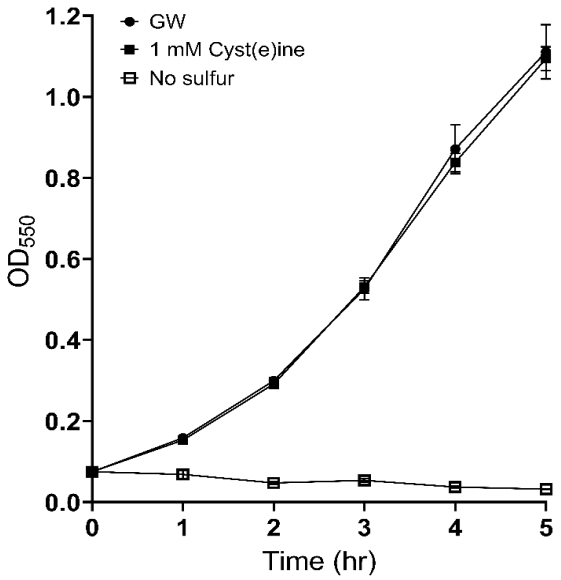


Figure 3.1. Optimal cyst(e)ine concentration for gonococcal growth. *N. gonorrhoeae* proliferation in 1 mM cyst(e)ine resulted in growth comparable to the GW control. The no sulfur control confirms that gonococcal growth in the modified GW medium is dependent on sulfur supplementation. Data are mean ± SEM from ≥5 biological replicates per condition.

3.1.1 Sensitivity of *N. gonorrhoeae* to exogenous cyst(e)ine

Given that cysteine is cytotoxic, a cysteine concentration curve was used to determine the optimal concentrations supporting *N. gonorrhoeae* growth and to define the cysteine toxicity threshold. Cultures were grown with cyst(e)ine concentrations ranging from 0.25 – 6 mM. For each condition, the growth rate, defined by doubling time during early exponential growth, alongside the capacity for high density growth, represented as maximum OD_{550} achieved, was determined and used to define growth response to cysteine concentration gradients (Figure 3.2).

Optimal gonococcal growth occurred at 1 mM cysteine, which resulted in growth comparable to that observed in the GW medium (Figure 3.2). At concentrations below 1 mM, cultures displayed a minor reduction in growth rate which was significantly reduced at cysteine concentrations below 0.5 mM (Figure 3.2 a). In addition, the ability of these cultures to reach high cell densities was significantly impaired, indicating that cyst(e)ine becomes growth-limiting under these conditions and that growth ceases once cyst(e)ine is depleted (Figure 3.2 b). Similarly, cyst(e)ine concentrations above 1 mM produced progressively slower growth with reduced culture densities in a concentration-dependent manner (Figure

3.2 a and b). Maximum culture densities were significantly reduced at all concentrations above 1 mM, while statistically significant reductions in growth rate were observed at concentrations > 2 mM. Thus, 2 mM cyst(e)ine is defined as the cyst(e)ine toxicity threshold for *N. gonorrhoeae* under these conditions.

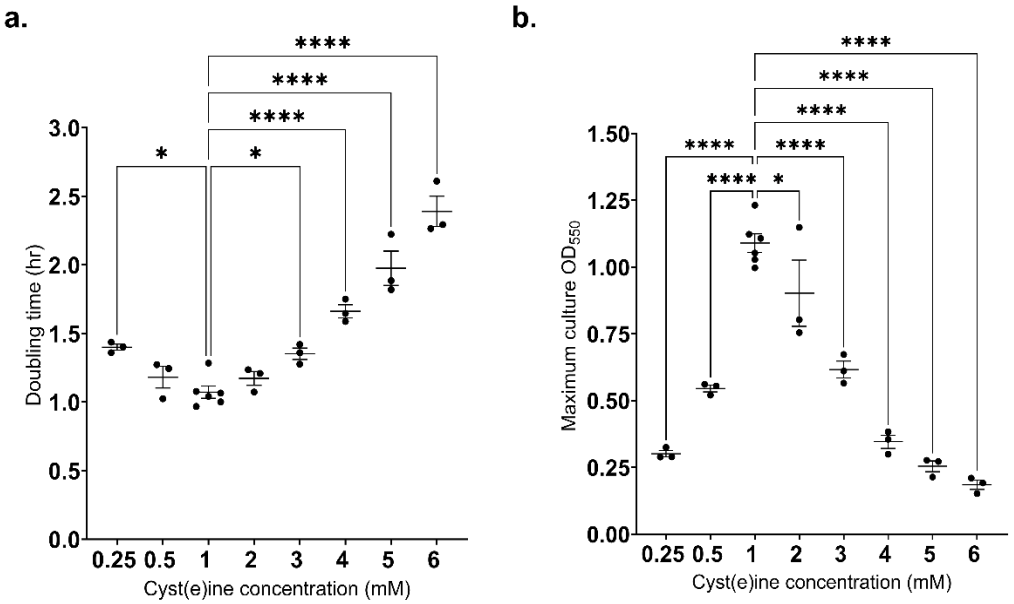


Figure 3.2. Cysteine tolerance in *N. gonorrhoeae*. *N. gonorrhoeae* WT was grown across a range of cyst(e)ine concentrations (0.25 – 6 mM). Growth at each cyst(e)ine concentration was compared to the optimal condition (1 mM) and quantified as (a) growth rate, determined by doubling time, and (b) culture growth capacity, measured as maximum OD_{550}. Statistical analysis was performed using ordinary one-way ANOVA with Dunnett's correction ($p < 0.05$). Data represents the mean ± SEM from 3 biological replicates per condition.

These results illustrate a narrow window of cysteine concentrations for optimal gonococcal growth and demonstrate that *N. gonorrhoeae* is sensitive to cyst(e)ine fluctuations. Notably, this cysteine toxicity threshold is below thresholds observed in several other pathogens including *C. difficile*, *H. pylori*, *M. pneumoniae*, and *S. pneumonia*e (Afzal et al., 2016; Devi et al., 2019; Großhennig et al., 2016; Gu et al., 2018), suggesting that *N. gonorrhoeae* might be limited in mechanisms for maintaining cysteine homeostasis.

3.1.1.1 Investigation of cysteine homeostasis mechanisms

Intracellular cysteine is present in the reduced form and, at elevated concentrations, contributes to cellular damage by driving Fenton chemistry (Park & Imlay, 2003; Smirnova et al., 2024). To mitigate this threat, tight regulatory mechanisms are

implemented alongside cysteine homeostasis mechanisms to balance cysteine acquisition strategies with cysteine degradation and/or export to avoid cysteine cytotoxicity (Kredich, 1992, 2008; Chonoles Imlay et al., 2015; Smirnova et al., 2019; Korshunov et al., 2020; Smirnova et al., 2024). Based on the relatively low cysteine tolerance of *N. gonorrhoeae,* putative mechanisms of cysteine homeostasis were explored.

Numerous export systems have been described in *E. coli* and include a variety of exporter types, some with specificity for cysteine and others with broader substrate specificity (Yamada et al., 2006; Ohtsu et al., 2010; Korshunov et al., 2020). In *N. gonorrhoeae,* the locus tag NGO00320 is annotated as an EamA family transporter, however, is predicted to be non-functional due to a frameshift mutation. Additional candidates for cysteine export were identified by pBLAST using *E. coli* proteins, including another potential EamA homolog (NGO1512) and a Bcr homolog (NGO0903). However, these homologs share low sequence similarity (20 – 30%) with their *E. coli* counterparts raising uncertainty regarding their functional conservation. This, together with the notable sensitivity of *N. gonorrhoeae* to cysteine, argues against cysteine export being a major or efficient mechanism of cysteine homeostasis.

As outlined in Section 1.4.2, bacteria possess a wide range of enzymes capable of degrading cysteine ranging from dedicated cysteine degradation enzymes to enzymes that can degrade cysteine as a secondary function (Table 3.1). A comprehensive search of the gonococcal genome for homologs of all known enzymes with cysteine degradation activity revealed that the gonococcus encodes no identifiable CDS, CD, CDO, CBS, CGL, or 3-MST homologs; indicating a lack of classical cysteine homeostasis mechanisms, which is consistent with the experimentally observed vulnerability to cysteine toxicity. Further, of the many enzymes with cysteine degradation as a secondary activity, only *cysK* is confirmed to be present in *N. gonorrhoeae* suggesting, overall, a minimal capacity for cysteine degradation.

Among the CSD superfamily, *N. gonorrhoeae* encodes only one member, *iscS*, which is a master enzyme that delivers sulfur to several partners involved in Fe-S cluster assembly, tRNA modification, cofactor biosynthesis, and NAD biosynthesis

(Das et al., 2021). In *E. coli* IscS has been implicated in cysteine degradation under reducing or anaerobic conditions (Wang et al., 2019). However, given its primary role in sulfur trafficking rather than detoxification, it remains unclear to what extent IscS may contribute to cysteine degradation in *N. gonorrhoeae,* and whether this is relevant to the aerobic conditions of the present study.

Together, these observations indicate that *N. gonorrhoeae* lacks robust canonical cysteine detoxification or degradation pathways, demonstrating a limited capacity for maintaining cysteine homeostasis which is reflected in the observed cysteine sensitivity. Future work should aim to verify cysteine homeostasis related functions for these cysteine efflux and degradation candidates.

3.2 Methionine synthesis and recycling

The relationship between cysteine and methionine can be bidirectional. In some pathogens, cysteine donates the sulfur required to synthesize methionine through the transsulfuration pathway (Ferla & Patrick, 2014). Conversely, methionine can be recycled back into cysteine through the methionine recycling pathway and the reverse-transsulfuration pathway (RTSP) and therefore acquired as a sulfur source when cysteine is scarce (Doherty et al., 2010; Lee et al., 2019; Rodionov et al., 2004; Seiflein & Lawrence, 2001, 2006; Wheeler et al., 2005). The relationship between methionine and cysteine biosynthesis pathways has not previously been investigated in *N. gonorrhoeae*. Consequently, it is unknown if cysteine is required for methionine synthesis, nor is it known if *N. gonorrhoeae* possesses the methionine- to - cysteine conversion route. The presence of the methionine-specific ABC transporter system, *metNIQ*, supports the ability of *N. gonorrhoeae* to acquire exogenous methionine. However, *N. gonorrhoeae* is incapable of utilizing methionine as the sole sulfur source. Therefore, given *N. gonorrhoeae* proliferates when cyst(e)ine is provided (in the absence of exogenous methionine), these results suggest that *N. gonorrhoeae* may synthesize methionine from cysteine but cannot recycle cysteine from methionine.

3.2.1 Methionine recycling pathway

Methionine recycling to cysteine requires two distinct pathways, the methionine recycling pathway and the RTSP. The methionine recycling pathway is required to first convert methionine to homocysteine, all genes required for this transformation

are encoded in the gonococcal genome (Table 3.1). However, no homologs of the CBS and CGL that make up the RTSP responsible for converting homocysteine to cysteine, are detected. This suggests *N. gonorrhoeae* lacks the ability to salvage cysteine from methionine, consistent with the experimental observation that *N. gonorrhoeae* cannot grow on methionine as a sulfur source. Interestingly, *C. difficile* cannot utilize methionine as a sulfur source but can utilize homocysteine, despite encoding the methionine recycling. Like *N. gonorrhoeae, C. difficile* lacks the RTSP, demonstrating that homocysteine utilization can occur independently of canonical pathways and thus warrants further investigation in *N. gonorrhoeae* (Dubois et al., 2016).

3.2.2 Methionine synthesis pathway

Given that in the absence of exogenous methionine cyst(e)ine can be used as a sole sulfur source for gonococcal growth, this indicates that cysteine can be used as a sulfur source for methionine synthesis. To determine how this occurs in *N. gonorrhoeae*, the gonococcal genome was searched for all possible genes involved in methionine biosynthesis (Table 3.1) and a putative methionine synthesis pathway was constructed.

In bacteria, methionine biosynthesis proceeds through three stages: acylation, sulfurylation, and methylation (Figure 3.3) (Ferla & Patrick, 2014). In the first two stages, homoserine is converted into homocysteine by sequential activation of homoserine (acylation) and subsequent addition of a thiol group (sulfurylation). The pathway to yield homocysteine is variable between species, with no clear canonical route persevering. All methionine synthesis pathways then converge at the final, and conserved stage of methylation, where methionine is synthesized from homocysteine.

Different pathogens employ various combinations of the following enzymes involved in acylation and sulfurylation. Homoserine is acylated to produce *O*-acetyl- or *O*-succinyl-L-homoserine (OAH or OSH), dependent on the acetyl donor (acetyl-CoA or succinyl-CoA), catalyzed by homoserine *O*-acetyltransferases (HAT) or homoserine *O*-succinyltransferases (HST), respectively (Figure 3.3, ①). HAT and HST are encoded for by two unrelated, and

phylogenetically distinct enzyme families, *metX* or *metA*. Originally, *metA* was associated with HAT or HST activity and *metX* was assumed to be exclusively HAT activity. However, biochemical and structural studies have shown that both families include enzymes with either activity, with substrate specificity dictated by active-site residues. As a result, *metA* and *metX* are frequently misannotated (Bastard et al., 2017; Ferla & Patrick, 2014).

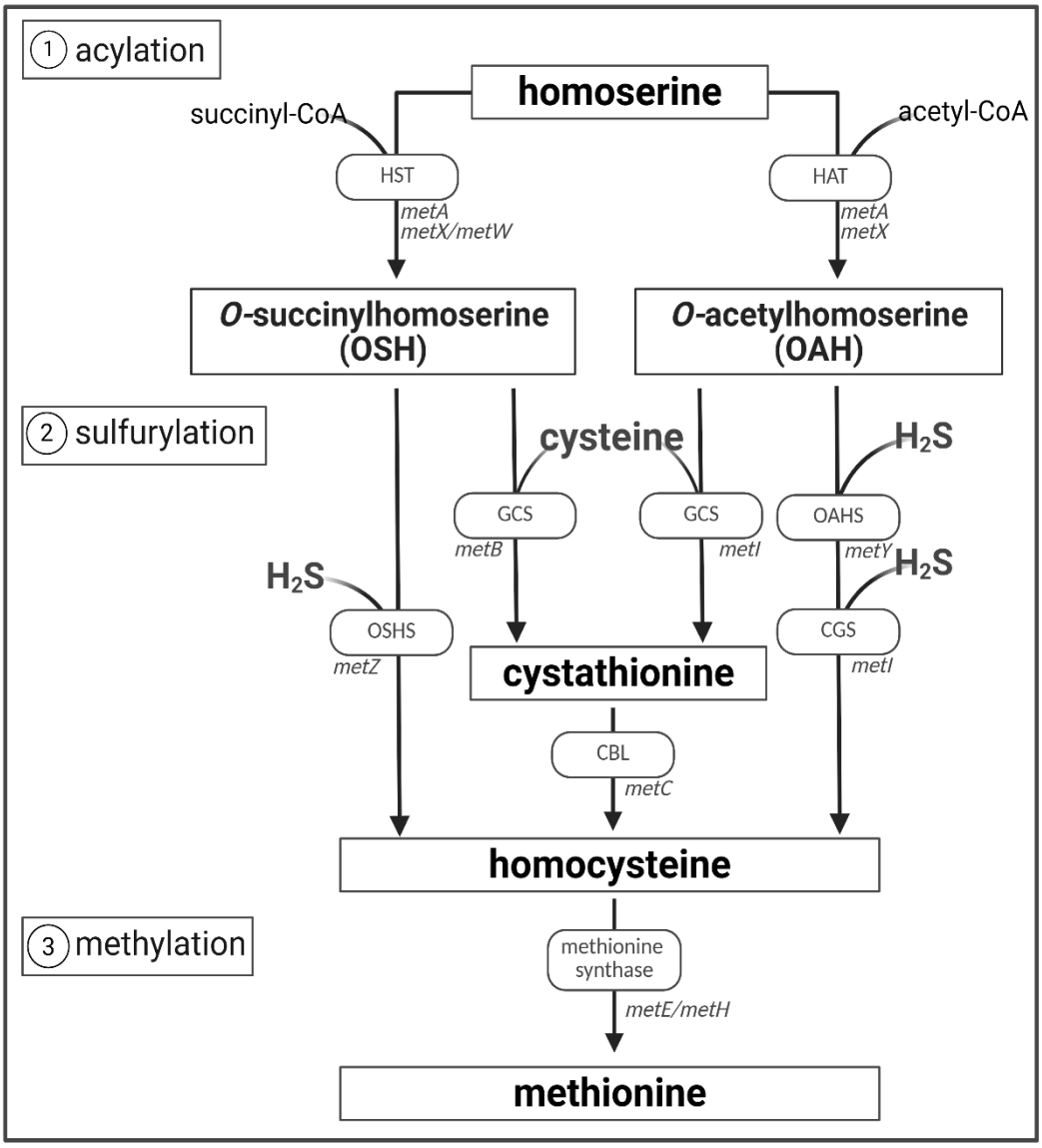


Figure 3.3. Pathways of methionine biosynthesis in bacteria. The synthesis of methionine proceeds via three main stages ① acetylation, ② sulfurylation, and ③ methylation. The possible reactions for each stage are depicted with the enzymes that catalyze them and their respective gene names. Abbreviations; HST; homoserine *O*-succinyltransferases, HAT; homoserine *O*-acetyltransferases, OSHS; *O*-succinylhomoserine sulfhydrylase, OAHS; *O*-acetylhomoserine sulfhydrylase CGS; cystathionine γ-synthase CBL; cystathionine β-lyase.

Sulfurylation of OAH or OSH then proceeds via two main routes, the direct or trans-sulfurylation pathway (Figure 3.3, ②). The direct sulfurylation pathway utilizes H_2S as the sulfur donor to form homocysteine in a single reaction. This reaction is catalyzed by *O*-succinylhomoserine sulfhydrylase (OSHS, *metZ*) or *O*-acetylhomoserine sulfhydrylase (OAHS, *metY*), depending on the form of activated homoserine. The trans-sulfurylation pathway, in contrast, requires cysteine as the sulfur donor and forms homocysteine in two-enzyme catalyzed reactions. OSH is first condensed with cysteine by cystathionine γ-synthase (CGS, *metB*) to form cystathionine, which is then cleaved by cystathionine β-lyase (CBL, *metC*) to release homocysteine. An alternative, dual functioning, CGS enzyme, encoded by *metI*, functions as a hybrid of these two pathways. Acting on OAH rather than OSH, MetI can accept either cysteine, as per the trans-sulfurylation pathway to produce cystathionine, or H_2S producing homocysteine as the sulfur donor as per the direct sulfurylation pathway. In the final step of methionine biosynthesis, homocysteine is converted to methionine (Figure 3.3, ③). Bacteria possess one, or both, methionine syntheses, encoded for by *metE* and *metH*, that catalyze this reaction, dependent or independent on the cofactor cobalamin, respectively.

As the route to methionine from homoserine can vary significantly between species, all known genes involved in methionine synthesis were searched for revealing that the *N. gonorrhoeae* genome contains the following set of methionine biosynthesis genes that comprise a complete pathway: *metX, metW, metZ,* and *metE*. An additional gene identified, *metB,* which appears in isolation of a complete pathway, is examined in section 3.2.2.1. The gene encoding *metX* is commonly annotated as HAT, however *in vitro* characterization of the gonococcal protein confirms HST activity, with substrate specificity for succinyl-CoA to convert homoserine to OSH (Bastard et al., 2017). The *metW* gene is co-transcribed with *metX* and appears critical for its function, although the exact function has not been elucidated (Bastard et al., 2017; Remmele et al., 2014). Following OSH formation, OSHS, encoded for by *metZ*, catalyzes the conversion of OSH to homocysteine, using H_2S as the sulfur donor. *N. gonorrhoeae* encodes only the cobalamin-independent methionine synthase, *metE*, which completes the pathway by converting homocysteine to

methionine. This pathway bypasses the cystathionine intermediate and predicts that the sulfur required for synthesis of methionine is H_2S, not cysteine.

Given that *N. gonorrhoeae* can grow in the presence of cyst(e)ine only, this suggests that cysteine must be degraded as a source of endogenous H_2S for *de novo* methionine biosynthesis under these conditions. Curiously, as previously outlined, *N. gonorrhoeae* has a limited capacity for cysteine degradation. At present, how H_2S is generated from cysteine for methionine biosynthesis remains unresolved but might involve CysK or IscS.

3.2.2.1 Structural prediction of an additional putative cysteine/methionine biosynthesis related enzyme

Interestingly, the *N. gonorrhoeae* FA1090 genome contains an additional putative methionine biosynthesis-related gene, *metB*, which encodes for CGS of the transsulfuration pathway, responsible the conversion of OSH to cystathionine. However, in the absence of the second enzyme of this pathway, CBL, which is required to convert cystathionine to homocysteine, it is not immediately obvious how *N. gonorrhoeae* might benefit from cystathionine production as besides being an intermediate cysteine to methionine conversion (and vice versa), no other functions for cystathionine have been reported in bacteria.

CGS belongs to the aspartate aminotransferase family of type I-fold PLP-dependent enzymes (Pfam PF01053), alongside other sulfur metabolism related proteins; cystathionine γ-lyase (CGL), methionine γ-lyase (MGL), and OAHS (Tran & Brown, 2022). These enzymes share substantial sequence and structural similarity despite catalyzing distinct β- and γ-elimination or substitution reactions. As such, members of this protein family are notoriously prone to misannotation. Considering this, and the likely relevance of this protein to the sulfur metabolism network in *N. gonorrhoeae*, we sought to manually refine the annotation of this gene via investigating structural differences to differentiate between the closely related proteins CBL and CGS. For this purpose, the FA1090 protein sequence was used as a query sequence to generate a high-confidence AlphaFold model (mean pLDDT = 97.3) of the gonococcal protein monomeric structure. This predicted structure was subsequently compared to published structures of the *E. coli* CBL (*Ec*CBL) and CGS (*Ec*CGS) proteins.

Although *Ec*CBL and *Ec*CGS are indistinguishable by active site residues, previous analysis revealed two regions of structural divergences between the two structures (Clausen et al., 1998; Messerschmidt et al., 2003). Region I, located in the C-terminal domain (residues 336–358 in *Ec*CGS numbering), forms a loop which protrudes further into the active site in *Ec*CGS, narrowing the active site cleft. Region II (residues 41-44 in *Ec*CGS numbering) lies in the N-terminal domain. In CGS, the arrangement is a short loop, while this CBL region consists of an extended loop with an additional α-helix. The predicted gonococcal structure was aligned to the *Ec*CBL (PDB: 1CL1) and *Ec*CGS (PDB: 1CS1) and inspection of gonococcal Region I and II (GC residues 336–357 and 39–42, respectively) aligns closely with *Ec*CGS in both regions and lacks the short α-helix found in CBL (Figure 3.4), supporting a CGS-type structure. However, CGS shares a high degree of structural and sequence similarity to the closely related protein, CGL, such that structure alone cannot reliably discriminate between CGS or CGL. Consequently, whether the gonococcal protein possesses CGS activity, CGL activity, or both cannot be resolved based solely on structural features.

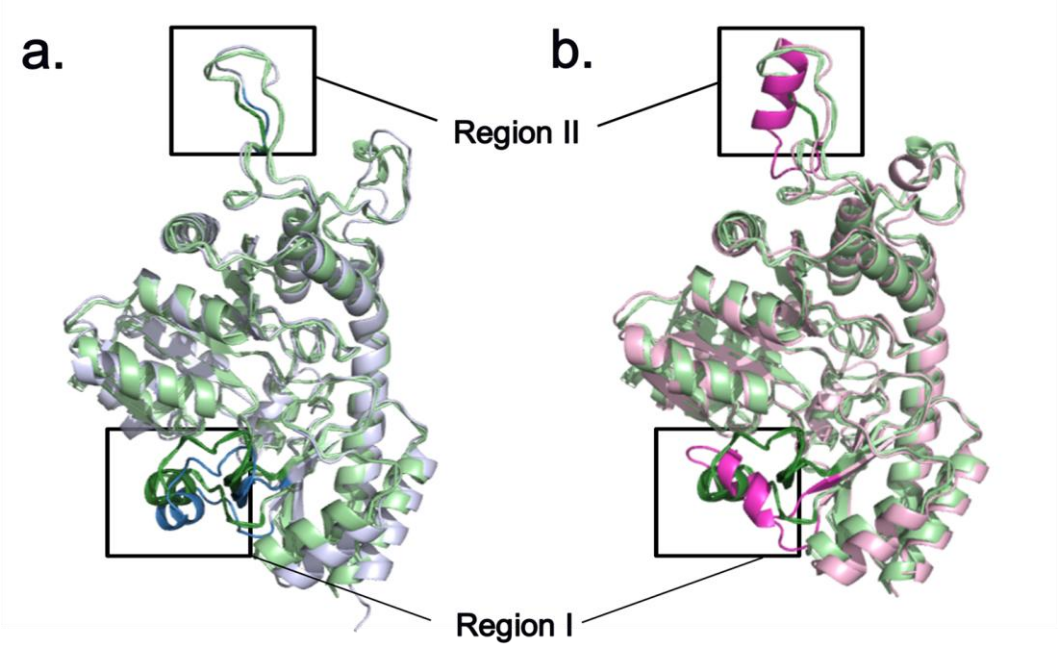


Figure 3.4. Predicted three-dimensional structure of gonococcal CGS (green) superimposed onto (a) *E. coli* CGS (blue) and (b) *E. coli* CBL (pink). Regions of divergence between *Ec*CGS and *Ec*CBL (Regions I and II) are indicated. The gonococcal structure was generated using AlphaFold (mean pLDDT = 97.3). *Ec*CBL (PDB: 1CL1) and *E*cCGS (PDB: 1CS1) structures were obtained from the Protein Data Bank.

Nevertheless, from the perspective of sulfur metabolism, the functional relevance of CGS activity in *N. gonorrhoeae* is unclear, as synthesis of cystathionine would not directly support methionine biosynthesis in the absence of CBL. On the other hand, CGL activity could be advantageous to the gonococcus by enabling utilization of cystathionine as a cysteine source or contributing to cysteine homeostasis and H_2S production via cysteine degradation. A further possibility is that the absence of both forward and reverse transsulfuration pathways indicates functional divergence from canonical CGS or CGL activity. In support of this, further inspection of the gonococcal structure revealed deviations from highly conserved active site residues, Glu325 and Arg106 (*Ec*CGS numbering), responsible for cystathionine anchoring. The corresponding residues in the gonococcal protein, Ser326 and Asp106 respectively, result in an inversion of the electrostatic interaction. Although the effect these substitutions have on cystathionine binding is not understood, they suggest altered substrate binding, raising the possibility of functional divergence.

At present, the structural features of the gonococcal PLP-dependent type I-fold protein favor a CGS annotation. However, whether the gonococcal protein retains canonical CGS or CGL activity, possesses bifunctional activity, or has divergent catalytic activity remains to be determined. Consequently, the putative function of this gene in the gonococcal sulfur metabolic network remains inconclusive and requires experimental investigation.

3.3 Gonococcal pathways for glutathione synthesis and recycling

Cysteine is an essential precursor for the synthesis of reduced thiols, including glutathione which has extensive roles in redox homeostasis and oxidative stress mitigation. Glutathione is the most abundant reduced thiol within the host and as such pathogens have evolved mechanisms to degrade glutathione as a nutrient source. Next, we investigated the extent of glutathione acquisition, synthesis, and degradation in *N. gonorrhoeae*.

3.3.1 Glutathione synthesis

Glutathione constitutes a powerful antioxidant system within *N. gonorrhoeae* and is thought to be critical for the gonococcal oxidative stress response (Seib et al., 2006). No homologs of characterized glutathione ABC transporter systems are

found in the gonococcal genome but *N. gonorrhoeae* does encode the genes for the canonical glutathione synthesis pathway in proteobacteria, *gshA* and *gshB*, suggesting that the intracellular pool of glutathione is established through synthesis (Table 3.1) (Masip et al., 2006).

3.3.2 Glutathione recycling

Genes encoding the canonical glutathione degradation pathway (*ggt* and the downstream peptidases, *pepA* and *pepN*) are present in the *N. gonorrhoeae* genome. Although *ggt* is transcriptionally active, it is functionally inactive due to a mutation that introduces a premature stop codon. This mutation is highly conserved in *N. gonorrhoeae* and as such, Ggt activity is routinely used to distinguish *N. gonorrhoeae* from *N. meningitidis*, which harbors an intact and functional Ggt protein (Takahashi & Watanabe, 2005). Homologs of alternative, non-canonical glutathione-degrading enzymes that catalyze initial GSH cleavage, such as ChaC in *F. tularensis* or PepT hypothesized to catabolize glutathione in *C. perfringens* were not identified in the gonococcal genome (Table 3.1). However, despite this *N. gonorrhoeae* was able to proliferate when glutathione is provided as the sole sulfur source (Figure 3.5).

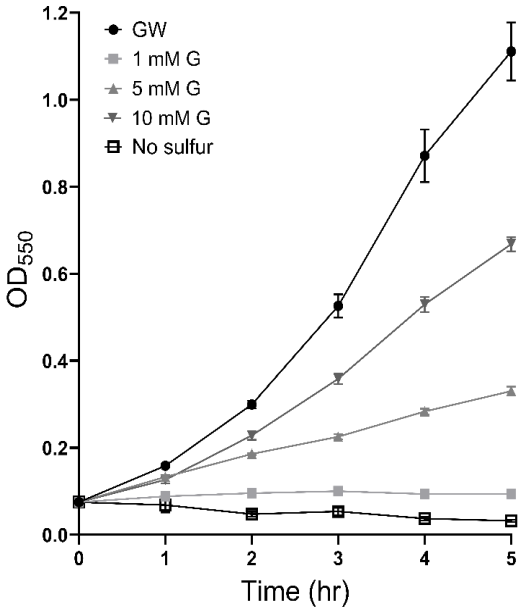


Figure 3.5. Growth of *N. gonorrhoeae* with glutathione (G) as the sole sulfur source. Growth curves of *N. gonorrhoeae* WT with glutathione as the sole source of sulfur at 1, 5, and 10 mM, compared to growth under GW control conditions. The no sulfur control confirms that gonococcal growth in the modified GW medium is dependent on sulfur supplementation. Data represents the mean ± SEM from minimum of 3 biological replicates per condition.

The growth capacity of the gonococcus with glutathione as the sole sulfur source increased in a concentration-dependent manner across the following physiologically relevant glutathione concentrations tested: 1, 5, and 10 mM (Figure 3.5). However, in all cases, the gonococcal cultures exhibited significantly reduced final culture densities relative to the GW control (Figure 3.6). Although a statistically significant reduction in growth rate was observed for 5 mM, and not 10 mM, glutathione (Figure 3.6 a), the modest reduction in growth rate for the 10 mM condition was associated with a measurable decreases in culture density, indicating a biologically meaningful growth limitation (Figure 3.6 b). Growth rate could not be reliably determined for the 1 mM condition as the viability of the culture over the time course used to determine doubling time is uncertain, given the limited extent of growth.

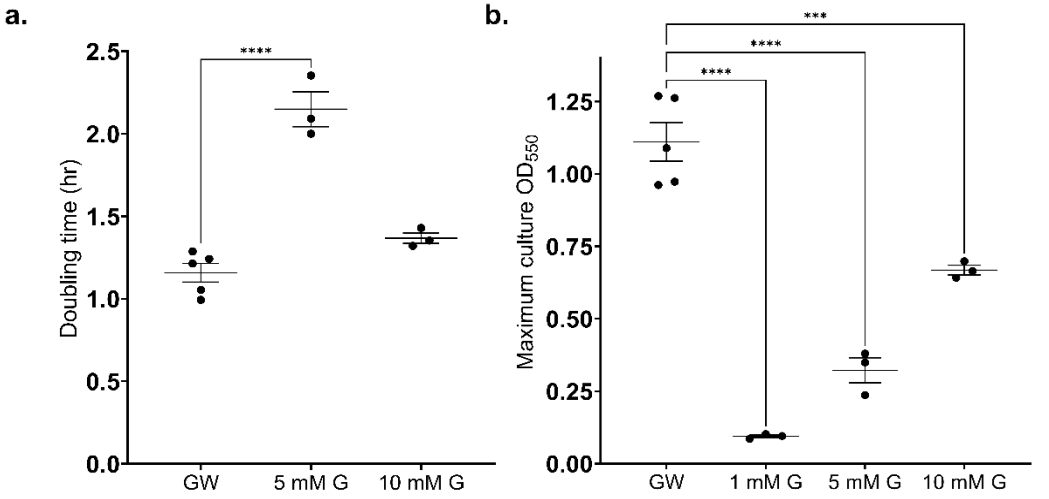


Figure 3.6. Growth metrics of *N. gonorrhoeae* grown in different glutathione (G) concentrations. The growth of *N. gonorrhoeae* WT was quantified as (a) growth rate, determined by doubling time, and (b) culture growth capacity, measured as maximum OD_{550} and compared to GW control. Statistical analysis was performed using ordinary one-way ANOVA with Dunnett's correction ($p < 0.05$). Data represents the mean ± SEM from a minimum of 3 biological replicates per condition.

Compared to growth in the cyst(e)ine only condition in which 1 mM cysteine was sufficient for optimal growth, at the highest glutathione concentration tested, which equates to 10 times the cysteine equivalent, growth was still sub-optimal. The observed reduced fitness for cultures grown in the presence of glutathione therefore likely reflects a metabolic cost or inefficiency associated with liberating cysteine from glutathione, suggesting that sulfur recovery from glutathione is rate-limiting for gonococcal growth. A similar metabolic restriction is seen in *C. jejuni* where,

like *N. gonorrhoeae*, growth on glutathione requires higher concentrations relative to growth on cysteine or cysteine containing dipeptides, including Cys-Gly, the product of initial glutathione cleavage, identifying initial glutathione cleavage as the limiting step (Vorwerk et al., 2014).

The pathway by which *N. gonorrhoeae* liberates cysteine from glutathione remains unresolved. Initial glutathione cleavage typically occurs in periplasm, with few reported exceptions (Lensmire et al., 2023). Subsequently, Cys-Gly is imported into the cytoplasm for further processing. In *N. gonorrhoeae,* it is unclear whether initial glutathione processing occurs in the periplasm or cytoplasm, although no dedicated glutathione transporters have been identified in the gonococcal genome suggesting extracellular cleavage. However, the existence of novel or alternative transport systems cannot be dismissed. Searches for putative dipeptide transporters that have been previously identified as facilitating Cys-Gly uptake returned two candidates: (i) a predicted major facilitator superfamily transporter (NGO1954), which exhibits low (~25%) sequence similarity to the proton-dependent oligopeptide transporter DtpA of *F. tularensis*, and (ii) a predicted peptide transporter (NGO1900) showing moderate (~54%) sequence similarity to the OPT-family transporter CptA from *C. jejuni* (Vorwerk et al., 2014; Ramsey et al., 2020). It is feasible that at least one of these systems could import Cys-Gly which could be further cleaved by peptidases, *pepN* and *pepA*.

Future work should aim to elucidate this mechanism by initially assessing gonococcal growth in the presence of glutathione cleavage products which would indicate whether cleavage occurs prior to transport. Should this be the case, the candidate genes for dipeptide transport could then be explored further. Identifying the genes required for utilizing glutathione as a sulfur source would advance the work herein by allowing for the generation of a non-glutathione utilizing mutant for the purpose of investigating glutathione acquisition during infection.

3.4 *De novo* cysteine biosynthesis and pathways of inorganic sulfur assimilation

N. gonorrhoeae has traditionally been defined as a cysteine auxotroph due to the absence of an inorganic sulfate assimilation pathway, and consequently, an inability to utilize sulfate and sulfite as a sulfur source for *de novo* cysteine biosynthesis,

consistent with our observations (Supplementary material 6.6). Taurine is a rarely used substrate for *de novo* cysteine biosynthesis was not considered relevant for *N. gonorrhoeae* given that taurine assimilation results in the production of sulfite, which *N. gonorrhoeae* cannot utilize and reduce to H_2S for cysteine biosynthesis. Alongside this, the genes required for taurine assimilation were not identified in the gonococcal genome (Table 3.1).

Evidence dating back to the 1980s suggests that thiosulfate can serve as an alternative sulfur source in the absence of exogenous cysteine, indicative of a functional *de novo* cysteine biosynthesis pathway (Le Faou, 1984). Despite this observation, the mechanism by which thiosulfate is assimilated in *N. gonorrhoeae* has not been elucidated. *N. gonorrhoeae* lacks the cysteine synthase isoform, CysM (OASS-B), that can accept either thiosulfate or H_2S as the sulfur donor for synthesis of cysteine. Instead, *N. gonorrhoeae* harbors the CysK enzyme which unequivocally demonstrates substrate specificity for H_2S, and not thiosulfate (McGarvie et al., 2025).

An alternative, CysM-independent pathway for thiosulfate assimilation has previously been described in *E. coli*, whereby thiosulfate reduction is mediated by a thiosulfate sulfurtransferase (TST) to produce sulfite and H_2S (Kawano et al., 2017, 2018). The *N. gonorrhoeae* genome encodes two putative TST, *str* (NGO0020) and *pspE* (NGO0369), predicted to localize to the cytoplasm and periplasm, respectively. Interestingly, periplasmic localization of PspE would circumvent the requirement for thiosulfate uptake prior to reduction as H_2S can passively diffuse across biological membranes (Kumar Pal et al., 2018). This is particularly notable as it is currently unclear as to whether *N. gonorrhoeae* can transport thiosulfate into the cytoplasm as components of the canonical thiosulfate/sulfate ABC transporter are either absent or predicted to be pseudogenes due to frameshift mutations (Hicks & Mullholland, 2018).

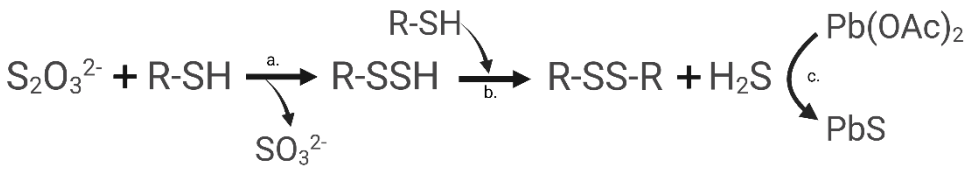
In the absence of CysM-dependent thiosulfate assimilation, we hypothesize that thiosulfate assimilation in *N. gonorrhoeae* occurs via TST-mediated reduction to H_2S, which can subsequently be incorporated into cysteine, and sulfite, which has previously been observed to accumulate in the culture medium proportional to gonococcal growth with thiosulfate (Le Faou, 1984).

3.4.1 Investigation of thiosulfate assimilation in *N. gonorrhoeae*

The contribution of the gonococcal sulfurtransferases, Str and PspE, to thiosulfate assimilation was investigated through biochemical characterization of enzyme activity to confirm thiosulfate reductase activity and analysis of growth phenotypes in the corresponding single and double genetic deletion strains in the presence and absence of thiosulfate.

3.4.1.1 Str and PspE are thiosulfate reductases, reducing thiosulfate to H_2S in the presence of various thiol acceptor substrates

Sulfurtransferase mediated thiosulfate reduction to sulfite and H_2S proceeds via two steps and requires a thiol acceptor substrate. The first step involves the enzyme catalyzed transfer of sulfur from thiosulfate to the thiol acceptor substrate (R-SH) forming a persulfide intermediate (R-SSH) and releasing sulfite (Equation 1 a). The thiol persulfide intermediate subsequently reacts spontaneously with a second thiol acceptor molecule (R-SH) to form a disulfide (R-SS-R) and release H_2S (Equation 1 b). To assess thiosulfate reductase activity of the gonococcal sulfurtransferases *in vitro*, Str and PspE (lacking the signal peptide) recombinant proteins were expressed in *E. coli*. The purified proteins were subsequently kinetically characterized via a continuous spectrophotometric assay which measures the production of H_2S, as lead sulfide (Equation 1 c) (Chiku et al., 2009). Kinetic characterization reveals both Str and PspE catalyze the reduction of thiosulfate to H_2S *in vitro,* confirming thiosulfate reductase activity in the presence of various of thiol acceptor substrates, including cysteine (CYS), glutathione (GSH), and dithiothreitol (DTT), demonstrating substrate promiscuity with respect to thiol acceptor substrates (Figure 3.7 and Figure 3.8).



Equation 3.1. Reaction scheme of kinetic assay. Thiosulfate is converted to sulfite and H_2S via a two-step pathway, comprising (a) an enzyme-catalyzed reaction and (b) a subsequent spontaneous reaction. In the presence of (c) lead acetate ($Pb(OAc)_2$), H_2S reacts to form lead sulfide (PbS), which can be quantified spectrophotometrically at 390 nm.

Comparable K_M values for thiosulfate are observed for Str and PspE, irrespective of the thiol acceptor substrate employed (Table 3.2). Conversely, affinities for the thiol acceptor substrate varied between the enzymes and substrates. Str exhibited the highest affinity for glutathione, whereas PspE displayed a preference for DTT. When thiosulfate concentrations were varied in the presence of GSH or DTT, both enzymes followed classical Michaelis-Menten kinetics (Figure 3.7), whereas when paired with cysteine, substrate inhibition was observed (Figure 3.8). This observed substrate inhibition of cysteine is of particular interest given that under conditions when cysteine is plentiful, bacteria preference cysteine acquisition over *de novo* cysteine biosynthesis strategies, and thus reducing the requirement for inorganic sulfur assimilation.

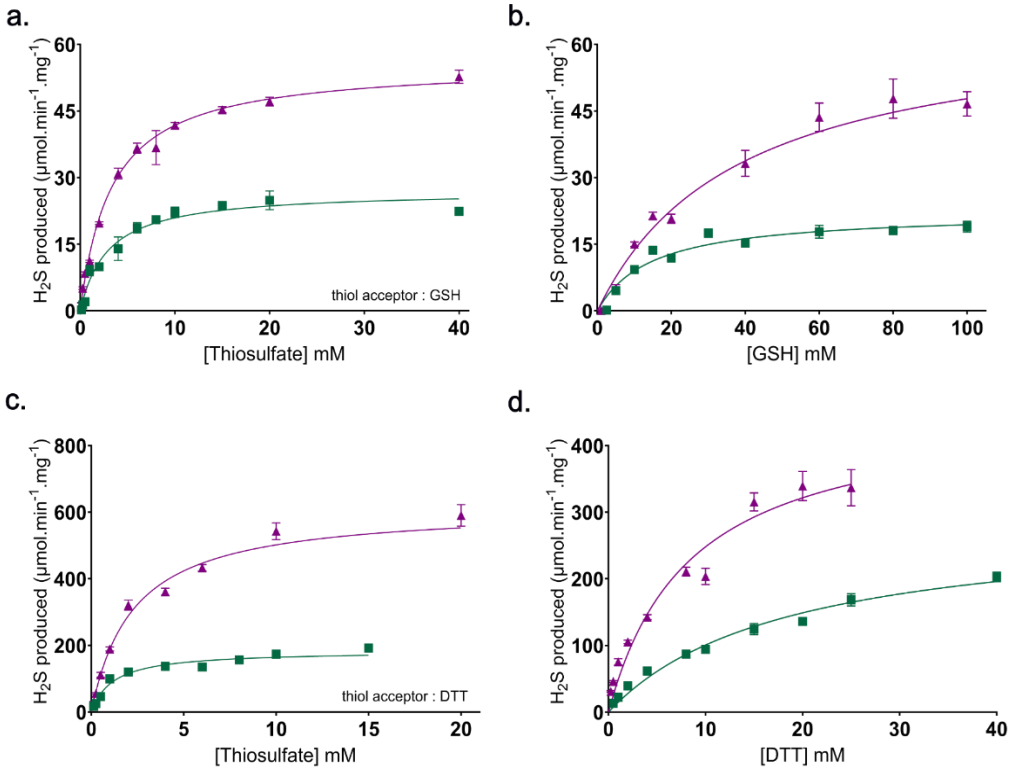


Figure 3.7. Michaelis-Menten Curves of Str (green) and PspE (purple) with thiol acceptor substrates GSH and DTT. Thiosulfate reductase activity was determined by varying thiosulfate concentrations (0.5 - 40 mM) at constant concentrations (a) 50 mM GSH and (c) 40 mM DTT and varying (b) GSH (0.5 - 100 mM) and (d) DTT (0.5 - 40 mM) concentrations at a constant thiosulfate concentration (50 mM). Dependence of rate on substrate concentrations was plotted and fitted with the Michaelis-Menten equation. ($R^2 > 0.9$) All data represents the mean ± SEM from at ≥ replicates.

When cysteine was varied at constant thiosulfate concentrations, activity was only detectable at relatively high cysteine concentrations (> 5 mM). As the gonococcal

cysteine toxicity threshold is 2 mM, it is highly unlikely cysteine represents a relevant thiol acceptor substrate for these proteins. We were unable to reliably determine kinetic parameters for cysteine as substrate saturation could not be achieved due to the formation of precipitate in the reaction mix at higher cysteine concentrations that interfered with data collection. Data of the lower concentrations that were collected is presented in the Supplementary material 6.7. Nonetheless, these results indicate that sulfurtransferases might interact differently with cysteine compared to alternative thiol acceptor substrates, although the reasons for this require further investigation.

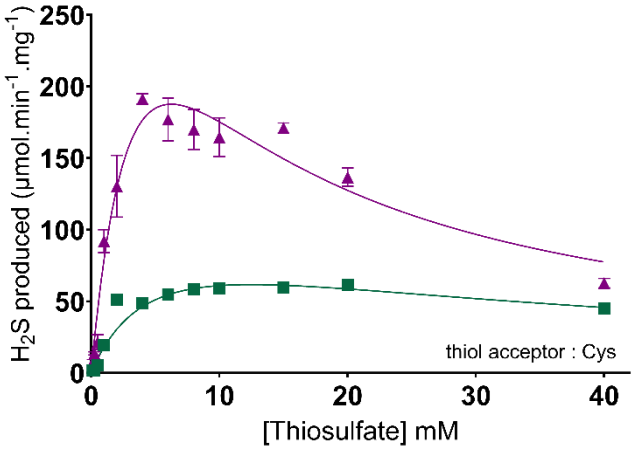


Figure 3.8. Thiosulfate concentration curve with cysteine as the thiol acceptor substrate. Thiosulfate reductase activity was determined by varying thiosulfate concentrations (0.5 - 40 mM) at constant concentrations of cysteine (40 mM). Dependence of rate on substrate concentrations was plotted and fitted with the Substrate-inhibition equation ($R^2 > 0.9$). All data represents the mean ± SEM from ≥ three replicates.

Comparison of Str and PspE revealed that PspE consistently exhibited greater reductase efficiency than Str, displaying 2 – 4-fold higher maximal velocities (V_{max}) and turnover rates (k_{cat}) irrespective of the thiol acceptor substrate tested (Table 3.2). For both enzymes, the highest catalytic rates were observed in the presence of DTT, which, although an abiotic and non-physiological substrate, is a strong reductant. One possible explanation for the elevated activity under these conditions is that, as the catalytic function of sulfurtransferase hinges on a redox-sensitive cysteine residue within the active-site and as DTT is a strong reductant, this may alleviate redox constraints influencing catalysis. Although the catalytic efficiencies reported here for glutathione supported activity are modest, they are orders of magnitude

higher than what has been previously recorded for the *E. coli* homologs, GlpE and PspE (Yu et al., 2023).

Together, these data indicate that Str and PspE possess overlapping catalytic capabilities with respect to thiosulfate reduction, while differing in efficiency, cellular localization, and thiol acceptor utilization, indicative of possibly overlapping and distinct functions within *N. gonorrhoeae.*

Table 3.2. Kinetic parameters of the gonococcal sulfurtransferases, Str and PspE.

Enzyme	K_M *(mM)*		K_i *(mM)*		V_{max}	k_{cat}	k_{cat} / K_M *(M-1.s-1)*	
	Thiosulfate	**Acceptor**	**Thiosulfate**	**Acceptor**	*µmol.min^{-1}.mg^{-1}*	*s^{-1}*	**Thiosulfate**	**Acceptor**
				thiol acceptor: glutathione[a]				
Str	2.91 ± 0.44	14. 67 ± 2.29	NA	NA	27.06 ± 1.11	6.45	2.21×10^3	3.61×10^2
PspE	3.32 ± 0.15	38.17 ± 5.63	NA	NA	66.034 ± 4.21	16.84	4.27×10^3	4.41×10^2
				thiol acceptor: dithiothreitol[a]				
Str	1.18 ± 0.137	18.45 ± 2.37	NA	NA	286.38 ± 17.83	68.25	3.71×10^4	3.70×10^3
PspE	2.22 ± 0.19	8.44 ± 1.50	NA	NA	607.18 ± 16.18	154.83	6.96×10^4	1.38×10^4
				thiol acceptor: cysteine[b]				
Str	5.38 ± 1.116	ND	29.05 ± 6.90	ND/NA	114.80 ± 12.83	27.36	4.27×10^3	ND
PspE	5.18 ± 2.08	ND	7.54 ± 3.13	ND/NA	498.77 ± 137.76	127.16	2.46×10^4	ND

[a]Michaelis–Menten kinetic model fitted to determine substrate parameters ($R^2 > 0.9$) [b]Substrate inhibition kinetic model fitted to determine substrate parameters ($R^2 > 0.9$). Kinetic assays fixed with either thiosulfate (50 or 40 mM for GSH and DTT respectively) or thiol acceptor substrate: glutathione (50 mM), dithiothreitol (40 mM), and cysteine (40 mM). Three to four parallel experiments were conducted to obtain the averages and standard deviations (n= 3 or 4), as applicable. The reaction mixtures contained 0.125µg for Str and PspE lacking the signal peptide (-SP). ND indicates the parameters could not be determined. NA indicates the parameters were not applicable

3.4.1.2 PspE contributes to thiosulfate and glutathione-dependent growth in *N. gonorrhoeae*

To determine the contribution of the sulfurtransferases in thiosulfate assimilation during gonococcal growth, single and double sulfurtransferase deletion strains (Δ*str,* Δ*pspE* and Δ*str:pspE*) were constructed to assess their ability to grow under conditions in which thiosulfate was provided as the available sulfur source. Deletion strains were confirmed by Nanopore sequencing however we acknowledge complementation strains are required to verify subsequent phenotypes. Unexpectedly, *N. gonorrhoeae* WT did not proliferate when thiosulfate (2 mM) was provided as the sole sulfur source under the conditions tested, contrary to earlier reports (Le Faou, 1984) (Figure 3.9 a and b). The basis for this discrepancy remains unclear but may reflect differences in medium composition or culture conditions. Specifically, in the original publication it is not entirely clear whether thiosulfate was the only available sulfur source, as glutathione was not specifically mentioned as omitted (Le Faou, 1984).

Given this uncertainty, and that sulfurtransferases require thiosulfate and a thiol acceptor substrate, such as glutathione, to reduce thiosulfate, the possibility that both substrates were required for WT gonococcal growth was considered. Notably, in the presence of both thiosulfate and glutathione growth was enhanced relative to conditions containing either substrate alone (Figure 3.9 b). Combinations of 2 or 5 mM thiosulfate (T) with 1, 2, or 5 mM glutathione (G) were assessed and revealed a growth increase in a glutathione-dependent manner, while remaining largely insensitive to further increases in thiosulfate concentration, further implicating glutathione as a limiting factor for gonococcal growth (Figure 3.9 a). An optimal combination of 2 mM thiosulfate and 5 mM glutathione (TG) supported growth comparable to the GW control, and significantly exceeded growth supported by 5 mM glutathione alone (Figure 3.9 a-d). This synergistic effect is consistent with a thiosulfate assimilation process that requires both substrates simultaneously.

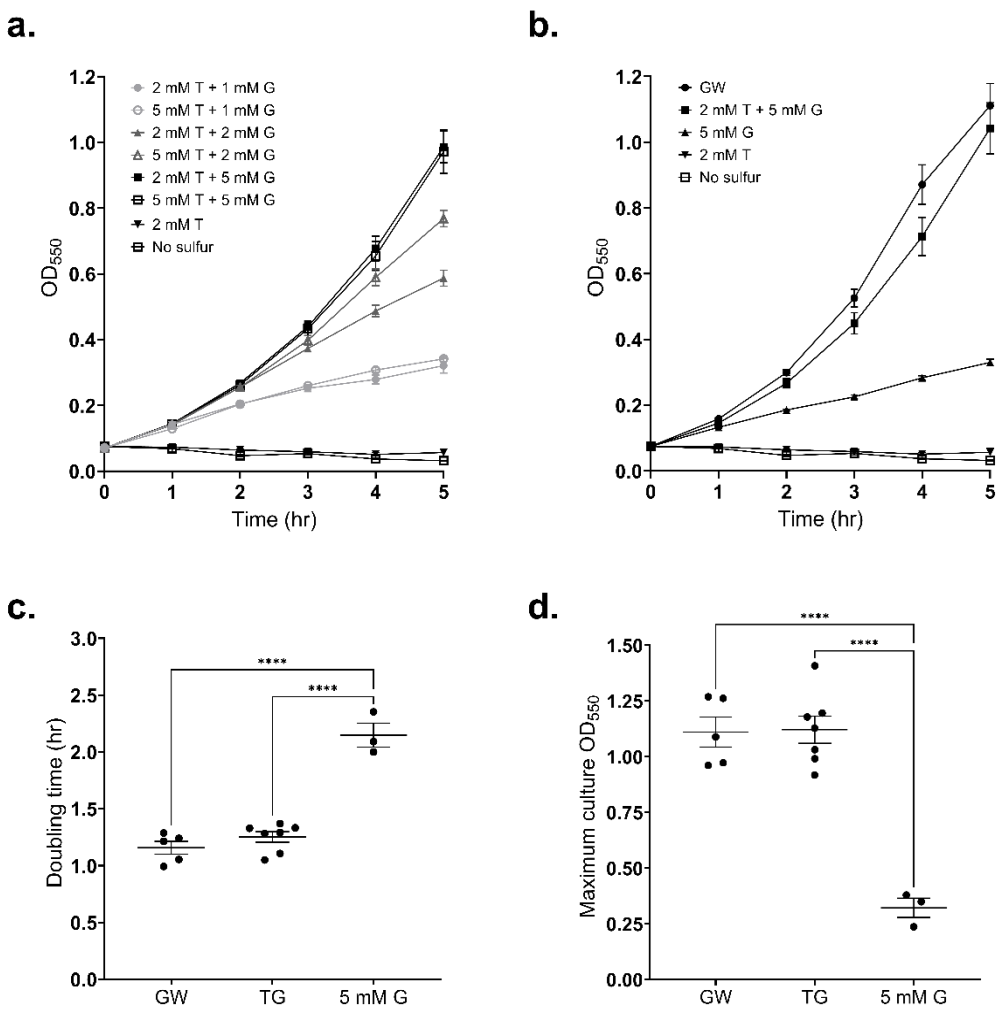


Figure 3.9. Growth of *N. gonorrhoeae* in the presence of thiosulfate and/or glutathione. (a) *N. gonorrhoeae* WT was grown in the presence of various combinations of thiosulfate (T) and/or glutathione (G), (b) optimal concentrations were determined to be 2 mM T supplemented with 5 mM G (labelled TG) which demonstrated significant increases in (c) growth rate and (d) growth yield, compared to 5 mM glutathione without thiosulfate. Statistical analysis was performed using ordinary one-way ANOVA with Dunnett's correction ($p < 0.05$). The no sulfur control confirms that gonococcal growth in the modified GW medium is dependent on sulfur supplementation. Data represents the mean ± SEM from ≥3 biological replicates per condition.

To determine if the sulfurtransferases are involved in such a mechanism, the growth of Δ*str,* Δ*pspE,* and Δ*str:pspE,* was compared with that of WT under both GW and the optimal TG condition. No growth defects were observed for any mutant strain in GW medium (Figure 3.10 a), indicating that sulfurtransferases are dispensable for growth in the absence of thiosulfate. In contrast, the growth rate of the Δ*pspE* and Δ*str:pspE* strains was significantly impaired under the TG condition relative to WT, whereas Δ*str* exhibited no growth defect (Figure 3.10 b and c). Further there was no difference in growth rate between the Δ*pspE* and Δ*str:pspE* mutants. These

phenotypes were additionally confirmed by viable cell counts (Supplementary material 6.8) and support a role for PspE, but not Str, in thiosulfate and glutathione-dependent growth, presumably through the reduction of thiosulfate to H_2S, which can subsequently be used for synthesizing cysteine. However, *pspE* deficient strains (Δ*pspE* and Δ*str:pspE*) demonstrate a modest, but significant, increase in growth rate compared to WT grown in 5 mM glutathione without thiosulfate (Figure 3.10 d), suggesting at least one additional PspE-independent process may contribute to growth under TG supplementation.

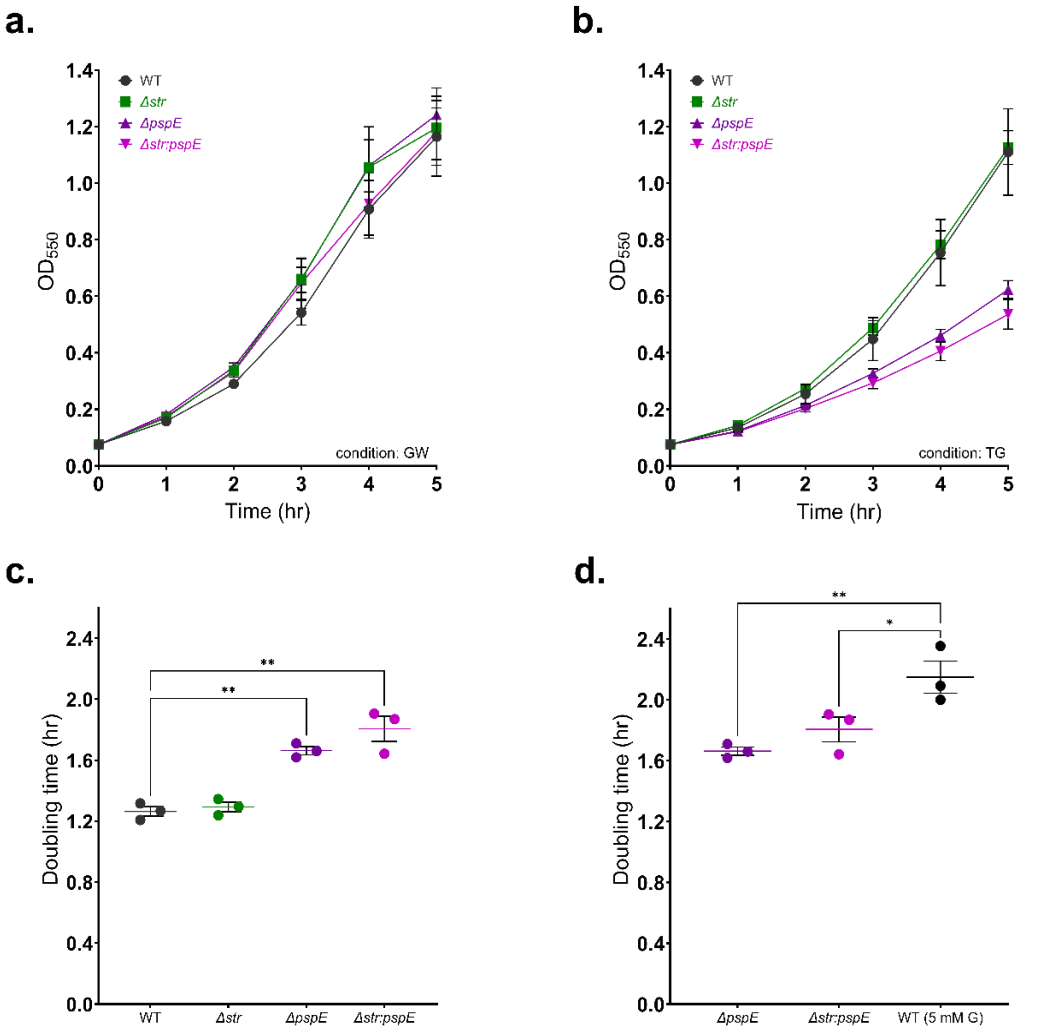


Figure 3.10. Growth of sulfurtransferase (Δ*str* (green), Δ*pspE* (purple), and Δ*str:pspE* (pink)) mutant strains compared to WT (black) in the presence of (a) GW and (b) thiosulfate (2 mM) and glutathione (5 mM) (TG). Growth rate of mutants compared to WT in the (c) TG (d) G (5 mM) only. Statistical analysis was performed using ordinary one-way ANOVA with Dunnett's correction ($p < 0.05$). Data represents the mean ± SEM from ≥3 biological replicates per strain.

Nonetheless, the observations that (i) extracellular glutathione is required for thiosulfate supported growth in WT, (ii) mutants deficient in the periplasmic *pspE* gene, but not the cytoplasmic *str* gene, are impaired in growth in conditions exogenous thiosulfate and glutathione are the available sulfur sources, and (iii) thiosulfate/sulfate ABC transporter appears compromised within *N. gonorrhoeae*, support a model in which thiosulfate assimilation in *N. gonorrhoeae* occurs, at least in part, in the periplasm mediated by PspE.

3.5 Methionine dependent growth enhancement

The rescue of growth observed when thiosulfate, which does not support gonococcal growth on its own, was combined with moderate concentrations of glutathione, which otherwise supports minimal growth, prompted an examination of whether other sulfur metabolites display similar cooperative effects. Sulfate and sulfite did not support gonococcal growth when provided as the sole sulfur source and supplementation with glutathione did not improve growth outcomes (Supplementary material 6.6).

In contrast, the addition of 2 mM methionine to 5 mM glutathione significantly enhanced growth relative to glutathione alone (Figure 3.11). Because *N. gonorrhoeae* lacks the methionine-to-cysteine reverse transsulfuration pathway, this growth enhancement is best explained by complementation of independent nutritional requirements rather than synergistic sulfur assimilation. Under methionine only conditions, the absence of a usable cysteine source likely represents the primary growth limiting factor. Therefore, when glutathione is supplied alongside methionine, it suggests that this limitation is alleviated, as presumably glutathione satisfies the requirement for cysteine, while methionine acquisition independently satisfies the cellular requirement for methionine. The resulting improvement in growth therefore reflects nutritional complementation rather than the synergistic mechanism when thiosulfate and glutathione are accessible.

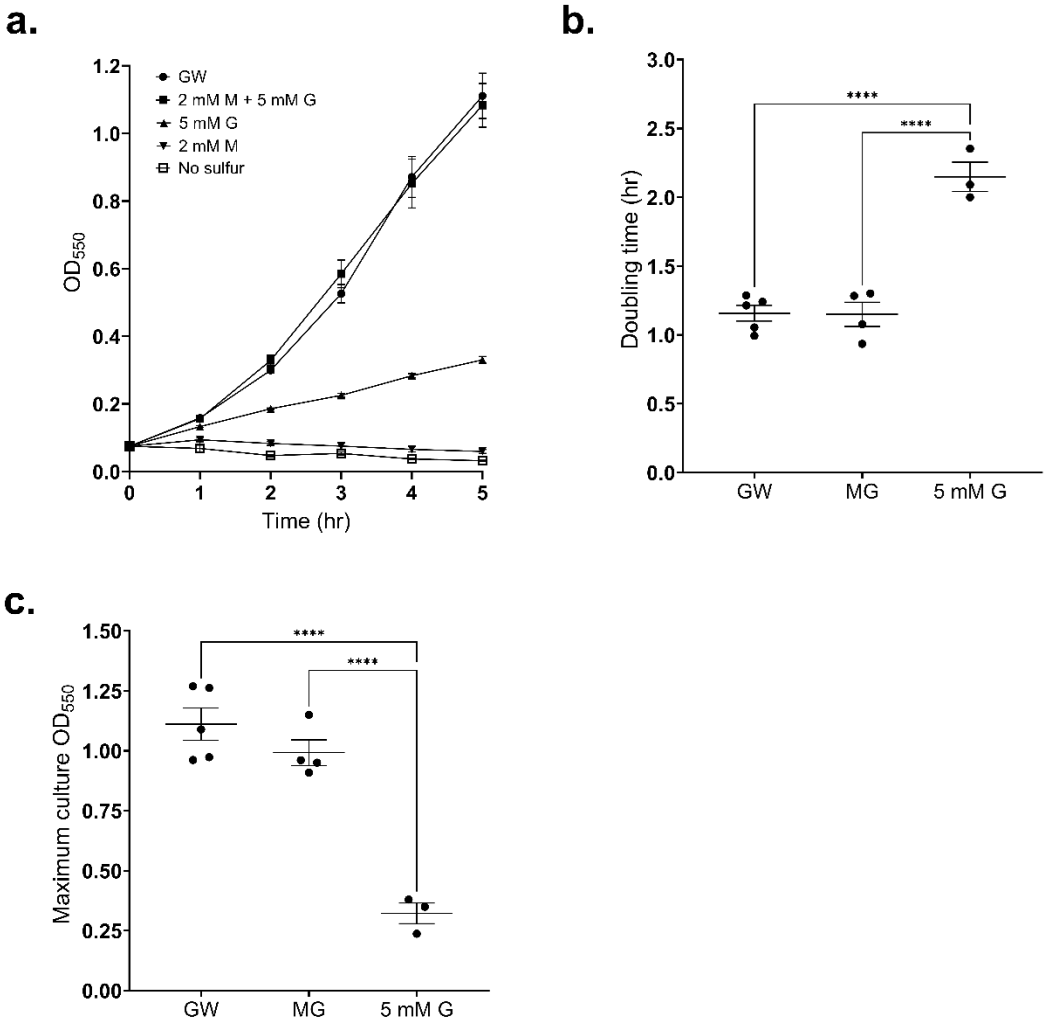


Figure 3.11. Growth of *N. gonorrhoeae* with glutathione and methionine supplementation. (a) Growth curves of *N. gonorrhoeae* WT grown in GW, methionine (M) only (2 mM), glutathione (G) only (5 mM), and combined (MG). Methionine supplemented growth differences in (b) growth rate, presented as doubling time and (c) growth capacity, determined by maximum culture OD_{550}, was compared to growth when glutathione was the sole sulfur source. Statistical analysis was performed using ordinary one-way ANOVA with Dunnett's correction ($p < 0.05$). The no sulfur control confirms that gonococcal growth in the modified GW medium is dependent on sulfur supplementation. Data represent the mean ± SEM ≥3 biological replicates per condition.

3.6 Cystine uptake is a major determinant of gonococcal growth and fitness during infection

This reconstruction of the sulfur metabolic network in *N. gonorrhoeae*, combined with the *in vitro* growth experiments, reveals at least three potential sulfur acquisition strategies for gonococcal growth: (i) acquisition of exogenous cysteine/cystine, (ii) recycling of glutathione, and (iii) thiosulfate assimilation. The

relative importance of these pathways during infection is yet to be elucidated. To begin to resolve this, the importance of cysteine and cystine acquisition were examined in the context of bacterial growth, survival, and infection. Genetic mutants lacking the substrate binding protein component for the cysteine and cystine ABC transporters, Δ*cjaA* and Δ*tcyA*, respectively, were constructed and deletion strains were confirmed by Nanopore sequencing, however we acknowledge complementation strains are required to verify subsequent phenotypes. Growth of these mutants was first examined in GW conditions to establish any growth defects.

Deletion of *cjaA* did not incur any growth defects in the GW medium, indicating that cysteine uptake is dispensable under this condition (Figure 3.12 a). In contrast, deletion of *tcyA* resulted in a complete loss of growth, suggesting that under the aerobic culture conditions tested, cystine is the primary available sulfur source. Furthermore, this observation supports TcyABC as the sole functional cystine transport system in *N. gonorrhoeae*, consistent with genomic predictions. Preliminary data (n< 3 biological replicates) suggests growth of Δ*tcyA* can be restored via chemical complementation with the alternative sulfur conditions, MG, TG, and cyst(e)ine at the concentrations previously established for WT. Although whether growth is as efficient as WT remains to be validated with additional biological replicates (Figure 3.12 b and c). Collectively, these data indicate that growth is abolished when uptake of the primary sulfur source, cystine, is prevented, and that alternative sulfur sources, including cysteine, can compensate when sufficiently accessible.

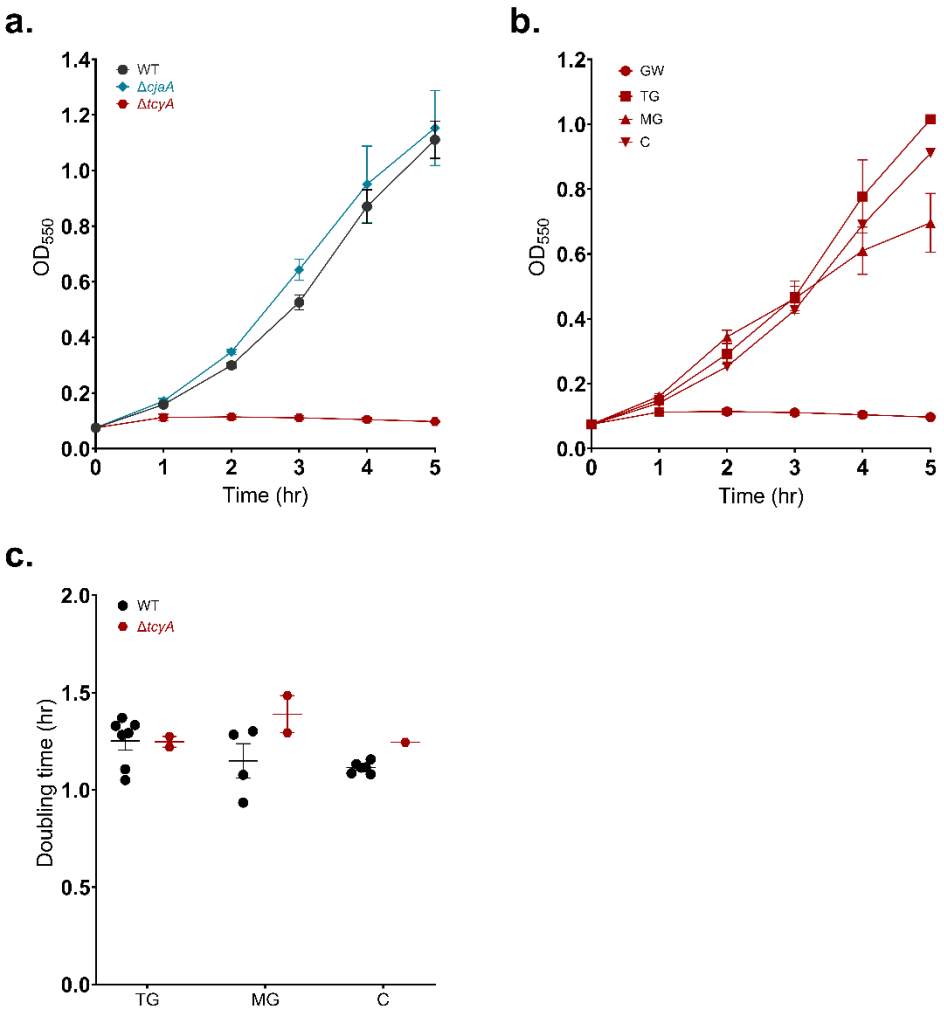


Figure 3.12. Growth of *N. gonorrhoeae* Δ*cjaA* (blue) and Δ*tcyA* (red) compared to WT (black). (a) Growth of *N. gonorrhoeae* WT, Δ*cjaA,* and Δ*tcyA* in GW. Data represents the mean ± SEM of 3 biological replicates per strain. (b) Growth of Δ*tcyA* in GW,thiosulfate and glutathione (TG), methionine and glutathione (MG), or freshly prepared cysteine (C). (c) Growth rate of Δ*tcyA* in the various conditions was compared to WT. Data represents the mean ± SEM 1 – 6 biological replicates per strain.

To assess the contribution of cysteine and cystine acquisition during infection, ME-180 endocervical epithelial cells were infected with WT, Δ*tcyA*, and Δ*cjaA* strains. The ability of the Δ*tcyA* strain to infect epithelial cells was severely attenuated, while no significant differences were observed for Δ*cjaA* compared to WT under the conditions tested. Given the ability of cysteine to restore growth of the Δ*tcyA*, this indicates that cystine is likely the dominant source of cysteine within the infection conditions. Most notably, the total viable population of the Δ*tcyA* strain declined substantially between the infection inoculum and the 4-hour time point, whereas the WT population increased between 3 – 8-fold over the same

period (Figure 3.13 a). This extreme population decline indicates a failure of Δ*tcyA* to survive and replicate under host cell culture conditions.

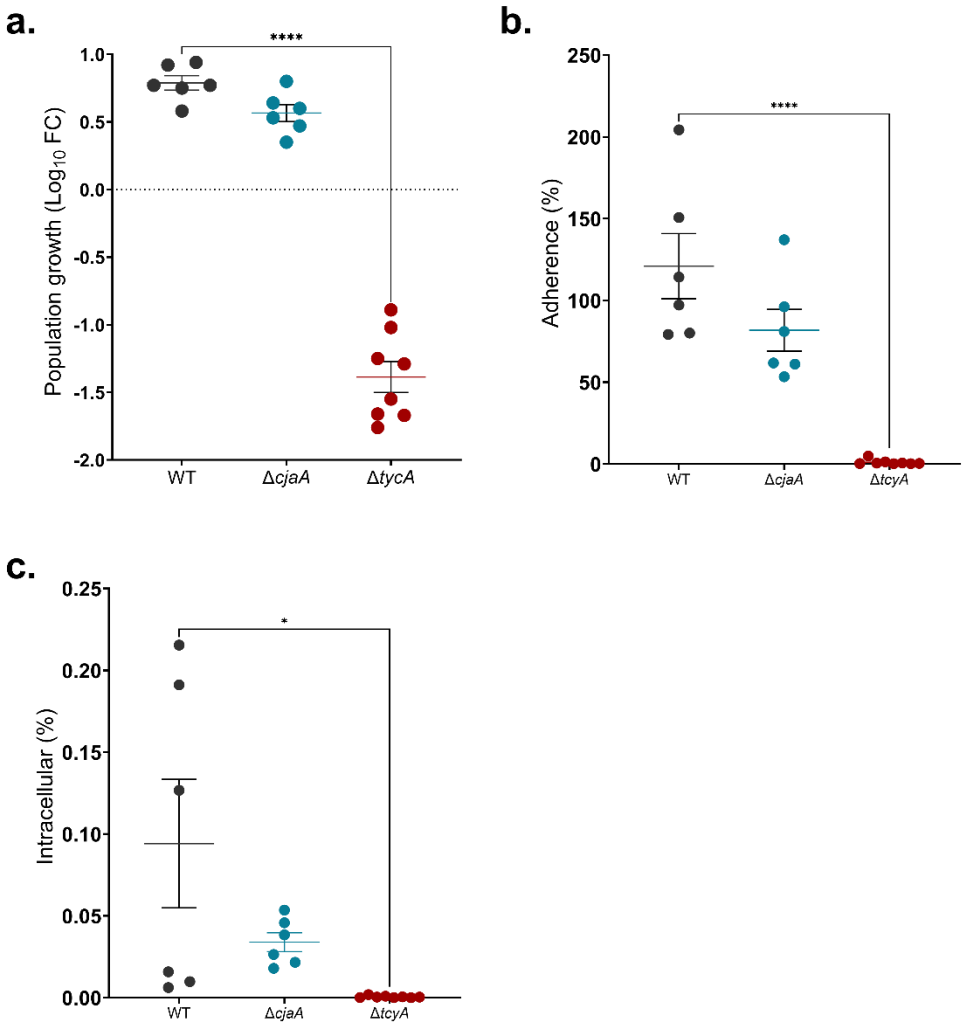


Figure 3.13. Host cell infection assays of *N. gonorrhoeae* Δ*cjaA* (blue), and Δ*tcyA* (red) strains compared to WT (black). (a) Population growth expressed as $\log_{10}$ fold change (Log_{10}FC) of total CFU at 4 hr post-infection relative to the inoculum (b) adherence and (c) invasion indices were determined 4 hr post-infection of ME-180 endocervical cell and are expressed as a percentage of the initial inoculum. Statistical analysis was performed using ordinary one-way ANOVA with Dunnett's correction ($p < 0.05$). Data represent mean ± SEM from ≥6 biological replicates per strain

Consistent with this defect, the capacity of Δ*tcyA* to adhere to epithelial cells was significantly reduced compared to WT (Figure 3.13 b). Furthermore, recovery of viable Δ*tcyA* bacteria from gentamicin-protected intracellular fractions was extremely low (<10 colony forming units (CFU)) (Figure 3.13 c). As such, it cannot be conclusively determined whether Δ*tcyA* bacteria successfully invaded epithelial cells or whether these counts reflect assay artefacts. However, given that epithelial

adhesion is a prerequisite for colonization and subsequent invasion, the striking reduction in cell-associated bacteria is consistent with a failure to establish infection and invade host epithelial cells.

Together, these results indicate that loss of cystine uptake, but not cysteine, results in profound infection defects that are driven primarily by an inability to maintain viability and proliferate. This suggests that the acquisition of cystine is limiting for gonococcal infection, specifically at the stage of colonization, under these conditions. However, as the exact sulfur composition within the infection assay media is unknown, we cannot yet exclude the possibility that the severity of the Δ*tcyA* phenotype is exacerbated by restricted access to alternative sulfur sources given that growth of Δ*tcyA* in liquid media can be chemically complemented *in vitro* by supplementation with sulfur sources, glutathione, thiosulfate, and cysteine. The infection medium, McCoys 5A, contains cysteine (0.26 mM) and glutathione (1.63 μM), and is further supplemented with 10% fetal bovine serum (FBS). FBS contains amino acids, cysteine-containing peptides, and glutathione, however the concentrations of these are unknown nor whether inorganic sources, such as thiosulfate, are present. Thus, additional experiments are required to determine whether the severe infection phenotype of cystine limitation is consequential to an absolute requirement for cystine during infection, or a limitation of alternative sulfur sources in the experimental conditions. This is inherently challenging to discern, given the sulfur metabolite availability *N. gonorrhoeae* encounters during natural infection in different niches are mostly undefined. Furthermore, it remains to be established if the requirement for extracellular cystine is mirrored in an intracellular environment. Should extracellular supplementation restore survival of the *tcyA* mutant, this question could be addressed via a gentamycin protection assay to investigate intracellular survival. Nonetheless, these data support the essentiality of sulfur acquisition for gonococcal infection outcomes *ex vivo*.

3.7 Str and PspE have non-redundant roles during epithelial cell infection

Finally, we examined whether Str and PspE contribute to gonococcal fitness during epithelial cell infection. Relative to WT, all sulfurtransferase deletion strains exhibited significantly reduced adherence to epithelial cells and reductions in

intracellularly recovered gonococci, indicating impaired host cell interactions (Figure 3.14 a and b). The observed attenuation of both Δ*str* and Δ*pspE* indicates that Str and PspE are not functionally redundant, as neither can compensate for the loss of the other. Instead, these results are consistent with distinct contributions of Str and PspE to growth and infection outcomes. As the double mutant consistently exhibited more severe attenuation, the combined presence of both sulfurtransferases is required for optimal infection outcomes. Consistently, only the Δ*str:pspE* strain exhibited a significant reduction in population growth compared to WT (Figure

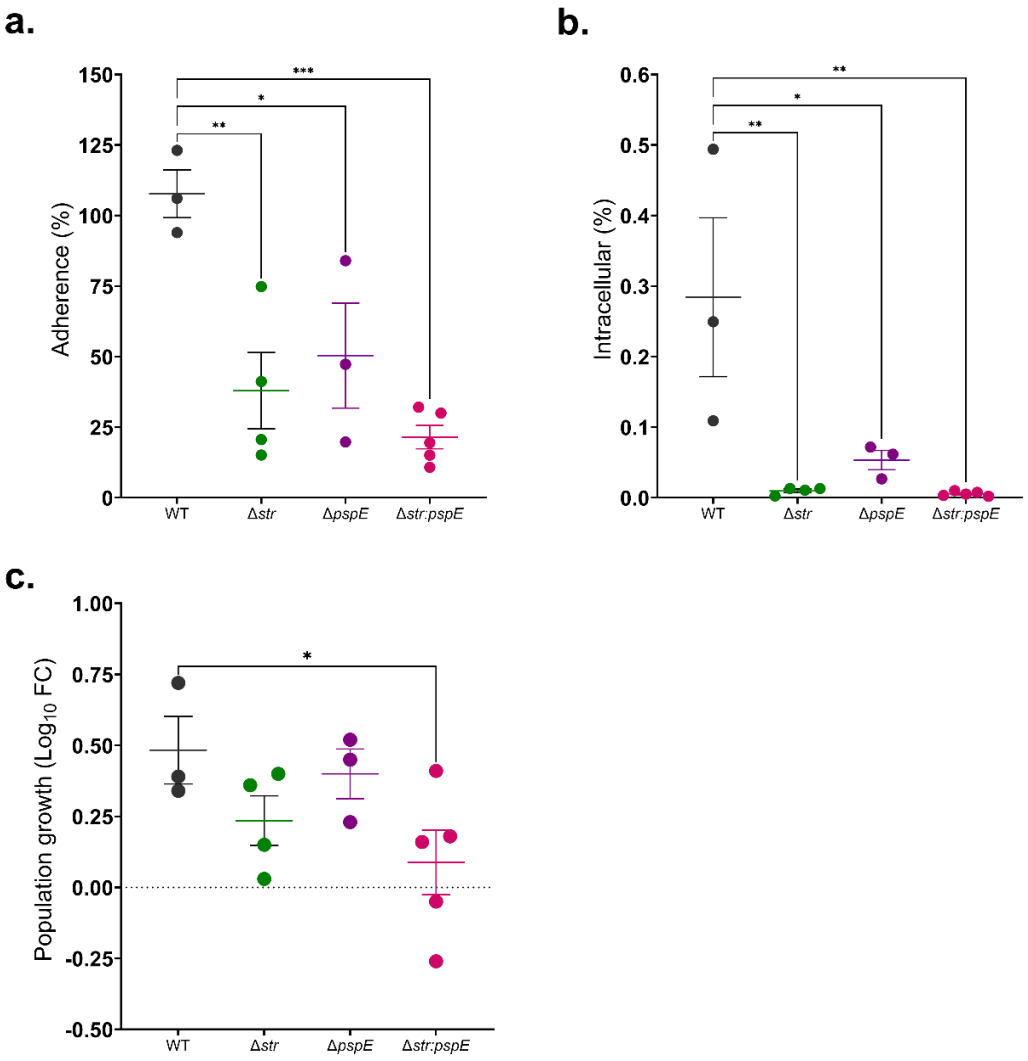


Figure 3.14. Host cell infection assays of *N. gonorrhoeae* Δ*str* (green), Δ*pspE* (purple), and Δ*str:pspE* (pink) strains compared to WT (black). (a) Adherence, (b) invasion, and (c) population growth indices were determined 4 hr post-infection of ME-180 endocervical cells. Adherence and invasion are expressed as a percentage of the initial inoculum. Population growth is expressed as $Log_{10}FC$ in total CFU at 4 hr post-infection relative to the inoculum. Statistical analysis was performed using ordinary one-way ANOVA with Dunnett's correction ($p < 0.05$). Data represent mean ± SEM from ≥3 biological replicates

3.14 c). These results further support non-functionally redundant physiological roles for these related proteins. However, the precise function of Str and PspE during infection remains undetermined. While *in vitro* growth experiments support a role for PspE in thiosulfate assimilation, it is currently unclear whether thiosulfate is available to *N. gonorrhoeae* under the epithelial infection conditions used here. Consequently, it cannot yet be determined whether the observed infection phenotype of PspE arises from defects relating to sulfur metabolism or alternative functions.

3.8 Discussion

The acquisition of essential nutrients is a fundamental for establishing infection. Compared to our understanding of how *N. gonorrhoeae* acquires nutrients such as carbon, iron, and zinc, the nutrient sulfur requirements and sulfur acquisition strategies of *N. gonorrhoeae* during infection are largely uncharacterized (Liyayi et al., 2023; Potter & Criss, 2024). A prerequisite to understanding the nutrient sulfur limitations *in vivo* is a comprehensive understanding of the gonococcal sulfur metabolic network. Once this is established, the conditional importance of these pathways during infection can then be evaluated. Given that the availability and speciation of sulfur metabolites is dependent on the host environment, which is largely unknown, these are particularly important considerations as to the plausibility of targeting pathways, such as methionine or cysteine biosynthesis, where the pathway endpoints might be directly acquired from the environment. The putative sulfur metabolic network within *N. gonorrhoeae* was constructed by integrating literature-informed bioinformatic searches for bacterial sulfur metabolism related pathways, alongside experimental evidence of gonococcal growth in the presence of several potential sulfur sources (Figure 3.15). Subsequently, we begin to elucidate the role of sulfur acquisition strategies in the context of gonococcal growth in liquid culture and epithelial cell infection.

Due to the extracellular environment being predominantly oxidizing, cysteine mainly exists as cystine, and as such, cystine acquisition is expected to be of significance importance to the pathogenesis of *N. gonorrhoeae* (Keevil et al., 1986). In agreement, the cystine substrate binding protein was previously shown to be upregulated upon adherence to epithelial cells, indicative of an important role

during colonization (Du & Arvidson, 2006). Herein, inhibiting cystine uptake via genetic deletion of the cystine substrate binding protein, Δ*tcyA*, resulted in a severe attenuated ability to adhere to and invade endocervical epithelial cells. These results further highlight that sulfur acquisition is essential not only for replication, but for survival as evident by extreme population decline. One caveat of this infection assay is that the exact sulfur composition of the infection media is unknown. It is therefore plausible that alternative sulfur metabolites are limiting, consequently artificially impairing the ability of Δ*tcyA* to survive. It remains to be assessed if supplementation of additional sulfur compounds could restore survival during the infection assays, as observed in the growth assays. However, it is not certain how additional supplementation would reflect the natural sulfur landscape *N. gonorrhoeae* encounters *in vivo*, as physiologically relevant concentrations and speciation of the sulfur metabolites within gonococcal niches are largely unknown. Nonetheless, these data support sulfur acquisition, particularly cystine, as a critical determinant for establishing infection under aerobic conditions. Furthermore, the experimental evidence presented here supports TcyABC as the single cystine uptake system in *N. gonorrhoeae*, consistent with genomic predictions, and in contrary to several other pathogens that employ multiple transporters to acquire exogenous cystine (Kim et al., 2012; Chonoles Imlay et al., 2015; Lensmire et al., 2020).

In contrast to cystine acquisition, the deletion of the cysteine transporter (*cjaA*) did not hinder gonococcal growth or infection outcomes under any of the conditions tested. This lack of phenotype possibly reflects limitations of the experimental conditions which is conducive to an oxidizing environment, under which cysteine is expected to be oxidized to cystine. These conditions would mask any contribution of cysteine acquisition to gonococcal fitness in the WT strain and render CjaA dispensable when cystine is accessible. Therefore, discerning the influence of CjaA-dependent cysteine transport during infection requires assessment under relevant conditions where cysteine is accessible.

Scenarios within the host in which cysteine is available to *N. gonorrhoeae* are not well-defined, owing to the highly dynamic nature of the host redox potential. Gonococci occupy multiple niches, which span a broad range of oxidation-

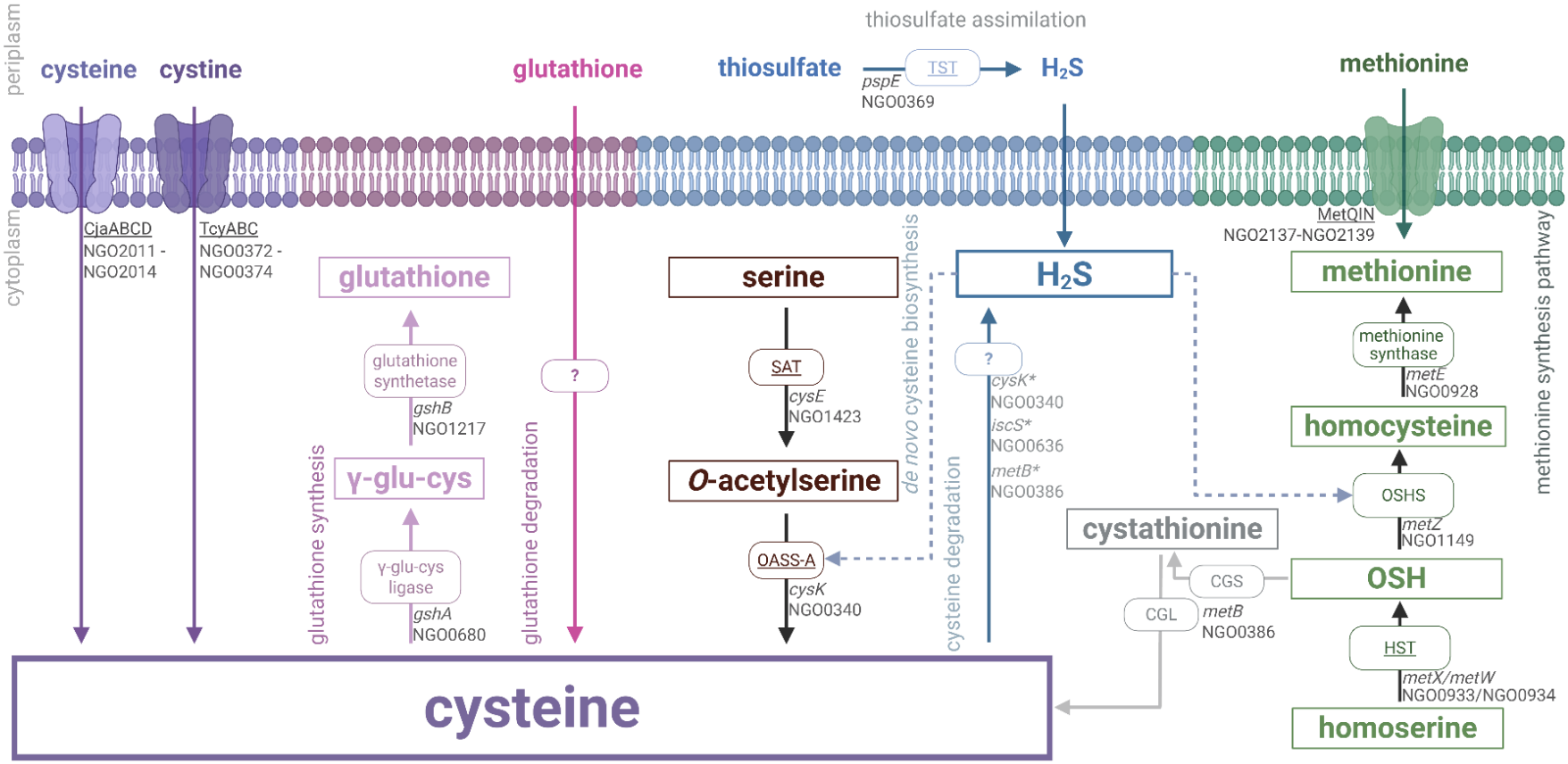


Figure 3.15. Overview of the putative sulfur metabolic network construction in *N. gonorrhoeae*. The sulfur metabolic network was constructed integrating growth experiments and literature based bioinformatic analysis. Enzymes underlined indicates where enzyme activity has been experimentally confirmed. Pathways in grey relate to the possible proteins encoded for by the *metB* gene. ? indicates that the genes/proteins involved are unidentified. * indicates candidate genes for reaction. Abbreviations: TST; thiosulfate sulfurtransferase, SAT; serine acetyltransferase, OASS-A; *O*-acetylserine sulfhydrylase, CGL; cystathionine γ lyase, CGS; cystathionine γ synthase, OSH; *O*-succinyl-L-homoserine, OSHS; *O*-succinylhomoserine sulfhydrylase, HST; homoserine *O*-succinyltransferases.

reduction potentials, under which cysteine speciation is likely to differ. For example, both the extracellular environment of the rectum and the host intracellular environment support reducing environments, favoring reduced cysteine (Ottaviano et al., 2008; Even et al., 2025). In contrast, during symptomatic infection of the male urogenital tract, an intense neutrophil infiltration is commonly observed, consequently generating a large degree of reactive oxygen species, conditions that would favor oxidation of cysteine to cystine (Smirnov et al., 2014; Walker et al., 2023). Furthermore, the redox potential within an anatomical site is also subject to fluctuations. This is exemplified within the female genital mucosa, where direct measurements of the vaginal redox potential demonstrates that redox potential can vary from moderately oxidizing to strongly reducing states, which is influenced by local microbial composition (Holmes et al., 1985). This redox heterogeneity encountered across and within gonococcal niches is expected to continuously influence cysteine speciation within the host and throughout gonococcal infection, possibly imposing selective pressure for retention of both high-affinity cysteine and cystine acquisition systems, even if individual transporters appear dispensable under laboratory conditions.

Irrespective of speciation, the import of exogenous cysteine and cystine likely constitutes an important sulfur acquisition strategy for *N. gonorrhoeae*. Notably, however, we demonstrate that *N. gonorrhoeae* is particularly sensitive to cyst(e)ine fluctuations, requiring a relatively high concentration of cyst(e)ine for optimal growth and exhibiting a low threshold for cyst(e)ine toxicity. Higher concentrations of cyst(e)ine were required to support equivalent growth when methionine was unavailable, 1 mM in cyst(e)ine only condition versus 0.65 mM in the GW medium which contained 0.1 mM methionine. As the proposed methionine synthesis pathway requires H_2S as the sulfur donor, rather than cysteine, this increased requirement for cysteine in the absence of methionine is feasibly explained by a metabolic bottleneck in acquiring H_2S from cysteine (Figure 3.16 a versus b). Consistently, we were unable to identify genes encoding dedicated cysteine catabolic enzymes. *N. gonorrhoeae* does encode several enzymes that have been shown in other organisms to possess cysteine desulfhydrase activity, as a secondary function, including CysK, IscS, and potentially the protein product of the *metB* gene, CGS or CGL (Awano et al., 2005; Wang et al., 2019; Pedretti et al., 2024). However,

cysteine degradation activity has not been studied for these gonococcal proteins, and therefore, the contribution of these proteins, if any, to cysteine degradation requires further investigation.

The sensitivity of *N. gonorrhoeae* to elevated cysteine concentrations is also consistent with absence of robust cysteine degradation enzymes. Although physiologically relevant concentrations of free cyst(e)ine across different host niches are largely unknown, limited reports of host cysteine levels indicate that typical cysteine concentrations are magnitudes lower than *N. gonorrhoeae* toxicity threshold (2 mM) within host environments (Stipanuk et al., 2006). While it is, therefore, unlikely that *N. gonorrhoeae* encounters lethal cysteine levels during natural infection, *N. gonorrhoeae* displays a reduced capacity to tolerate exogenous cysteine compared to gastrointestinal and respiratory pathogens, such as *S.* Typhimurium*, C. difficile, S. pneumoniae, M. pneumoniae*, and *H. pylori* (Lee et al., 2006; Afzal et al., 2016; Dubois et al., 2016; Großhennig et al., 2016; Díaz-Yáñez et al., 2020). These pathogens possess robust cysteine catabolic mechanisms, which become activated at physiologically relevant concentrations, and allow them to withstand exogenous cysteine concentrations 2 to 16-fold greater than *N. gonorrhoeae.* The elevated cysteine toxicity thresholds observed in these pathogens likely reflect pathogen-specific requirements shaped by niche adaptation and the exploitation of cysteine catabolism for virulence-associated functions. In *Salmonella*, cysteine degradation is important for intracellular survival in the gastrointestinal tract, where luminal and intracellular cysteine concentrations fluctuate widely in response to dietary intake, necessitating robust buffering and detoxification systems (Díaz-Yáñez et al., 2020). In other pathogens, such as *M. pneumoniae* and *H. pylori*, cysteine catabolism leads to the production of the volatile sulfur compound, H_2S, which is postulated to contribute to tissue damage and disease progression (H. Lee et al., 2006; Großhennig et al., 2016; Kawahara et al., 2020). These pathways, while primarily contributing to virulence, are likely to confer increased tolerance to exogenous cysteine. *N. gonorrhoeae*, in contrast, is not likely exposed to sudden fluctuations in cysteine concentrations, nor is *N. gonorrhoeae* known to produce volatile sulfur compounds, offering a potential explanation for the observed differences in cysteine tolerance and absence of robust cysteine degradation enzymes, between *N. gonorrhoeae* and these pathogens.

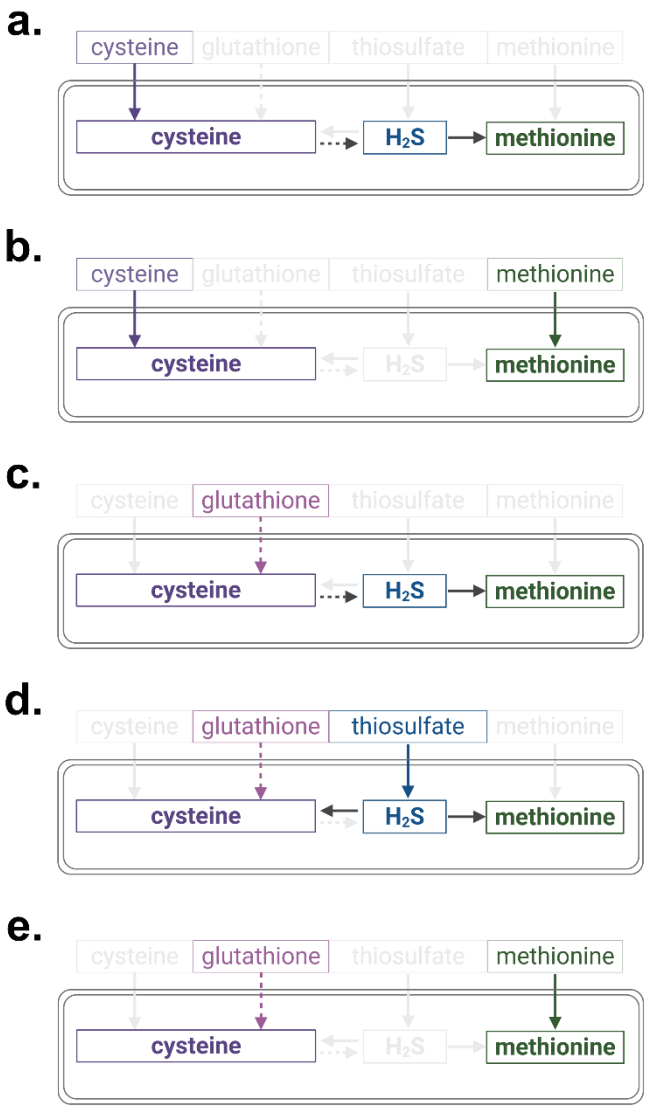


Figure 3.16. Schematic representation of how intracellular cysteine and methionine requirements are met under different growth conditions. Growth conditions represented are (a) cysteine as the sole sulfur source (b) cysteine and methionine (GW control) (c) glutathione as the sole sulfur source (d) thiosulfate and glutathione (e) methionine and glutathione. Dotted lines represent predicted metabolic constraints.

In the absence of cysteine, pathogens may assimilate inorganic sulfur compounds, sulfate and thiosulfate, for *de novo* cysteine biosynthesis, and/or recycle the organic sulfur compounds, methionine, glutathione, and taurine. Such metabolic flexibility is considered an important driver of pathogenic success and supports pathogens that navigate various niches with changing sulfur compositions. In contrast to some pathogens that harbor comprehensive sulfur metabolic networks, *N. gonorrhoeae* appears limited in its sulfur acquisition strategies, lacking the genes required to assimilate sulfate, recycle methionine, utilize taurine, or degrade glutathione (Table

3.1). Thus far, *N. gonorrhoeae* has been reported to use only thiosulfate as an alternative sulfur source (Le Faou, 1984). Herein, we expand on the known repertoire of sulfur acquisition strategies in *N. gonorrhoeae* and provide evidence that *N. gonorrhoeae* can proliferate when glutathione is supplied as the sole sulfur source in the absence of cysteine, despite the absence of canonical glutathione catabolizing enzymes. This outcome highlights gaps in our understanding of sulfur acquisition and demonstrates the limitations of relying solely on genomic inference in the absence of experimental validation.

Glutathione supported gonococcal growth in liquid culture in a concentration-dependent manner. However, at the highest concentration of glutathione tested, growth was significantly less efficient compared to cysteine and GW growth conditions, indicating that cysteine liberation from glutathione is a limiting factor for gonococcal growth *in vitro* (Figure 3.16 c). While glutathione utilization may not be highly efficient, this metabolic constraint may nevertheless confer a biological advantage by providing slow and steady, low level cysteine supply, preventing excessive intracellular cysteine accumulation, which given the absence of robust cysteine degradation pathways and observed cysteine sensitivity in *N. gonorrhoeae*, could mitigate cysteine induced toxicity and help manage intracellular redox potential. Interestingly, the range of glutathione concentrations required to support gonococcal growth is consistent with host intracellular concentrations, suggesting that glutathione may constitute an important sulfur source for *N. gonorrhoeae* during intracellular survival and replication, where cysteine concentrations are tightly regulated due to cytotoxicity (Guoyao et al., 2004; Stipanuk et al., 2006). Although it has been demonstrated that *H. pylori* accesses host intracellular glutathione pools to support extracellular growth and colonization (Baskerville et al., 2023), the inability of Δ*tcyA* mutants to survive when cultured with ME-180 cells is inconsistent with such a mechanism, suggesting that use of glutathione as a sulfur source might be most relevant in intracellular environments where glutathione is abundant.

In addition to utilizing cysteine and glutathione, *N. gonorrhoeae* has retained the necessary enzymes for *de novo* cysteine biosynthesis, CysE and CysK. Recent characterization of these proteins in *N. gonorrhoeae* supports the canonical functionality of these enzymes (Oldham et al., 2022; McGarvie et al., 2025). The

H_2S required for cysteine biosynthesis via CysK can freely diffuse across biological membranes; although it is usually acquired via the reduction of oxidized sulfur species, such as sulfate, as H_2S is both chemically unstable under oxidative environments and restricted within the host as it is highly toxic. *N. gonorrhoeae* is among several pathogens that have lost the capacity for sulfate assimilation but can grow when thiosulfate is provided as sole sulfur source (Le Faou, 1984; André et al., 2010; Lithgow et al., 2004; Vorwerk et al., 2014; Lensmire & Hammer, 2019; Berude et al., 2024), yet requires H_2S as the sulfur donor for cysteine biosynthesis via CysK. To date, the biochemical and genetic basis for this observation has remained mostly unresolved. One alternative thiosulfate assimilation pathway has been described in *E. coli* that might provide the missing link to how such pathogens acquire sulfide from thiosulfate; a sulfurtransferase-mediated thiosulfate reduction process resulting in reduced species, sulfite and sulfide. In *E. coli,* this is mediated by the thiosulfate sulfurtransferase, GlpE (Kawano et al., 2017, 2018).

Unexpectedly, thiosulfate did not support growth under the conditions tested, contrary to previous reports (Le Faou, 1984). Thiosulfate did, however, enhance growth when supplied in combination with glutathione, indicative of a synergistic relationship consistent with sulfurtransferase activity. The gonococcal sulfurtransferases, Str and PspE, both demonstrate thiosulfate reductase activity *in vitro*, reducing thiosulfate to H_2S in the presence of various thiol acceptor substrates, including glutathione. Despite this apparent catalytic redundancy, partial attenuation of growth under thiosulfate and glutathione supplementation was observed only in the Δ*pspE* mutants. We therefore propose a model in which thiosulfate assimilation in *N. gonorrhoeae* occurs, at least in part, in the periplasm mediated by PspE. We hypothesize that this pathway contributes to growth under these conditions by supplying H_2S required for cysteine biosynthesis, and potentially more broadly to sulfur metabolism, as H_2S is the predicted sulfur donor for methionine based on the pathway reconstruction presented here (Figure 3.15). Consistently, methionine supplementation also supports growth when provided in combination with glutathione (Figure 3.16 e). As methionine cannot be used as sulfur source, this indicates that cysteine and methionine impose independent constraints on growth. In this context, H_2S derived from thiosulfate could plausibly

alleviate both constraints by supplying H_2S upstream of cysteine and methionine biosynthesis, thus bypassing the need to derive H_2S indirectly from cysteine.

This PspE-mediated thiosulfate reduction proposed pathway contrasts with the thiosulfate assimilation pathway described in *E. coli*, where the cytoplasmic sulfurtransferase GlpE, which is homologous to Str, has been implicated in thiosulfate utilization (Kawano et al., 2017). One possible explanation for these observed differences is that the thiosulfate/sulfate uptake system in *N. gonorrhoeae* is compromised, necessitating periplasmic processing. As thiosulfate import is an ATP-dependent process, the pathway in *N. gonorrhoeae* presents the most efficient route for acquiring H_2S from thiosulfate. Notably, in both *N. gonorrhoeae* and *E. coli*, TST-mediated thiosulfate reduction only partially accounts for thiosulfate assimilation via non-canonical (CysM-independent) pathways, indicating that additional, sulfurtransferase-independent mechanisms likely operate in parallel in both organisms (Kawano et al., 2017).

The physiological relevance of thiosulfate as a sulfur source during gonococcal pathogenesis is uncertain. In the host, the majority of thiosulfate is generated endogenously through mitochondrial oxidation of toxic H_2S, a process that occurs primarily within metabolically active host cells (Kies & Hammer, 2022). This intracellular availability, together with the synergism observed with intracellularly relevant glutathione concentrations, is consistent with a potential role for TST-mediated thiosulfate utilization within intracellular niches. Alternatively, given the promiscuous nature of sulfurtransferases, it remains plausible that unidentified extracellular substrates could support PspE-mediated thiosulfate reduction in the absence of glutathione, although such substrates have not yet been described.

Regardless of the uncertainty surrounding thiosulfate availability during infection, Δ*pspE* exhibits attenuated adherence to and invasion of epithelial cells, indicating that PspE is important for optimal infection outcomes under the conditions tested. The loss of PspE was not functionally compensated by Str (or vice versa), supporting distinct or only partially overlapping roles for these sulfurtransferases during epithelial cell infection. Given that the TST are multifunctional proteins with proposed roles in sulfur metabolism, H_2S homeostasis, disulfide-bond formation, and iron–sulfur cluster formation and repair (Spallarossa et al., 2001; Bordo & Bork,

2002; Cheng et al., 2008; Chng et al., 2012; Yu et al., 2023), it cannot yet be determined whether the infection defects observed in Δ*pspE* reflect impaired sulfur acquisition or disruption of other processes essential for survival in host-associated environments. Although we do demonstrate that PspE produces H_2S from thiosulfate *in vitro* and is required for optimal growth under thiosulfate-supplemented conditions, together supporting a putative role in thiosulfate assimilation, direct evidence of PspE-produced H_2S during growth or infection is currently lacking. Consequently, whether the observed attenuation during infection reflects a reduced capacity for thiosulfate assimilation and subsequent sulfur limitation remains unresolved. Moreover, even if PspE-mediates H_2S production during infection, its downstream fate, whether channeled into synthesis of sulfur-containing amino acids or contributing to alternative physiological functions, would require further experimental validation.

The reconstructed sulfur metabolic network presented here demonstrates that sulfur acquisition in *N. gonorrhoeae* is more nuanced than what is implied by its prior classification as a cysteine auxotroph. The gonococcus relies on cysteine and cystine acquisition, and under conditions of cysteine limitation, glutathione and thiosulfate sulfur sources may be used. However, the restricted efficiency and synergetic requirements of these alternative pathways indicate that sulfur acquisition strategies might be limited and context dependent. This constrained sulfur metabolic capacity is curious given the relevance of cysteine limitation within the host and situates sulfur availability as a key determinant of gonococcal fitness during infection.

By defining the sulfur acquisition strategies available to *N. gonorrhoeae*, this provides the foundation under which their conditional relevance can begin to be investigated to understand both how sulfur acquisition promotes different stages of infection and to infer the sulfur metabolite landscape encountered across different host niches. Importantly, as a host-restricted pathogen, these findings highlight that apparent metabolic constraints may reflect pathoadaptive tuning to host environments, underscoring the need to consider environmental metabolite accessibility when evaluating sulfur metabolic pathways as potential therapeutic targets.

4 | Chapter Four

Transcriptional adaptations to cysteine limitation

Remarkably, *Neisseria gonorrhoeae* inhabits a wide range of host niches and survives hostile conditions during infection, colonizing anatomically distinct genital mucosa of men and women, in addition to alternative anatomical locations such as the nasopharynx, conjunctiva, and rectum (Miller, 2006). The nutrient landscapes within these niches are shaped by several host factors and resident microbiota and as such are expected to vary sustainably both within and between individuals (Edwards & Butler, 2011; Quillin et al., 2018). Moreover, *N. gonorrhoeae* invades and replicates in various intracellular environments including epithelial cells, macrophages, and neutrophils, environments that impose distinct metabolic constraints. The speciation and accessibility of sulfur metabolites encountered by the gonococcus is expected to vary as it transitions between these diverse environments during infection. However, despite this diversity of sulfur landscapes, in Chapter Three we determined that *N. gonorrhoeae* possesses a relatively limited sulfur metabolic repertoire. In addition to cysteine and cystine, *N. gonorrhoeae* was only capable of proliferating when glutathione was provided as the sole sulfur source, albeit at a reduced efficiency compared to growth on cysteine. We further demonstrated that although thiosulfate and methionine did not support growth alone, when paired with glutathione optimal growth was restored. However, mechanisms that enabled growth under these various, cysteine-independent, growth conditions were unable to be fully elucidated in the bioinformatic based reconstruction of the sulfur metabolic network in *N. gonorrhoeae.* Particularly, mechanisms that enable gonococcus to utilize glutathione as a sulfur source in the absence of cysteine, and whether thiosulfate and methionine facilitate growth by providing unique or distinct nutrient supplementation are not understood.

In this Chapter, we endeavored to apply an integrated multi-omics approach combining transcriptomics and metabolomics analyses across cysteine-independent conditions to investigate how proliferation is supported in the absence of cysteine. More broadly, we further sought to elucidate transcriptional changes that underpin adaptations to cysteine limitation, given the importance of this sulfur source for

gonococcal survival and the extreme relevance of cysteine limitation within host environments.

To this end, *N. gonorrhoeae* WT was cultured in four sulfur conditions: (1) C: cysteine only (1 mM), (2) G: glutathione only (5 mM), (3) TG: thiosulfate (2 mM) and glutathione (5 mM), and (4) MG: methionine (2 mM) and glutathione (5 mM). These concentrations were selected based on prior growth optimization work indicating that 1 mM cysteine supports robust gonococcal growth. Growth with 5 mM glutathione and 2 mM methionine or thiosulfate were chosen as they produced comparable growth rates to 1 mM cyst(e)ine. The glutathione only condition was maintained at 5 mM both to ensure consistency across comparisons, and as a significant reduction in growth occurred, to explore whether this metabolic constraint incurs additional stress responses or survival features resulting from sulfur-limited induced reduced growth. For these experiments, cultures were scaled up to generate sufficient biomass for RNA-seq and metabolite profiling. As a result, growth kinetics deviated slightly from those shown in Chapter Three, with faster doubling times, likely driven by increased aeration in these cultures. Cells were harvested at mid-late exponential phase (2.5 hr), and total RNA along with intracellular and extracellular metabolites was collected for downstream analysis. Growth curves corresponding to these cultures are shown in the Supplementary material 6.9.

However, due to limitations in the metabolomics data processing pipeline (later discussed in Section 4.5) the resulting metabolite profiles generated in this study could not be confidently interpreted. Accordingly, this chapter focuses on the transcriptomic analysis of gonococcal adaptation to different sulfur landscapes.

4.1 Global impact of sulfur sources on the transcriptional landscape

The global transcriptional impact of the different growth conditions on gene expression was assessed via principal component analysis (PCA), which demonstrated high reproducibility between biological replicates and revealed strong condition-dependent clustering, indicating that sulfur source drives distinct global transcriptional responses (Figure 4.1 a). PC1 accounted for 63% of total variation, with a further 20% explained by PC2. Across the first two principal

components, the four sulfur conditions are separated into three distinct clusters. Notably, the C and TG conditions clustered together. Conversely, the MG and G conditions form separate, non-overlapping clusters, indicative of transcriptional landscapes that are distinct from each other and from the TG/C cluster. The distinct clustering of the cysteine-independent growth conditions indicates that broadly similar transcriptional responses did not occur in the absence of cysteine, rather transcriptional responses varied depending on the alternative sulfur metabolites available. Furthermore, the close clustering of the TG and C conditions suggest that in the absence of cysteine, thiosulfate and glutathione may fulfil sulfur requirements in a manner that reflects cysteine acquisition.

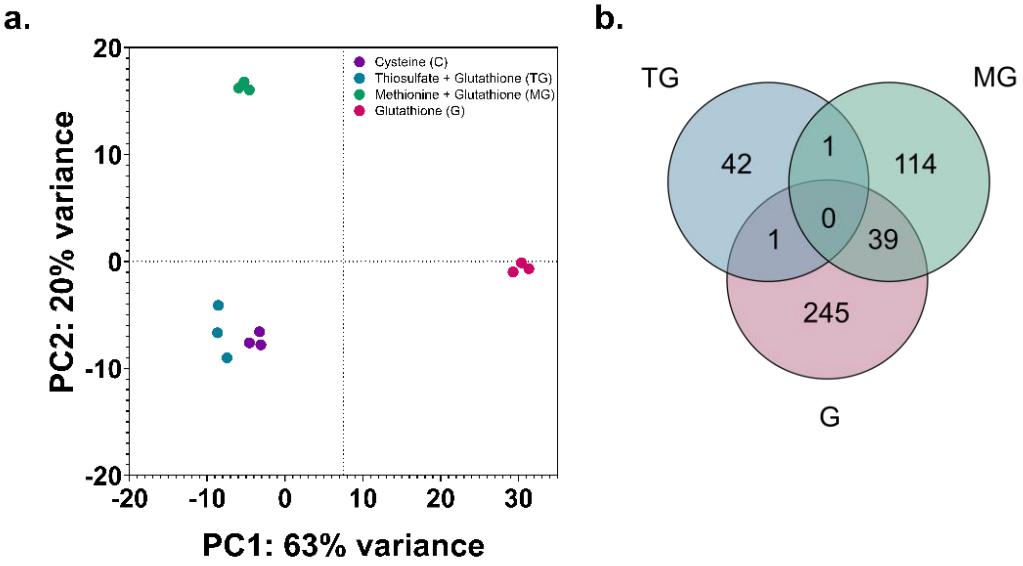


Figure 4.1. Magnitude of transcriptional changes in response to sulfur source. (a) PCA plot of global transcriptional responses to cysteine (C, purple), thiosulfate and glutathione (TG, blue), methionine and glutathione (MG, green) and glutathione only (G, pink). Data represents three biological replicates. (b) Venn diagram of differentially expressed genes (compared to cysteine as the control) concordantly expressed between the cysteine-independent growth conditions.

Consistent with this clustering, compared to the cysteine only control, relatively few differentially expressed genes (Log2 fold-change (L2FC) > 1, p_{adj} value < 0.05) were identified in the TG condition, whereas growth with MG and G incurred substantially more differentially expressed genes. Moreover, there was minimal overlap of genes concordantly differentially expressed between the conditions, with most of this overlap occurring between MG and G (Figure 4.1 b). In contrast, TG shared minimal overlap with the other cysteine-independent growth conditions.

Surprisingly, none of the differentially expressed genes exhibited conserved expression patterns across all three cysteine-independent conditions, indicating that transcriptional responses to cysteine limitations are not uniform. Together, these results suggest that in the absence of cysteine, the broader sulfur landscape encountered influences transcriptional responses.

4.2 Regulation of genes involved in sulfur metabolism

Next, we examined the differentially expressed genes involved in sulfur metabolism of the cysteine-independent growth conditions to assess how sulfur requirements are fulfilled in the absence of cysteine. As expected, several genes involved in sulfur metabolism were differentially expressed across the different sulfur conditions (Table 4.1). Notably, expression of the cystine ABC transporter (*tcyABC*), sulfate ABC transporter (*sbp, cysATW*) and the cysteine synthesis enzyme OASS (*cysK*) were induced in the MG and G conditions. This transcriptional response is reminiscent of a classical sulfur starvation response observed in many other pathogens and suggests the gonococcus attempts to overcome sulfur limitations by increasing cystine uptake and cysteine biosynthesis (Guédon & Martin-Verstraete, 2007; André et al., 2010; Afzal et al., 2016). Although we observe an upregulation of the sulfate transport system, the *sbp* gene, responsible for sulfate recognition and binding, and the permease subunit, *cysT*, are both interrupted by a frameshift mutation and are predicted to be pseudogenes (Hicks & Mullholland, 2018). Furthermore, experimental evidence presented in Chapter Three confirms the gonococcus cannot use sulfate as sulfur source. As such, the upregulation of these genes can be attributed to existing regulatory mechanisms of sulfate acquisition, despite this pathway being no longer functional in *N. gonorrhoeae*.

Table 4.1. Sulfur metabolism genes differentially expressed under cysteine limitation.

Locus Tag				L2FC		
FA19	**FA 1090**	**Gene**	**Gene product**	**TS**	**MG**	**G**
VT05_RS00420	NGO0340	*cysK*	cysteine synthase A	-2.99	2.09	2.75
VT05_RS00585	NGO0372	*tcyA*	amino acid ABC transporter substrate-binding protein	ns	2.25	2.00
VT05_RS00590	NGO0373	*tcyB*	amino acid ABC transporter permease	ns	2.09	1.83
VT05_RS00595	NGO0374	*tcyC*	amino acid ABC transporter ATP-binding protein	ns	1.96	1.74
VT05_RS00980	NGO0445	*cysA*	sulfate/molybdate ABC transporter ATP-binding protein	ns	2.06	2.24
VT05_RS00990	NGO02385	*cysT*	sulfate ABC transporter permease subunit (pseudogene)	ns	1.83	1.92
VT05_RS00985	NGO0446	*cysW*	sulfate ABC transporter permease subunit	-1.36	1.76	2.04
VT05_RS03480	NGO04615	*sbp*	sulfate ABC transporter substrate-binding protein (pseudogene)	ns	1.96	2.23
VT05_RS09695	NGO2011	*cjaD*	amino acid ABC transporter permease	-1.36	1.28	ns
VT05_RS09700	NGO2012	*cjaC*	amino acid ABC transporter permease	-1.58	1.91	ns
VT05_RS09705	NGO2013	*cjaB*	amino acid ABC transporter ATP-binding protein	-1.58	2.28	ns
VT05_RS09710	NGO2014	*cjaA*	amino acid ABC transporter substrate-binding protein	-1.27	2.35	ns

Genes differentially expressed are defined as L2FC >1, padj < 0.05 as determined by DESeq2 analysis. L2FC values are relative to cysteine-only control; positive values indicate higher expression under test conditions. Locus tags for N. gonorrhoeae FA19 are provided alongside corresponding FA1090 locus tags. ns; indicates not significantly different compared to cysteine-only control.

Given this canonical response to sulfur starvation in the MG and G conditions, here forth we recognize MG and G as conditions of 'sulfur starvation'. Conversely, the TG condition did not elicit a similar sulfur starvation response, indicating that in the absence of cysteine, thiosulfate and glutathione as alternative sulfur sources are sufficient to satisfy cellular sulfur demands, further supporting the notion that cysteine auxotrophy in *N. gonorrhoeae* better reflects a conditional dependence on cysteine, rather than an absolute requirement for exogenous cysteine specifically.

Unexpectedly, several genes involved in sulfur acquisition and assimilation demonstrated expression patterns across the cysteine-independent growth conditions that are not explained by a response to sulfur starvation, including the cysteine biosynthesis gene, *cysK* and cysteine transporter (*cjaABCD*). Although the upregulation of *cysK* under MG and G is consistent with sulfur starvation, we surprisingly observe an approximate eight-fold reduction in *cysK* expression in the TG condition. Despite not inducing a sulfur starvation response, this severe downregulation was unexpected, as it was anticipated that *de novo* cysteine biosynthesis would play a substantial role in the growth of *N. gonorrhoeae* in this condition based on the hypothesis that thiosulfate would be reduced to H_2S and subsequently incorporated into cysteine by the enzymatic action of CysK.

In addition to cysteine biosynthesis, CysK is known to possess several moonlighting functions, including cysteine catabolism, antibiotic resistance, regulation, biofilm formation, and contact dependent inhibition mechanisms (Campanini, Benoni, et al., 2015). In several pathogens, CysK has been implicated in contributing to cysteine detoxification under conditions of cysteine excess (Awano et al., 2005; Dubois et al., 2016; Gu et al., 2018). As cysteine is present in excess in the control condition (C), it is possible that the expression of *cysK* may be elevated to contribute to mechanisms of cysteine degradation, thereby inflating baseline expression levels. Consequently, the apparent downregulation of *cysK* in the TG grown gonococci could reflect a reduced requirement for cysteine detoxification in the absence of exogenous cysteine. In contrast, the upregulation of *cysK* in MG and G remains consistent with sulfur-limiting conditions, a response that is notably absent in TG (Figure 4.2 a).

Adding weight to this hypothesis is the absence of canonical cysteine degradation pathways in *N. gonorrhoeae* (as highlighted in Chapter Three), with *cysK* being among few genes identified with possible cysteine degradation capabilities. Furthermore, similar observations of increased expression of *cysK*, and *cysE*, expression in response to cysteine is observed in *C. difficile* (Dubois et al., 2016).

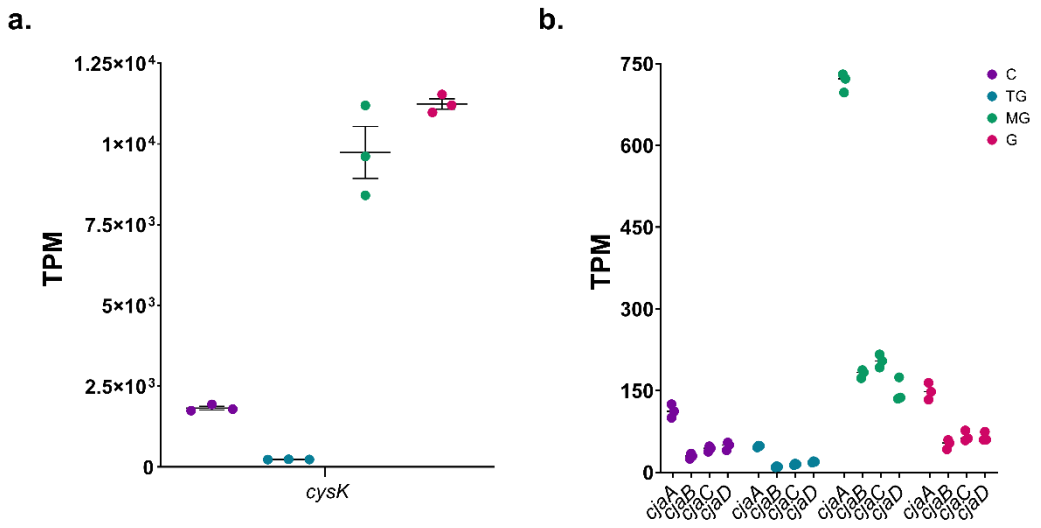


Figure 4.2. Normalized expression counts (TPM) of (a) *cysK* and (b) *cjaABCD* transcripts across the different sulfur conditions; cysteine (purple), TG (blue), MG (green), and G (pink). Data represents mean ± SEM of 3 biological replicates.

In contrast to this study, we do not observe the concordant regulation of *cysK* and *cysE*. However, *cysE* in *N. gonorrhoeae* is constitutively expressed (Remmele et al., 2014). While the exact role of CysK in cysteine homeostasis, whether contributing to cysteine synthesis and/or degradation, and contexts under which these divergent functions may be activated, requires further experimental validation, these results nonetheless demonstrate that expression of *cysK* is sensitive to fluxes in sulfur availability.

The cysteine transporter (*cjaABCD*) also demonstrated a distinct expression pattern inconsistent with sulfur starvation across the cysteine-independent conditions. Transcription of *cjaABCD* was induced in the MG condition, repressed in the presence of TG, and was not differentially regulated in glutathione only, indicating that *cjaABCD* is not regulated as part of a canonical sulfur-limitation response in *N. gonorrhoeae* (Figure 4.2 b). Compared to our understanding of the regulation of other genes involved in sulfur acquisition, much less is known regarding the regulation of *cjaABCD*, owing in part to the rare occurrence of this transporter

among pathogens. However, few transcriptomic studies conducted in other pathogens have demonstrated the upregulation of the cysteine transporter in response to acidic conditions in *H. pylori*, and, in contrast to our observations, sulfur starvation in *S. pneumoniae* (Merrell et al., 2003; Afzal et al., 2016). Although it is worth noting that the *cjaA* homolog in *S. pneumoniae* is truncated, with unknown consequences.

Although *N. gonorrhoeae* encodes the canonical sulfur-responsive transcriptional regulator, *cysB*, commonly found in Gram-negative bacteria, the sulfur regulon controlled by CysB is currently undefined. Therefore, it is unknown if *cjaABCD* is regulated in response to cysteine limitation in *N. gonorrhoeae*, as the other sulfur acquisition genes appear to be, however this currently appears unlikely. Regardless of CysB-mediated regulation, *cjaABCD* in *N. gonorrhoeae* is repressed by the global regulator, MtrR, which has recently shown to be modulated by host steroid hormones (Folster et al., 2009; Hooks et al., 2024). As no other sulfur acquisition genes are regulated by MtrR, this difference in transcriptional regulation may explain the incongruent expression of *cjaABCD* under these conditions, although the physiological consequences behind the differential expression of the cysteine transporter under MG and TG conditions remains unknown.

4.3 Differentially expressed genes in response to sulfur starvation

To further understand the broader effect of cysteine-limitation on the regulation of gene expression in *N. gonorrhoeae*, we examined the transcriptional changes associated with sulfur starvation. As growth in the MG and G conditions both elicited a sulfur starvation response in *N. gonorrhoeae*, while displaying different growth kinetics, the genes concordantly expressed between these two conditions are considered associated with sulfur starvation (Table 4.2).

In addition to those involved in sulfur metabolism discussed above, a further 30 genes were identified as differentially expressed in response to sulfur starvation. Among these, several intriguing links between sulfur starvation and processes involving one carbon metabolism, transport, translation, redox homeostasis, and competence are notable.

4.3.1 Methionine synthesis and one carbon metabolism

An interesting observation in the transcriptomic response to sulfur limitation is the concordant downregulation of *metF* and *metE*. MetF catalyzes the NAD(P)H-dependent conversion of 5,10-methylene-tetrahydrofolate (THF) to 5-methyl-THF, thereby committing one-carbon units to methionine biosynthesis. MetE subsequently consumes 5-methyl-THF to convert homocysteine to methionine in the terminal step of methionine biosynthesis. Interestingly, *metE* is downregulated under sulfur limitation, despite contrasting exogenous methionine availability between the two conditions, methionine abundance (MG) and absence of exogenous methionine (G), indicating that the regulation of *metE* is not driven by exogenous methionine availability.

In bacteria, expression of *metE* is typically under the control of the repressor and/or activator proteins, MetJ and MetR, respectively. MetJ functions as a global repressor of the methionine biosynthesis pathway (except for *metH* if applicable), whereas MetR regulates only the terminal step pertaining to expression of *metE* and/or *metH* (Weissbach & Brot, 1991; Leyn et al., 2014). Consistent with the distribution of MetJ and MetR across proteobacteria, *N. gonorrhoeae* does not encode a *metJ* homolog but does encode a *metR* homolog that is highly conserved with the MetR protein in *N. meningitidis* (Leyn et al., 2014).

MetR belongs to the LysR-type transcriptional regulators and activates gene expression in response to intracellular homocysteine concentrations. The core MetR regulon comprises only *metR* and *metE,* while additional genes vary considerably between taxa (Leyn et al., 2014). Given the concordant downregulation of *metE* and *metF* and the close genomic proximity of these genes within *N. gonorrhoeae*, it is interesting to note that *metF* is a well-conserved gene regulated by MetR (Leyn et al., 2014). However, as MetR responsive genes have not been characterized in *N. gonorrhoeae* or related species, it remains unclear whether *metE* and *metF* are co-regulated in *N. gonorrhoeae.* Nevertheless, the repression of *metE* under sulfur limitation is plausibly explained by reduced intracellular homocysteine resulting from a reduction in sulfur availability and consequently flux through this pathway.

As MetF and MetE sit at the metabolic intersection between the folate and methionine cycles, their coordinated repression may signify broader perturbations

in folate-dependent one carbon metabolism, reducing the commitment of one-carbon units towards methionine biosynthesis. However, whether this reflects redistribution of one-carbon units within one carbon metabolism following reduced methionine biosynthesis or a decreased demand for downstream metabolites, such as SAM remains unclear. Furthermore, as many folate-mediated reactions are dependent on NAD^+/NADH balance, perturbations in methionine biosynthesis under sulfur limitations may have broader implications for cellular redox homeostasis, although the direction and extent of this would require further investigation (Shetty & Varshney, 2020).

4.3.2 Transporters

Several genes encoding diverse transport systems were differentially expressed under sulfur starvation. Notably, these included the efflux transporter *mtrF*, the metal-associated transport genes *mntC* and *tdfJ*, and NGO16850, encoding a queuosine precursor transporter.

mtrF encodes an antibiotic efflux pump best known for mediating resistance to sulfonamide antimetabolites that target one carbon metabolism. Beyond antibiotic resistance, MtrF exports endogenous metabolites such as *p*-aminobenzoic acid, a key precursor for folate biosynthesis (Shafer et al., 2016). This upregulation of *mrtF* therefore aligns with the previously observed perturbations in one carbon metabolism, rather than functions relating to antibiotic stress, given the context of the experimental conditions herein (Folster & Shafer, 2005; Su et al., 2015; Shafer et al., 2016).

In parallel, genes involved in the acquisition of manganese (*mntC*) and zinc (*mntC* and *tdfJ*), were downregulated in response to sulfur limitation, indicating a reduced requirement for specific transition metals. While sulfur metabolism is inherently linked to iron homeostasis due to the prominent role of iron-sulfur clusters in cellular functions, the impact of sulfur on zinc and manganese homeostasis is less intuitive. In *N. gonorrhoeae, mntC* and *tdfJ* are repressed by peroxide responsive transcriptional regulator, PerR, linking the expression of these genes to metal-dependent oxidative stress defense mechanisms (Seib et al., 2006). Therefore, the repression of these genes under sulfur limitation might reflect a state of decreased oxidative stress compared to that encountered in the cysteine-only control.

Finally, sulfur starvation was accompanied by upregulation of NGO1685, which encodes a queuosine (Q) precursor transporter. NGO01685 was upregulated alongside the Q biosynthesis gene *queF*, indicative of increased Q biosynthesis (Díaz-Rullo & González-Pastor, 2023). Q is a modification of the wobble base in tRNAs that decode NA(C/U) codons, which functions to regulate translation via influencing translational efficiency and fidelity. Q based modifications in pathogens are beginning to be elucidated as contributing to important regulatory mechanisms for virulence (Díaz-Rullo & González-Pastor, 2023). Interestingly, a recent publication in *S. mutans* demonstrated that Q biosynthesis is dramatically influenced by nutrient availability, although the exact nutritional cues responsible remains unidentified (Jaroch et al., 2025). The upregulation of Q biosynthesis genes under conditions of sulfur starvation herein raises the possibility that sulfur, or sulfur-containing compounds, might be involved.

4.3.3 Translation

Several genes encoding translational proteins were downregulated including translation initiation factor-3 (*infc*), genes involved in ribosomal biogenesis and RNA processing (*era* and *rnc*) and 5S ribosomal proteins (*rpmE* and *rpmJ*). Expression of translational proteins are influenced by growth rates and metabolic states, with faster growth rates and nutrient replete states supporting greater abundances of translational machinery (Schmidt et al., 2016). As the downregulation of these genes occurs independent of growth states, this suggests that sulfur limitation might impose metabolic constraints that alters resource allocation, potentially away from translations processes (Hidalgo & Utrilla, 2020).

4.3.4 Stress responses and redox homeostasis

Given the central role of sulfur in redox homeostasis and oxidative stress response, it is somewhat surprising that few genes involved in stress responses were differentially regulated under sulfur limitation, none of which have been functionally characterized in *N. gonorrhoeae*. Expression of *adhP,* another PerR responsive gene, encoding an alcohol dehydrogenase was significantly induced under sulfur starvation. AdhP has been implicated in mitigating oxidative stress potentially through the modulation of NADPH/$NADP^+$ pools, suggesting a possible link between sulfur starvation and alterations in intracellular redox (Briolat &

Reysset, 2002; Echave et al., 2003). In contrast, other genes typically associated with oxidative stress and thiol protection, *mdaB* and NGO0057, encoding a NAD(P)H-dependent oxidoreductase and protein disulfide oxidoreductase respectively, were repressed under sulfur starvation (Du et al., 2005). Notably, these transcriptional responses occurred in the absence of a broader canonical oxidative

Table 4.2. Genes concordantly differentially expressed in response to sulfur starvation conditions, MG and G.

Locus Tag				L2FC	
FA19	**FA 1090**	**Gene**	**Gene product**	**MG**	**G**
Amino acid biosynthesis					
VT05_RS03315	NGO0844	*argB*	acetylglutamate kinase	1.01	1.06
VT05_RS03735	NGO0928	*metE*	5-methyltetrahydropteroyltriglutamate-homocysteine *S*-methyltransferase	-1.94	-1.32
Cofactor & vitamin biosynthesis					
VT05_RS07790	NGO1684	*queF*	preQ(1) synthase	2.11	2.91
VT05_RS03740	NGO0929	*metF*	methylenetetrahydrofolate reductase	-2.58	-1.42
Translation					
VT05_RS12050	NGO0260	*era*	GTPase Era	-1.27	-1.32
VT05_RS12045	NGO0259	*rnc*	ribonuclease III	-1.79	-1.12
VT05_RS00190	NGO0296	*infC*	translation initiation factor IF-3	-1.25	-1.17
VT05_RS03745	NGO0930	*rpmE*	type B 5S ribosomal protein L31	-1.32	-1.25
VT05_RS03750	NGO0931	*rpmJ*	type B 5S ribosomal protein L36	-1.27	-1.42
DNA replication/modification/recombination/repair					
VT05_RS00555	NGO0367	*radA*	DNA repair protein	1.01	1.02
VT05_RS11085	NGO0078	*dnaE*	DNA polymerase III subunit alpha	-1.01	-1.06
Transport					
VT05_RS07795	NGO1685		queuosine precursor transporter	2.22	2.76
VT05_RS05965	NGO1368	*mtrF*	AbgT family antimetabolite efflux transporter	2.17	2.24
VT05_RS10990	NGO00320	*eamA*	EamA family transporter (pseudogene)	1.67	1.12
VT05_RS08225	NGO09360		MFS transporter	1.44	1.34
VT05_RS05895	NGO1355		sodium-dependent transporter	-1.08	-3.04
VT05_RS11570	NGO0168	*mntC*	metal ABC transporter solute-binding protein, Zn/Mn family	-1.82	-1.27
VT05_RS05075	NGO1205	*tdfJ*	S100A7-binding protein	-2.72	-1.88
Competence					
VT05_RS04910	NGO1177	*comP*	DNA-binding type IV pilus protein	1.12	1.47

VT05_RS04915	NGO1178	*comE1*	DNA-binding competence protein	1.14	1.34
VT05_RS05605	NGO1304	*comE2*	DNA-binding competence protein	1.22	1.08
VT05_RS09045	NGO1902	*comE3*	DNA-binding competence protein	1.22	1.10
VT05_RS07870	NGO09005	*comE4*	DNA-binding competence protein	1.27	1.17
Redox homeostasis					
VT05_RS06395	NGO1442	*adhP*	alcohol dehydrogenase	3.15	1.97
VT05_RS06530	NGO1473	*mdaB*	NAD(P)H-dependent oxidoreductase	-4.54	-3.50
VT05_RS10965	NGO0057		protein disulfide oxidoreductase	-1.72	-1.03
Protein folding					
VT05_RS06295	NGO1422	*grpE*	nucleotide exchange factor	1.25	1.78
Uncharacterized & conserved family domain proteins					
VT05_RS10780	NGO0022		cupin domain-containing protein	1.43	1.71
VT05_RS04365	NGO1049		DUF4198 domain-containing protein	-2.04	-1.58
VT05_RS11560	NGO0166		Meta-pathway of phenol degradation family protein	-1.99	-1.05
Hypothetical					
VT05_RS02410				1.11	1.13

Genes differentially expressed are defined as L2FC >1, padj < 0.05 as determined by DESeq2 analysis. L2FC values are relative to cysteine-only control; positive values indicate higher expression under test conditions. Locus tags for N. gonorrhoeae FA19 are provided alongside corresponding FA 1090 identifiers where available.

stress responses (Quillin et al., 2018), suggesting that sulfur limitation in *N. gonorrhoeae* may primarily perturb intracellular redox balance.

4.3.5 Competence genes

An unexpected observation the in transcriptomic response to sulfur limitation was the upregulation of five competence-associated genes, *comP* and *comE* 1 – 4. These proteins are essential for natural transformation in *N. gonorrhoeae* and act in conjunction with the T4P machinery, while remaining functionally independent of T4P biogenesis and canonical pilus-associated behaviors, such as motility and adhesion (Wolfgang et al., 1999; I. Chen & Gotschlich, 2001). ComE proteins bind incoming extracellular DNA in the periplasm following pilus-mediated uptake. This facilitates the stabilization and transfer of incoming DNA into a DNase-resistant compartment during transformation. ComP is a minor type IV pilin that functions as a species-specific DNA uptake sequence (DUS) receptor within the T4P, preferentially binding DUS-containing DNA and thereby promoting efficient and selective DNA capture during pilus retraction (Berry et al., 2013).

Regulation of competence among naturally transformable bacteria is highly variable and species-specific, with environmental stresses such as DNA damage, nutrient limitation, starvation, and high population density reported to be common triggers for expression of competence systems (Vesel et al., 2025). In *N. gonorrhoeae*, competence is unusual in that it is considered constitutive and is maintained throughout all phases of growth (Hamilton & Dillard, 2006). The coordinated upregulation of *comP* and all *comE* across both conditions suggests that specific DNA-uptake components can be subject to transcriptional regulation under specific conditions, potentially functioning to fine-tune DNA uptake efficiency and bias uptake toward homologous DUS containing DNA sequences.

How this modulation might enhance gonococcal survival under sulfur limitation remains unclear, and it is further uncertain whether this reflects a direct response to sulfur starvation or secondary stress associated with sulfur limitation. DNA uptake systems have been linked to several survival functions beyond homologous recombination, including nutrient scavenging and genome maintenance and repair (Vesel et al., 2025). The nutrient scavenging hypothesis is based on observations that starvation can induce competence in some species and that extracellular DNA

represents a potential source of carbon, nitrogen, and phosphorus (Finkel & Kolter, 2001; Vesel et al., 2025). However, this is largely debated due to the high energetic cost and mechanistic complexity of DNA uptake, as well as the presence of dedicated proteins, such as ComE, which function to stabilize incoming DNA and promote entry into a DNase-resistant state, which would conflict catabolic mechanisms required for nutrient scavenging.

Alternatively, competence may enhance survival by facilitating genome maintenance by increasing access to homologous DNA templates that could repair or replace damaged genetic material. Such a role would be consistent with selective upregulation of DNA uptake components, particularly *comP* which preferentially recognizes DNA containing the *Neisseria* species DUS (Berry et al., 2013). Consistent with this hypothesis, expression of DNA repair gene, *radA* is also upregulated under sulfur limitation. In *N. gonorrhoeae*, *radA* is predicted to be essential and is highly expressed; however, its specific function has not been experimentally characterized (Remmele et al., 2014). In *S. pneumoniae*, RadA supports homologous recombination by facilitating RecA-driven single-stranded DNA recombination over extended genetic distances (Marie et al., 2017). The gonococcal RadA homolog shares approximately 47% protein similarity with its pneumococcal counterpart, suggesting potential functional conservation. Given that gonococcal RecA operates in concert with multiple accessory factors to mediate DNA repair, natural transformation, and pilus antigenic variation (Mehr & Seifert, 2002), the concurrent upregulation of *comP*, *comE* genes, and *radA* may reflect a coordinated response that enhances genome maintenance capacity under sulfur-limited conditions.

4.4 Condition-specific transcriptional responses to cysteine-independent growth conditions

In the absence of cysteine, broadly conserved transcriptional response across TG, MG, and G were not observed. Instead, overlap of differentially expressed genes was minimal, leaving most transcriptional responses unique to the individual conditions. We therefore focused next on differentially expressed genes to define the transcriptional responses distinguishing TG, MG, and G.

4.4.1 Transcriptional response to thiosulfate and glutathione

The TG grown gonococci exhibit the least transcriptional changes among the cysteine-independent growth conditions, closely resembling the cysteine transcriptome offering further support that thiosulfate (in the presence of glutathione) may compensate for cysteine limitation and fulfil sulfur requirements in a comparable manner to cysteine. We originally hypothesized that this is achieved through the cysteine biosynthesis pathway, in which H_2S is derived from reducing thiosulfate (Figure 4.3 a). However, the transcriptomic results revealed downregulation of cysteine biosynthesis gene, *cysK*, in the TG condition. While this is plausibly explained by alternative moonlighting functions of the CysK, these observations warrant further investigation into the function of CysK, and more broadly cysteine biosynthesis, for gonococcal growth in the presence of exogenous thiosulfate.

Overall, the transcriptome of the TG grown gonococci was broadly similar to cysteine grown gonococci, with a total of 44 genes differently expressed, 42 of which were unique to this condition (Figure 4.3 b). Most of these genes were downregulated and are located to prophage islands in the genome (Geslewitz & Seifert, 2025), in addition to two genes relating to transport and one hypothetical gene. Only four genes were identified as being upregulated in the thiosulfate and glutathione condition; *glnD*, *pykA*, and two genes involved in transcriptional regulation, *nmlR* and NGO0797.

NmlR is a MerR-like regulator with postulated involvement in the defense against disulfide stress in *N. gonorrhoeae* (Jackson et al., 2010; Kidd et al., 2005). NmlR regulates itself and three other genes in *N. gonorrhoeae*; (i) a glutathione-dependent dehydrogenase, *adhC* that plays a role in protection against nitric oxide stress in other organisms (Seib et al., 2006), however is disrupted by a frameshift mutation in *N. gonorrhoeae*, (ii) aCPx-type ATPase, *copA*, involved in efflux of copper ions linked to protection against nitrosative stress (Djoko et al., 2012) and (iii) a thioredoxin reductase, *trxB* that confers protection against oxidative or disulfide stress in other pathogens (Ballal & Manna, 2010; Higgs et al., 2025). However, the transcription levels of these NmlR-regulated genes were not significantly altered compared to cysteine grown gonococci.

The other transcriptional regulator upregulated in this condition, NGO0797, is uncharacterized in *N. gonorrhoeae* and located within an operon alongside *glnD*, which was also upregulated. *glnD* encodes the nitrogen responsive regulatory protein, uridylyltransferase, involved in the post-translational modification of glutamine synthesis, hinting at subtle changes in nitrogen metabolism in the presence of thiosulfate (van Heeswijk et al., 1993).

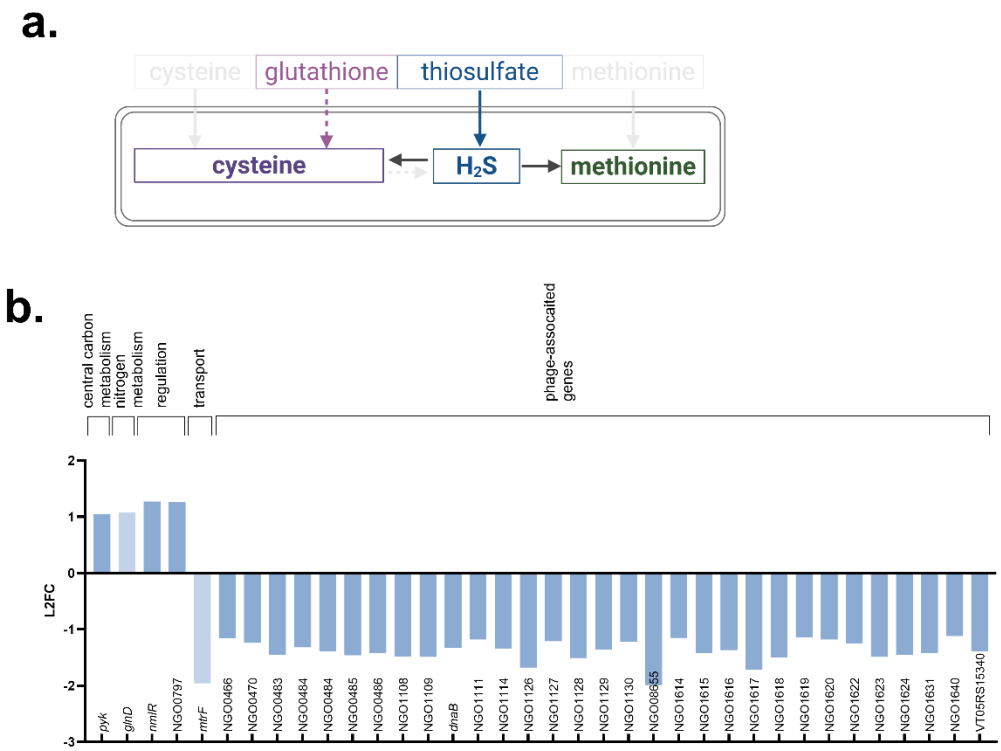


Figure 4.3. Transcriptional responses unique to thiosulfate and glutathione grown gonococci (a) schematic representation of proposed fulfilment of cysteine and methionine requirements during growth in the presence of thiosulfate and glutathione. Dotted lines represent proposed metabolic constraints. (b) Condition-specific differentially expressed genes (L2FC >1, $p_{adj} < 0.05$) as determined DESeq2 analysis.

Lastly, *pyk*, encoding pyruvate kinase, was upregulated in TG grown cells. Pyruvate kinase catalyzes the final step of the glycolysis pathway, converting phosphoenolpyruvate (PEP) into pyruvate and consequently producing ATP. As discussed, we hypothesize altered levels of H_2S in this condition, which is a known inhibitor of aerobic respiration (Borisov & Forte, 2021). In this context, *pyk* could be upregulated to compensate for loss of ATP generation (Borisov & Forte, 2021). However, it would be paramount to this interpretation to examine the production of H_2S in this condition.

4.4.2 Transcriptional responses to methionine and glutathione

As established in Chapter Three, exogenous methionine is not essential for gonococcal growth, nor can methionine be utilized as a sulfur source. We postulated that the restoration of growth by methionine when supplemented with glutathione reflects the alleviation of methionine biosynthesis, which required sulfur derived from glutathione through cysteine and H_2S intermediates (Figure 4.4 a). Although the MG condition differs from the cysteine control with respect to both sulfur availability and the presence of exogenous methionine, transcriptional responses attributable to sulfur limitation were identified as genes co-expressed between the MG and G conditions and are not considered further here. To isolate transcriptional effects associated with methionine availability from those driven by sulfur limitation, we examined the 114 genes uniquely differentially expressed in the MG (Figure 4.4 b). Compared to the cysteine-only control, in which exogenous methionine was unavailable, the addition of methionine to the culture medium resulted in notable changes in the expression of genes involved in core metabolic processes, the translational machinery, and interbacterial antagonism. These are discussed further below, following commentary on the impact of exogenous methionine on the regulation of the methionine biosynthesis pathway.

4.4.2.1 Methionine biosynthesis and once carbon metabolism

The methionine biosynthesis genes have been previously identified as non-essential but highly expressed under normal growth conditions (Remmele et al., 2014). Curiously, the genes involved in methionine synthesis (*metX, metW,* and *metZ*) or uptake were not differentially expressed in response to excess exogenous methionine availability, with normalized expression levels (TPM) remaining similar across the various conditions (Figure 4.4 c). As previously discussed, *metE*, was identified as being down regulated under sulfur limitation, irrespective of exogenous methionine. This suggests that methionine biosynthesis *N. gonorrhoeae* is controlled at the terminal step of the pathway, presumably due to the absence of MetJ regulatory programming.

Consistent with the concurrent downregulation of *metE* alongside *metF*, MG grown gonococci further exhibit changes in the expression of genes involved in one carbon metabolism including the upregulation of *folD* and *gcvH*, further supporting the

notion that availability of sulfur metabolites influences flux through one carbon metabolism.

4.4.2.2 Central carbon metabolism and amino acid biosynthesis

In the MG condition, several genes involved in central carbon metabolism were upregulated, including genes involved in the TCA cycle (*gltA, sucD, and mqo*), glycolysis (*gap* and *pgk*), and the pentose phosphate shunt (*tal*). Under glucose-replete, aerobic growth conditions, such as those here, *N. gonorrhoeae* exhibits a bifurcate metabolism, in which only a minor fraction of acetyl-CoA derived from glucose catabolism enters the TCA cycle (Steiner et al., 2023). Instead, glucose-derived carbon is preferentially routed toward fatty acid and amino acid biosynthesis, with excess acetyl-CoA secreted via acetate overflow metabolism, enabling less efficient ATP production, at the advantage of lower protein burden (Steiner et al., 2023). In contrast, the TCA cycle is primarily fueled by alternative carbon sources, predominantly proline and glutamate, which enter the cycle as α-ketoglutarate (α-KG), effectively bypassing the oxaloacetate-to-α-KG section of the TCA cycle (Potter & Criss, 2024).

In this context, the upregulation of *gltA*, which commits acetyl-CoA to the TCA cycle via citrate synthesis, is notable and indicates an increased routing of carbon into the TCA cycle under methionine-replete conditions. Examination of the normalized transcript abundance (TPM) of all genes involved in central carbon metabolism across all four sulfur conditions further supports a distinct expression pattern in which TCA cycle genes are elevated when exogenous methionine is available (MG) compared to methionine-limited conditions (C, TG, and G) (Figure 4.4 c). Together, these data indicate that access to exogenous methionine may influence central carbon metabolism primarily through increasing flux through the TCA cycle. However, it remains unresolved whether this methionine-dependent effect arises due to an increased demand for reducing equivalents, ATP production, or the generation of metabolic intermediates.

Interestingly, these alterations in central carbon metabolism are accompanied by the induced expression of several genes involved in amino acid biosynthesis including *proA*, *glnA, ilvE,* and *argD/argJ/argB* required for the synthesis of proline, glutamate, branched acid amino acids (leucine, isoleucine, and valine) and

arginine, respectively. Notably, *ilvE*, *gdhA*, and *argD* catalyze the reversible reactions involving the interconversion between glutamate and α-KG, closely linking these to the TCA cycle, specifically at the a-KG node. The induction of these amino acid biosynthetic genes is not explained by amino acid limitation, as these amino acids are readily available in the growth medium. Instead, the concurrent upregulation of these specific biosynthesis genes alongside the TCA cycle genes is consistent with an increased flux through the TCA cycle, perhaps to manage overflow of TCA cycle intermediates, such as α-KG.

4.4.2.3 Regulation of translational machinery

Another interesting observation in the MG condition is a coordinated downregulation of 14 genes related to proteins important for translation, including several 30S and 50S ribosomal proteins, ribosome maturation factors, and translation-associated functions (Figure 4.4 b). Such a response is typically associated with reduced growth rates or a persistence state and is also observed during the stringent response in *N. gonorrhoeae,* which is often activated in response to nutrient limitation (Beste et al., 2007; Talà et al., 2023) Interestingly, despite signatures of sulfur starvation, gonococci grown in this condition did not incur reduced growth suggesting a capacity to metabolically compensate for sulfur-limitation. To this point, this substantial downregulation of translational machinery is intriguing and may reflect a re-allocation of resources as ribosomal production is a major cellular investment to manage growth under nutrient limitation (Schmidt et al., 2016; Hidalgo & Utrilla, 2020).

4.4.2.4 Interbacterial competition

Multiple adhesion family (Maf) genomic islands encode the *Neisseria* specific MafA/B/I polymorphic toxin-antitoxin system, comprising of the toxin (MafB), its conjugate immunity protein (MafI), and the outer membrane-associated adhesion protein (MafA) which facilitates toxin secretion, alongside additional genes of unknown functions. Seven genes located within the gonococcal Maf genomic islands (1, 3 - 5) were downregulated in the MG condition, including genes encoding toxins and immunity proteins, in addition to genes of unknown function (Baerentsen et al., 2022). Although MafB toxins have been implicated in interbacterial competition, the functions of many MafB toxins, including the one downregulated here, along with the other genes encoded within Maf-islands and the

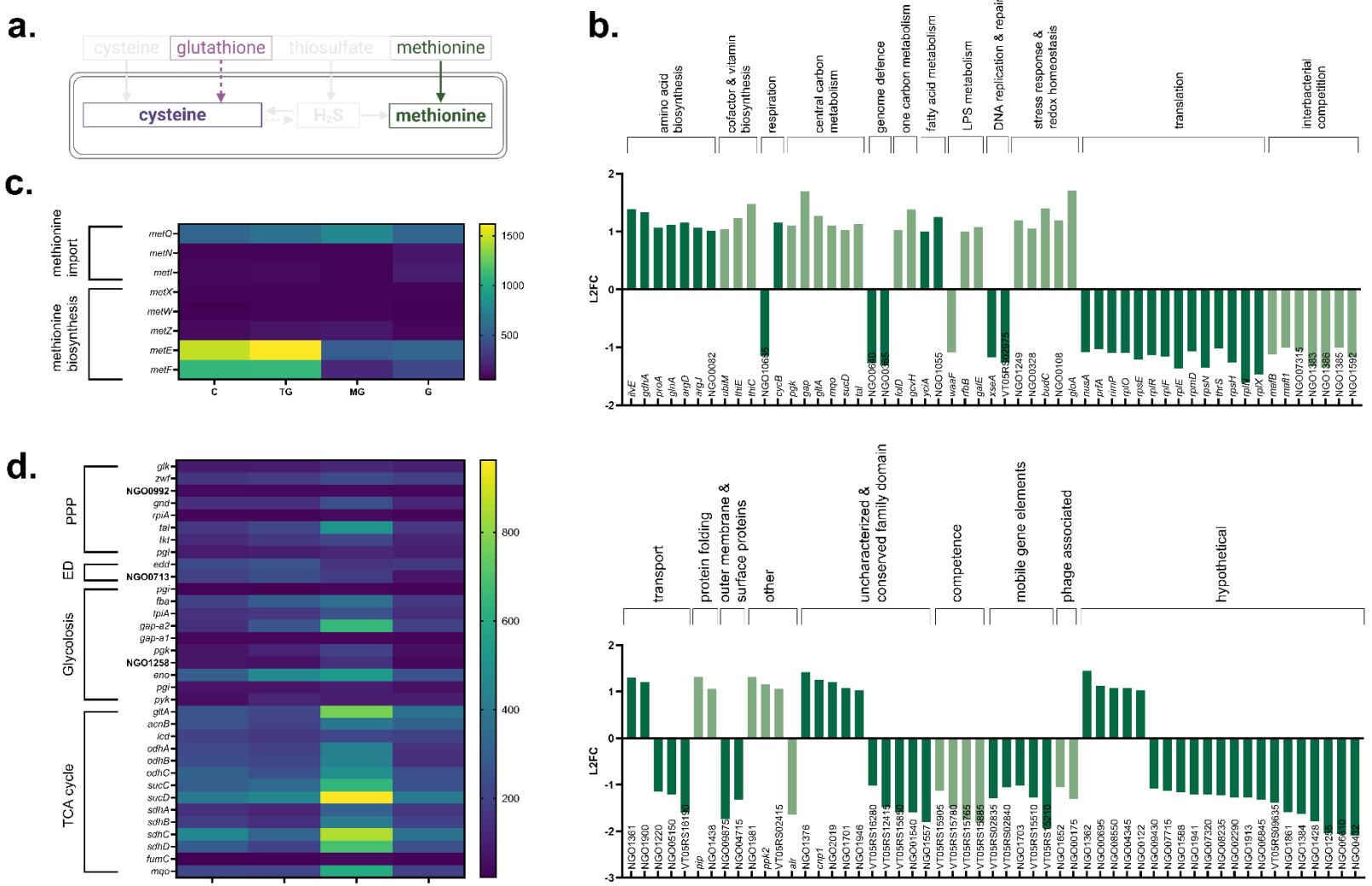


Figure 4.4. Transcriptional responses unique to methionine and glutathione grown gonococci (a) schematic representation of proposed fulfilment of cysteine and methionine requirements during growth in the presence of methionine and glutathione. Dotted lines represent proposed metabolic constraints. (b) Condition-specific differentially expressed genes (L2FC >1, $p_{adj} < 0.05$) as determined DESeq2 analysis. (c and d) Expression trends of the genes involved in (c) methionine import and biosynthesis (d) central carbon metabolism across the different sulfur condition.

Figure 4.4. continued… Normalized gene counts (TMP) clustered within the respective pathways: PPP; Pentose phosphate pathway, ED; Entner–Doudoroff, Glycolysis, and TCA cycle. Data represents mean values of three biological replicates.

regulatory mechanisms governing their gene expression are undefined. Consequently, the functional significance of their differential expression here remains unclear but may suggest that expression of Maf genes may be responsive, directly or indirectly, to fluctuations in nutrient levels. Alternatively, these downregulations could simply reflect a reduced investment in energetically costly, non-essential, processes under these conditions.

4.4.1 Transcriptional response to growth in glutathione

As representing a condition of both cysteine and methionine limitation (Figure 4.5 a) and reduced growth, it is unsurprising that the glutathione only condition elicited the most substantial transcriptional changes among all conditions tested, with 245 genes uniquely differentially expressed. It is known that conditions of amino acid starvation trigger drastic changes in the transcriptome, which is often coordinated via the stringent response which specifically associated with slowed growth or persistent-like states (Zhu & Dai, 2023). Given the absence of amino acids, cysteine and methionine, and the slow growth phenotype, we were curious as to whether the transcriptomic response resembled that of the gonococcal stringent response which has been recently characterized (Talà et al., 2023). The transcriptome of the gonococcal stringent response is characterized by large genomic changes that reflect significant upregulations of amino acid metabolism and downregulations ribosome biogenesis and protein synthesis, central carbon metabolism, and the electron transport chain and oxidative phosphorylation. Intriguingly, we did not observe major changes in these functional categories and observe only minimal overlap (~ 33 %) between genes that are differentially expressed here (matched L2FC values), and those observed induced during an active stringent response (Figure 4.5 b). This suggests that the transcriptional landscape observed here in response to sulfur amino acid limitation is not explained by the canonical nutrient responsive stringent response.

Due to the substantial transcriptional response within this condition (Figure 4.6), we subsequently consider overarching trends within this dataset, rather than

individual genes or pathways, except for three genes directly relevant to sulfur metabolism. Notably, prophage-associated genes comprise approximately one third of the differentially expressed genes and are therefore considered in further detail. Finally, we highlight the potential impact of the differential expression of two toxin–antitoxin (TA) systems, given the inherent involvement of TA systems in stress response.

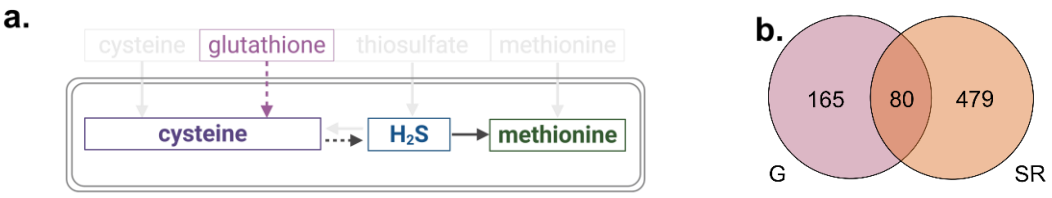


Figure 4.5. Schematic representation of glutathione- only condition and comparison to the stringent response. (a) schematic representation of proposed fulfilment of cysteine and methionine requirements during growth in the presence of glutathione. Dotted lines represent proposed metabolic constraints. (b) Venn diagram of concordant differentially expressed genes between the glutathione only condition (G) and previous published stringent response (SR).

4.4.1.1 Genes involved in sulfur metabolism

In addition to the sulfur metabolism genes previously discussed (Section 4.2), an additional three genes of interest were uniquely differentially expressed *N. gonorrhoeae* grown under cysteine and methionine limitation: *metI*, *metN*, and *pspE*.

Components of the methionine ABC transporter *metI* and *metN* were upregulated, potentially reflecting a canonical adaptive response to nutrient limitation, in which bacteria increase expression of high-affinity transport systems to enhance scavenging in response to nutrient deprivation (Davies et al., 2021). Although *metQ,* the SBP component, was not differentially expressed, the relative transcript abundance (TPM) remained higher than that of *metI* and *metN,* suggesting that *metQ* may not be limiting for transport (Figure 4.4 c). MetQ is a lipoprotein and localizes both the periplasm for methionine uptake, and has also reported to be surface-exposed, where it may contribute to adhesion (Semchenko et al., 2017). The increased expression of *metI* and *metN* may therefore be sufficient to enhance the methionine transport capacity independent of additional *metQ* expression.

Also differentially expressed was *pspE*, which encodes the periplasmic thiosulfate sulfurtransferase which reduces thiosulfate to H_2S *in vitro* (Section 3.4.1.1). In this condition, *pspE* was downregulated, unaccompanied by expression changes in the other genes encoded within the same operon, suggesting that regulation of *pspE* may be, at least partially, independent from the operon consistent with observations in *E. coli* (Darwin, 2005). Although we propose PspE to contribute to sulfur metabolism, this function is unlikely to be relevant to this condition or the cysteine-only control to which it was compared due to the absence of thiosulfate in both conditions. As a promiscuous enzyme, PspE may contribute to several physiological functions not limited to sulfur acquisition, including alternative sulfur transfer reactions, the generation of cellular sulfane sulfur which contributes to oxidative stress resistance, and disulfide bond formation involved in proteins folding (Cheng et al., 2008; Chng et al., 2012; Yu et al., 2023). Given that the extent of PspE functions in *N. gonorrhoeae* has not been elucidated, the basis for this downregulation remains unclear but could reflect direct or indirect consequences of an overall reduction in sulfur availability.

4.4.1.2 Broad trends in the transcriptome in response to cysteine and methionine limitation

Given the significant growth defect, a substantial reduction in the transcript abundance of translation-related genes, specifically ribosomal genes, were anticipated as these are strongly, and consistently, associated with reduced growth phenotypes and is an indicator of reduced metabolism (Gyaneshwar et al., 2005; Kim et al., 2025). Surprisingly, however, we observe no significant differences in the expression of genes involved in translational processes unique to glutathione grown cells despite the significant slow growth phenotype. However, alternative canonical signatures of slow growth are reflected in the transcriptome (Beste et al., 2007; Dressaire et al., 2008; Bechhofer & Deutscher, 2019). These include the downregulation of genes involved in cellular respiration (*norB*, *nqrABCDEF*, *nuo*NMLHG, *petA*, *atpEFHAD*), DNA replication (*ligA* and NGO1226), nucleotide metabolism (*dcd* and *pyrH*), RNA metabolism (*orn*, NGO0607, NGO1261), and cell division (NGO0317). Additionally, genes pertaining to outer membrane related proteins and processes were downregulated including genes involved in cell wall and envelope biosynthesis (*pglBHO, galU, murD*), outer membrane and surface

proteins (NGO1802*, pld,* NGO1739*, nspA*), LOS biosynthesis (*lptA, lgtG,* NGO06735, NGO1710), fatty acid and lipid biosynthesis (*lpxD* and *fabZ*), and protein secretion (NGO1955). Together, these observations are consistent with diminished respiration and energy production during reduced growth and a subsequent reduction in energetically costly processes, potentially resulting in alterations in the surface complexity of glutathione grown gonococci.

Other functional groupings that have numerous altered genes were those involved in proteostasis, nutrient acquisition, and stress responses (Figure 4.6). Majority of these proteins are uncharacterized in *N. gonorrhoeae*, leaving the nature of the relationship between these genes and cysteine and methionine depletion unclear. Furthermore, the genes within these categories exhibit variable expression patterns indicative of a complex transcriptional response which is underscored by the differential expression of ~1/5th (7 out of 34) of the transcriptional regulators found in *N. gonorrhoeae.* However, these regulators remain poorly characterized, limiting interpretation.

4.4.1.3 Prophage

Curiously, a significant proportion of genes upregulated under the glutathione-only condition localize to the three most complete double-stranded DNA prophage islands (Φ1 – Φ3) in the *N. gonorrhoeae* FA1090 genome (Geslewitz & Seifert, 2025). The functional role of prophage activation in *N. gonorrhoeae* remains poorly understood, however several studies have reported differential expression of prophage-associated genes under specific conditions, including anaerobic growth, female genital tract infection, and exposure to hydrogen peroxide, suggesting that phage-encoded genes may contribute to important gonococcal functions (Isabella & Clark, 2011; McClure et al., 2015; Quillin et al., 2018). Approximately 90 phage-associated genes were upregulated in the glutathione-only condition representing the most extensive prophage-associated transcriptional response described in *N. gonorrhoeae* to date and the most highly differentially expressed genes within the dataset herein. This response included upregulation of a cI-type repressor ortholog encoded by NGO1630, which has recently been shown to be essential for gonococcal survival (Geslewitz & Seifert, 2025). CRISPRi-mediated repression of NGO1630, as well as two additional cI-type repressor orthologs (NGO0479 and NGO1116), induces prophage gene expression, production of bacteriophage-like

virions, and extensive cell death within two hours of growth, demonstrating that these repressors are required to maintain prophage suppression and prevent phage-mediated killing of the gonococcus (Geslewitz & Seifert, 2025).

Under the conditions examined herein, the gonococci appeared to be viable and consistently proliferating, based on consistently increasing OD measurements. However, given the severe attenuation of growth rate in this condition it would be important to additionally confirm this with viable cell counts to distinguish whether cells were consistently proliferating or whether the cell culture better resembles a persistent-like state. Nevertheless, the data support a limited capacity to grow, albeit reduced, which indicates that a full prophage induction is unlikely to have occurred given that even partial attenuation of cI-type repressor resulted in rapid cell death and phage particle production within a similar timeframe (Geslewitz & Seifert, 2025).

Given the lethality associated with gonococcal prophage activation, the pronounced induction of prophage-associated genes observed herein is notable and raises fundamental questions regarding the intracellular cues and mechanisms that trigger the expression of prophage genes in *N. gonorrhoeae*. Environmental conditions and host physiological states are recognized as cues contributing phage activation into lytic cell cycle, most notably cellular stress related to DNA damage and consequently the activation of the bacterial SOS response, alongside other SOS-independent cues, such as oxidative stress (Makky et al., 2021). These canonical cues do not offer an obvious explanation here, as *N. gonorrhoeae* lacks a classical SOS response and no overt transcriptional signatures of oxidative stress were identified. In the present study, the attenuation of growth in the presence of glutathione is attributed to a metabolic limitation, with liberation of cysteine from glutathione to be rate limiting for growth. Under these conditions, cysteine and methionine limitations may induce a stressed state that destabilize prophage repression either directly or indirectly through intracellular perturbations. Whether these perturbations are sensed through metabolic or redox imbalance or canonically through DNA damage is uncertain. It also remains to be determined whether prophage induction contributes directly to the observed growth attenuation or instead represents a consequence of cellular stress as it is not possible to resolve

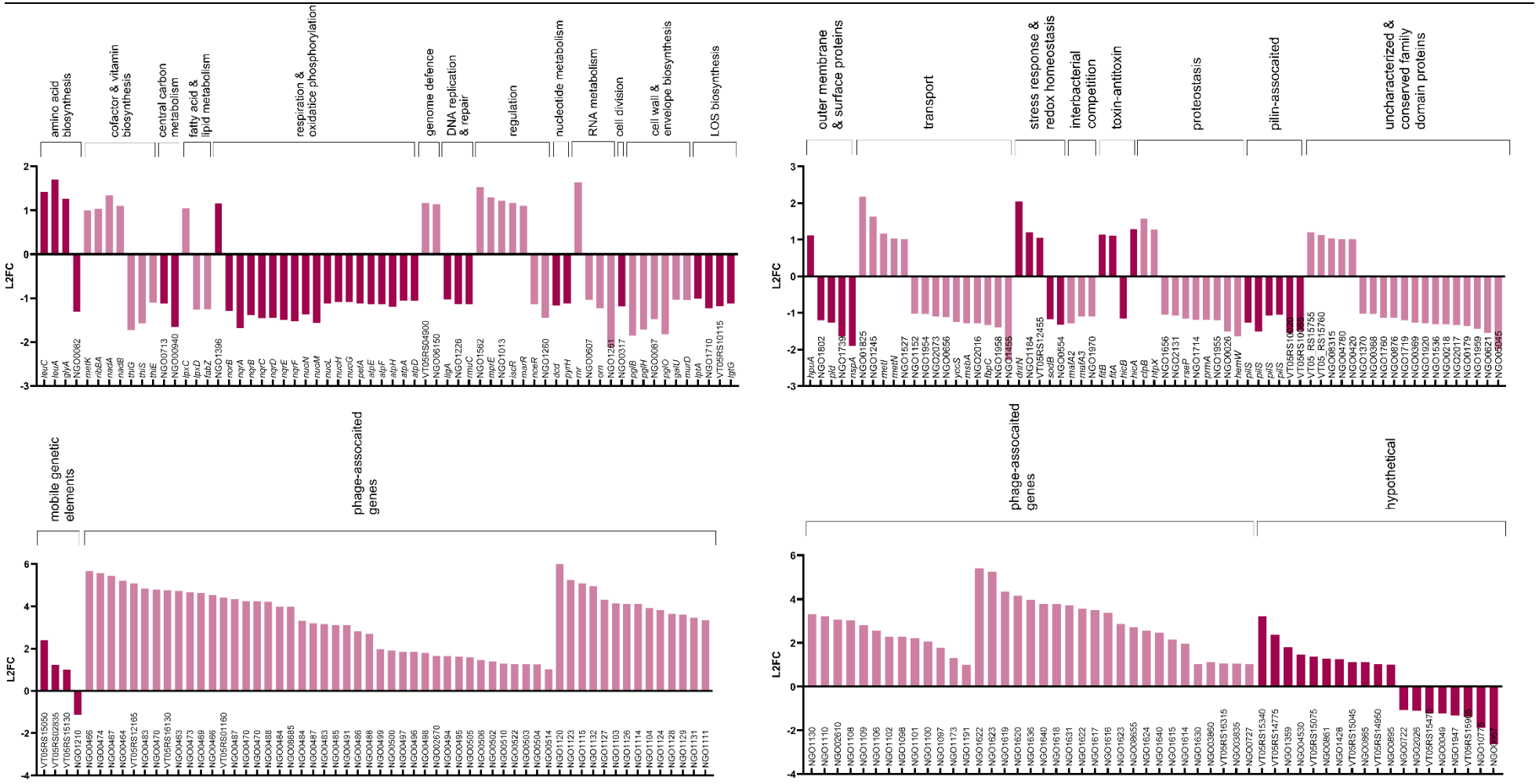


Figure 4.6. Differentially expressed genes unique to gonococci grown with glutathione as the sole sulfur source. Condition-specific differentially expressed genes (L2FC >1, $p_{adj} < 0.05$) as determined DESeq2 analysis.

whether this transcriptional response reflects a coordinated population-wide sub-lethal state or stochastic induction within a subpopulation of the gonococcal culture (Ibrahim & Aranjani, 2026).

Nonetheless, this significant transcriptional response indicates that severe nutrient limitations may represent a useful condition to further examine the induction mechanisms behind the resident phages and the gonococcal mechanisms of phage-defense.

4.4.1.4 Toxin-antitoxin systems

Type II TA systems contribute to a plethora of diverse functions including stress response, phage counter defense, mobile genetic elements maintenance, and biofilm formation (Chan et al., 2023; Ostyn et al., 2025). Given the central importance of TA systems, specifically to conditions of stress, it is interesting that two gonococcal TA systems were differentially expressed under cysteine and methionine depletion, HicAB and FitAB. Although the functions of these systems in *N. gonorrhoeae* have not been extensively characterized, limited evidence does support their involvement in pathogenesis (Hopper et al., 2000; Holley et al., 2025).

In *N. gonorrhoeae*, the HicAB TA system, comprising of the HicA toxin (NGO1628) and the HicB antitoxin (NGO1628), has recently been shown to facilitate growth arrest when over-expressed, promote biofilm formation, and enhance fitness *in vivo* (Bagabas et al., 2025; Holley et al., 2025). *hicAB* is encoded within the gonococcal prophage island ϕ3, in proximity to the cI repressor (NGO1630), the expression of which was upregulated in the present study. In addition, the *hicA* gene is upregulated without changes in the expression of *hicB* (NGO1627) potentially indicative of un-neutralized toxin. Furthermore, an additional gene annotated as a *hicB* antitoxin (NGO0723) which is genetically unlinked to the *hicAB* cassette (NGO1627 - NGO1628) previously studied, was also downregulated and, therefore, is unlikely to dampen the activity of HicA, supporting the possibility of free HicA protein to exert toxin effects.

Interestingly, studies in other bacteria demonstrated the induced expression of *hicA* by amino acid and carbon starvation and has been linked to antiphage defense (Gerdes, 2024; Jørgensen et al., 2009). In this context, the concordant expression of *hicA* and prophage genes in response to amino acid starvation raises the

possibility that HicAB as a possible phage-counter defense mechanism in *N. gonorrhoeae*.

In addition to *hicA*, the expression of another type II TA system, *fitAB*, was induced under cysteine and methionine limitation. Early studies on this system in *N. gonorrhoeae* demonstrated its involvement in promoting intracellular survival by slowing intracellular replication (Hopper et al., 2000). Inducing growth-arrest to facilitate survival under stress, in this case host cell invasion, is characteristic of TA systems. Given the slow growth phenotype in this condition, it is interesting that *fitAB* expression is altered. However, as both *fitA* and *fitB* are upregulated this may not necessarily translate to increases in toxin activity and therefore could instead reflect stress response that is either directly or indirectly related to amino acid starvation, rather than a functional contributor to growth arrest.

4.5 Metabolomic experiments

To complement the transcriptomic analysis, metabolomic experiments were undertaken to investigate in the intracellular and extracellular metabolome of gonococci cultures grown in cysteine-dependent and -independent growth conditions, C, TG and MG. The glutathione-only condition was excluded from metabolomic experiments as sufficient biomass could not be obtained due to the slow growth phenotype and consequent low-density cultures.

Targeted metabolomic experiments were performed in conjunction with University of Auckland (UoA) Mass Spectrometry Hub, who developed the protocols and provided gas chromatography–mass spectrometry (GC–MS) equipment, analytical pipelines, and GC-MS expertise. Briefly, the experimental conditions for *N. gonorrhoeae* culturing and metabolite extractions were adapted for *N. gonorrhoeae* and performed in-house. Subsequently, the extracted metabolites were transported the University of Auckland Mass Spectrometry facility where methyl-chloroformate- (MCF) derivatization and GC–MS protocols were conducted, followed by the UoA analytical pipeline (Smart et al., 2010). The analytical pipeline included peak detection, spectral deconvolution, metabolite annotation against a manually procured reference library, and data clean-up before generating a summary output table containing annotated metabolites and their corresponding peak abundances across all samples.

The metabolite summary table contained compound annotations, retention time information, match factors, total identification (ID), and peak intensity values for each sample, among other information (Supplementary material 6.10). Initial examination of this dataset revealed several inconsistencies that limited confidence in the automated peak identification workflow. The first concern identified within the summary table related to the "total ID" metric, representing the number of detections for a given metabolite across the dataset. In multiple instances this value exceeded the total number of biological samples analyzed, making it unclear how detections were being counted within the pipeline. In cases where the "total ID" number was lower than the number of samples analyzed, the output did not specify which individual samples contained the detected metabolite, preventing reliable determination of metabolite presence across conditions. To confidently determine which samples contained the metabolite of interest, the raw spectra was manually inspected to identify metabolite peaks.

Inspection of the raw GC–MS spectra subsequently revealed additional ambiguities in peak annotation. For several compounds, multiple peaks were annotated as the same metabolite despite occurring at clearly distinct retention times. This raised uncertainty regarding whether these represented derivatization artefacts, multiple spectral features associated with the same compound, or independent peaks that had been aggregated during automated processing. In other cases, peak intensity values were reported in the summary output despite the absence of a clearly identifiable peak at the expected retention time in the corresponding raw spectra. Furthermore, sulfur compounds were sometimes detected in media-only control samples where the compound was not expected to be present, suggesting potential miss-annotation, background signal, or limitations in the automated peak assignment procedure.

Accurate metabolomic interpretation is contingent upon robust peak detection, validated metabolite annotation, and clear tracking of metabolite presence across samples. As such, these inconsistencies precluded confident biological interpretation of the dataset. Resolving these issues would require redevelopment and expert validation of the data processing pipeline to ensure correct peak identification and quantification. As this could not be completed within the timeframe of this thesis, the metabolomic dataset is not interpreted further here. Future work will focus on reprocessing the raw GC–MS data using an

independently validated analysis pipeline to enable reliable metabolite identification and downstream biological interpretation.

4.6 Summary and conclusions

Given the relevance of cysteine limitation within host environments, understanding how *N. gonorrhoeae* adapts to restricted cysteine availability is central to understanding how sulfur requirements are met during infection. In this chapter we sought to further elucidate how the gonococcus proliferates in the absence of cysteine and to identify the pathways that support the utilization of alternative sulfur sources. In particular, the bioinformatic reconstruction alone could not explain how the gonococcus proliferates when glutathione is provided as the sole sulfur source. More broadly, we sought to understand how sulfur limitation affects intracellular homeostasis.

Intriguingly, cysteine limitation did not elicit a uniform transcriptional response across the cysteine-independent growth conditions. Instead, transcriptional responses were condition-specific, demonstrating that adaptation to cysteine limitation is shaped by the availability of alternative sulfur-containing metabolites and that these are not functionally interchangeable. Among the cysteine-independent conditions, the transcriptome of *N. gonorrhoeae* grown with thiosulfate and glutathione most closely resembled the transcriptome of cysteine-grown gonococci, indicating that together these sulfur sources facilitate growth and fulfil sulfur requirements in a manner comparable to cysteine. In contrast, the MG and G conditions elicited a transcriptional response consistent with sulfur starvation, indicating that glutathione is an inefficient sulfur source and that methionine does not compensate for cysteine deficiency, consistent with the proposed sulfur metabolic network in Chapter Three. Examination of the differentially expressed genes involved sulfur metabolism, specifically *cysK* and the cysteine transporter *cjaABCD*, revealed regulatory patterns more nuanced than anticipated, underscoring that current models of sulfur metabolism are incomplete. Furthermore, no gene candidates were identified among the concordant differentially expressed genes between the MG and G conditions that might explain how gonococcus proliferates using glutathione as a source of sulfur in these conditions.

During conditions of sulfur starvation (MG and G), a small subset of genes was concordantly differentially expressed, indicative of transcriptional responses associated with sulfur-limitation. Interestingly, these changes do not reflect overt stress responses but instead suggest subtle alterations in redox homeostasis. However, an overall lack of characterization of gonococcal proteins limits a comprehensive interpretation here. An interesting observation in the responses associated with sulfur starvation was the upregulation genes involved in competence, suggesting these constitutively expressed systems maybe be subject to additional regulation in response to conditions of nutrient limitation.

In addition to shared responses, the MG and G conditions incurred mostly condition-specific transcriptional changes. In the MG condition, these included upregulation of genes involved in central carbon metabolism, amino acid biosynthesis, and one-carbon metabolism, alongside downregulation of translational machinery and non-essential processes such as interbacterial competition. One explanation for these trends is resource reallocation that enables maintenance of growth under sulfur starvation when methionine is available. The limitation of both methionine and cysteine (G condition) was associated with overarching trends in the dataset that reflect reduced energy metabolism and energetically costly processes consistent severe growth impairments. The most prominent observation in the glutathione-grown gonococcal transcriptional landscape, however, was the upregulation of a substantial number of genes located within prophage islands. Consistent with growth observations that glutathione is a poor sulfur source in the absence of alternative sulfur metabolites, this indicates that severe nutrient limitation might destabilize prophage repression in *N. gonorrhoeae*.

Collectively, the evidence presented in this chapter demonstrates that sulfur source availability influences transcriptional responses in *N. gonorrhoeae* in a metabolite specific manner. More specifically, in response to sulfur starvation, the gonococcus does not appear to overcome this limitation through diverse acquisition strategies but instead adjusts cellular processes to compensate for inefficient sulfur acquisition via resource reallocation, where possible. This metabolic inflexibility with respect to sulfur acquisition positions cysteine acquisition, and potentially *de novo* cysteine biosynthesis, as central constraints on gonococcal physiology. As an exquisitely adapted human pathogen, this raises further questions regarding

cysteine accessibility within gonococcal niches, suggesting that cysteine may be more available than currently assumed, or that the gonococcus possesses additional, yet unidentified, mechanisms to access host cysteine.

5 | Chapter Five

Conclusions

5.1 Thesis summary

As a host-restricted organism, *N. gonorrhoeae* has an absolute dependance on its human host to fulfil nutrient requirements. Accordingly, targeting pathways that are central to acquiring nutrients is a promising avenue to combat the threat of extensively drug-resistant *N. gonorrhoeae*. Sulfur acquisition contributes crucially to pathogenesis by facilitating survival, biofilm formation, resistance to oxidative stress and virulence (Alkhuder et al., 2009; Álvarez et al., 2015, 2015; Bachman et al., 2015; Brenner et al., 2022; Chevalier et al., 1999; Du & Arvidson, 2006; Gebhardt et al., 2015; Grifantini et al., 2002; Hatzios & Bertozzi, 2011; Kim et al., 2012; Lensmire et al., 2023; Lithgow et al., 2004; Man et al., 2020; Pinto et al., 2013; Ren et al., 2017; Seiflein & Lawrence, 2001; Senaratne et al., 2006; Takahashi & Watanabe, 2005; Turnbull & Surette, 2008; Vaval Taylor et al., 2023; Xayarath et al., 2009). Some of these pathways, namely *de novo* cysteine biosynthesis, has been proposed as ideal target candidates and inhibitor development is underway in several pathogens, including *N. gonorrhoeae* (Hicks et al., 2022; Tao et al., 2024; Oldham et al., 2025). However, the efficacy of such strategies is contingent on whether pathogens are restricted in their ability to access pathway end products throughout infection. Elucidating vulnerable pathways therefore requires a thorough understanding of what pathways are conditionally essential and the conditions they become relied upon.

In this thesis we investigate the sulfur nutritional requirements of *N. gonorrhoeae*, addressing fundamental questions regarding the metabolic flexibility employed by the gonococcus with respect to sulfur acquisition and how the gonococcus adapts to conditions of cysteine- and sulfur-limitation. Given the variability of sulfur metabolism across pathogens (as outlined in Chapter One), this warranted manual curation of the gonococcal sulfur metabolic network, which was undertaken by combining bioinformatic analyses, *in vitro* kinetic assays, and experimental growth curve data of WT and mutant strains.

We confirm that *N. gonorrhoeae* can directly uptake exogenous cystine and cysteine. However, cysteine limitation is expected to be frequently encountered

during infection as its cytotoxic nature prevents accumulation within the host. Despite this, *N. gonorrhoeae* possesses limited alternative sulfur acquisition strategies. In the absence of cyst(e)ine, only glutathione independently supported gonococcal proliferation, providing the first evidence that identifies glutathione as a feasible sulfur source for *N. gonorrhoeae*. However, growth on glutathione was significantly attenuated, which could be restored by further supplementation with methionine or thiosulfate. This indicates that the use of glutathione is constrained by downstream metabolic limitations, likely imposed by inefficient liberation of cysteine from glutathione, as well as an inability to effectively degrade cysteine to generate H_2S required for methionine biosynthesis.

We propose that supplementation of glutathione with thiosulfate restores growth through the reduction to H_2S, which is partly mediated in the periplasm by the thiosulfate sulfurtransferase, PspE, thus simultaneously providing substrates for the *de novo* synthesis of cysteine and methionine and alleviating both metabolic constraints. However, inconsistently, expression of *cysK* encoding the final enzyme in the cysteine biosynthesis pathway, was downregulated during growth in this condition. While this is feasibly explained by the nuanced behavior of CysK involving moonlighting functions, which we hypothesize to be cysteine degradation in the cysteine-replete control condition, these data warrant further investigations into the role of *de novo* cysteine biosynthesis in *N. gonorrhoeae.*

We identified several cysteine-independent growth conditions and analyzed the transcriptome in these conditions to investigate responses to cysteine limitation and sulfur starvation. Interestingly, the transcriptome was highly variable across the cysteine-independent conditions, with no unifying transcriptional signatures identified. Minimal differences in the transcriptome of TG grown gonococci suggest that together, these sulfur compounds fulfil sulfur requirements in a comparable manner to cysteine. Further examination of the transcriptional landscape during conditions that induced sulfur starvation (MG and G) revealed that rather than implementing diverse strategies to utilize alternative sulfur sources, the gonococcus adapts to compensate for sulfur insufficiencies, and this compensation may be shaped by the broader nutrient landscape, as seen in the MG transcriptome. However, under conditions where sulfur compound accessibility is further restricted resulting in growth attenuation (G), these compensatory

mechanisms appear inadequate. Under these circumstances, strong induction of prophage genes suggests that nutrient limitation may destabilize prophage repression, rather than eliciting canonical stress responses. Thus, our findings demonstrate that contrary to our expectations, *N. gonorrhoeae* exhibits a limited capacity to compensate for cysteine limitation through the utilization of alternative acquisition strategies, utilizing only glutathione in its absence, and potentially thiosulfate for *de novo* cysteine biosynthesis, although the latter requires further validation. This metabolic inflexibility positions cystine, and possibly cysteine, acquisition as a central constraint on gonococcal physiology which is further emphasized by the drastic attenuation of gonococcal mutant strains defective in cystine uptake to survive, replicate, and colonize epithelial cells under aerobic conditions. Although it remains to be determined whether this inability to establish infection reflects an absolute requirement for cystine or a limitation of the experimental conditions, this significant phenotype nonetheless underscores the essential role of sulfur acquisition during early stages of pathogenesis.

It remains an open question as to whether this requirement for cystine is consistent throughout pathogenesis, across infection at distinct anatomical sites and during occupation of intracellular niches. Furthermore, the paradox that *N. gonorrhoeae* appears predominantly dependent on host-derived cystine/cysteine within ostensibly cysteine-limiting host environments either underscores the major gap in our understanding of sulfur landscapes *in vivo* or suggests the gonococcus employs additional unidentified mechanisms to access host cysteine. These questions are pivotal to refining our understanding of gonococcal nutrient requirements throughout infection and thus informing the feasibility of future endeavors to target sulfur acquisition strategies in the pursuit of novel antimicrobials.

5.2 Future perspectives

To support the proposed sulfur metabolic network herein, experiments aimed at validating hypothesized pathways and resolving enzyme functions are required and have been addressed throughout this thesis. Most notably, these include quantification of H_2S production across sulfur conditions, assessing growth on sulfur pathway intermediates (cystathionine, homocysteine, and cysteine dipeptides), clarification of cysteine degradation pathways, and defining the role of

CysK during cysteine-replete and cysteine-limited growth. Beyond this, a major advance would come from stable isotope labelling which would enable direct tracking of sulfur flux from exogenous sources. Currently, the understanding of sulfur metabolism not only in *N. gonorrhoeae*, but more broadly across bacterial pathogens, is largely inferred from growth experiments, mutant experiments, and transcriptional responses. Thus, stable isotope labeling stands to offer valuable insights into how sulfur moves through metabolic networks as responding to exogenous sulfur availability. Furthermore, within the context of the work presented here, isotope-labelled sulfur compounds including cysteine, glutathione, and thiosulfate, would enable direct quantification of downstream metabolites and provide crucial information regarding the utilization of pathways that underpin cysteine homeostasis. Furthermore, when integrated with our transcriptomic and, once resolved, metabolic analyses, stable-isotope labelling would provide information that bridges the gap between gene expression, metabolite abundances, and specific pathway utilization. Collectively, this will provide a comprehensive dataset of sulfur flux under different sulfur landscapes.

Another question that has emerged from the literature and this work is how pathogens utilize glutathione in the absence of canonical pathways. The inability to identify a plausible mechanism herein and by others highlights a gap in our fundamental understanding of organosulfur utilization, restricting comprehensive assessments of the importance of glutathione utilization throughout infection. Functional genomic approaches, such as transposon mutagenesis or CRISPR interference screening under glutathione-dependent growth conditions would provide an unbiased approach to identify essential genes for survival under these conditions, with the potential to uncover novel sulfur acquisition mechanisms which may be further applicable to several clinically relevant pathogens.

Ultimately, pathway verification and identification would subsequently enable a detailed characterization of how these strategies contribute to infection-relevant phenotypes. This would involve expanding the panel of mutants defective in sulfur acquisition strategies and assessing their capacity to form biofilms, colonize host cells, survive under oxidative and antibiotic induced stress, colonize host cells and survive within different intracellular niches. This, paired with *in vivo* infection models would unravel the relative importance of each pathway to host infection and

the stages during pathogenesis under which they are dependent upon. Thus, advancing our knowledge on how *N. gonorrhoeae* prioritizes, acquires, and depends upon distinct sulfur compounds across host environments and throughout pathogenesis.

6 |Chapter Six

Supplementary material

6.1 List of bacterial strains and plasmids

Table 6.1. List of bacterial strains and plasmids.

Strains and Plasmids	Description
Strains	
E. coli BL21	General cloning host for protein expression
N. gonorrhoeae FA19	Wild-type
N. gonorrhoeae Δ*str*	*str* gene replaced with an Erm^R casette
N. gonorrhoeae Δ*pspE*	*pspE* gene partially replaced with a Kan^R cassette (without terminator)
N. gonorrhoeae Δ*str:pspE*	*str* gene in Δ*pspE* strain replaced with an Erm^R casette
N. gonorrhoeae Δ*cjaA*	*cjaA* gene replaced with a Kan^R cassette
N. gonorrhoeae Δ*tcyA*	*cjaA* gene replaced with a Kan^R cassette
Plasmids	
str-pET28α	pET28α expression vector encoding *str* with an N-terminal His-tag
pspE-pET28α	pET28α expression vector encoding *pspE* with an N-terminal His-tag and without the signal peptide

Emr^R; erythromycin resistance cassette including the promoter and terminator, unless specified otherwise. Kan^R; kanamycin resistance cassette including the promoter and terminator.

6.2 Graver-Wade media composition

Table 6.2. Media components and concentrations in the GW media and the modified version.

Compound	Concentration (mM)	GW	Modified
Components of the M199 medium base			
Adenosine 5'-phosphate	0.000576	Y	Y
Adenosine 5'-triphosphate	0.001653	Y	Y
alpha Tocopherol phos. Na salt	0.000018	Y	Y
Ammonium Bicarbonate	17	Y	Y
Ascorbic Acid	0.000284	Y	Y
Biotin	0.000041	Y	Y
Calcium Chloride (anhyd.)	1.801802	Y	Y
Cholesterol	0.000517	Y	Y
Choline chloride	0.003571	Y	Y
D-Calcium pantothenate	0.000021	Y	Y
Deoxyribose	0.003731	Y	Y
D-Glucose (Dextrose)	42.55	Y	Y
Ferric nitrate	0.001733	Y	Y
Folic Acid	0.000023	Y	Y
Glutathione (reduced)	0.000163	Y	-
Guanine hydrochloride	0.001596	Y	Y
Hypoxanthine	0.247239	Y	Y
i-Inositol	0.000278	Y	Y
L-Alanine	0.280899	Y	Y
L-Arginine	0.714754	Y	Y
L-Aspartic acid	0.225564	Y	Y
L-Cystine	0.083009	Y	-
L-Cysteine	0.000626	Y	-
L-Glutamic acid	0.510204	Y	Y
L-Glutamine	4.084932	Y	Y
L-Glycine	0.666667	Y	Y
L-Histidine	0.104190	Y	Y
L-Hydroxyproline	0.076336	Y	Y
L-Isoleucine	0.305344	Y	Y
L-Leucine	0.458015	Y	Y
L-Lysine	0.382514	Y	Y
L-Methionine	0.100529	Y	-
L-Ornithine	0.039	Y	Y
L-Phenylalanine	0.151515	Y	Y
L-Proline	0.347826	Y	Y
L-Serine	0.238095	Y	Y
L-Threonine	0.252101	Y	Y
L-Tryptophan	0.049020	Y	Y

L-Tyrosine disodium salt dihydrate	0.22	Y	Y
L-Valine	0.213675	Y	Y
Magnesium sulfate	0.803383	Y	-
Menadione (Vitamin K3)	0.000058	Y	Y
Niacinamide	0.000205	Y	Y
Nicotinamide adenine dinucleotide	0.01	Y	Y
Nicotinic acid (Niacin)	0.0002	Y	Y
Oxaloacetate	0.252	Y	Y
Para-Aminobenzoic Acid	0.000365	Y	Y
Potassium Chloride	5.33	Y	Y
Pyridoxal hydrochloride	0.00012	Y	Y
Pyridoxine hydrochloride	0.00012	Y	Y
Riboflavin	0.00003	Y	Y
Ribose	0.00333	Y	Y
Sodium Acetate	5.510	Y	Y
Sodium Chloride	117.24	Y	Y
Sodium L-lactate	13	Y	Y
Sodium Phosphate monobasic	1.014	Y	Y
Spermine	0.919	Y	Y
Thiamine hydrochloride	0.099	Y	Y
Thymine	0.002	Y	Y
Tween 80®	Infinity	Y	Y
Uracil	0.3007	Y	Y
Vitamin A (acetate)	0.0003	Y	Y
Vitamin D2 (Calciferol)	0.0003	Y	Y
Xanthine-Na	0.0017	Y	Y
Additional components			
Glucose	37	Y	Y
Magnesium Chloride	0.80	-	Y
Ammonium Bicarbonate	17	Y	Y
Sodium Acetate anhydrous	4.90	Y	Y
L- glutamine	3.40	Y	Y
L-arginine	0.38	Y	Y
Hypoxanthine	0.25	Y	Y
Uracil	0.30	Y	Y
Oxaloacetate	0.25	Y	Y
Thiamine hydrochloride	0.10	Y	Y
L-ornithine	0.04	Y	Y
NAD	0.01	Y	Y
Sodium D-L lactate	13.00	Y	Y

Y: compound present at the concentration indicated. – Compound omitted in media composition

6.3 Genetic manipulation specifications

Provided here are the genetic constructs used for genomic manipulation to generate the deletion strains used throughout this work and the conditions used to PCR amplify construct required for the genetic manipulation protocol.

Table 6.3. Genetic construct specifications for PCR amplification.

Construct	5' primer	3' primer	Annealing temp °C	Extension time
str	TCGGATTGTGATGCC	AGGGAATTGGGGTGC	55	60 s
pspE	GTATGCCGTCTGAAAGCC	GCACTGATTTACGCCGTT	55	1min 10 s
cjaA	GTTTTCAGACGGCATCGCC	CGAAATACAGGCCGAACAGC	55	1 min 10 s
tcyA	TGAATCCGCCGTTTGCATA	TTTTGGAAAATACCGCCGG	57	1 min 20 s

Table 6.4. PCR cycling conditions for deletion strain construct amplification.

Step	Temperature (°C)	Time (min)	Cycles
Initial denaturation	95	15:00	1
Denature	95	0:20	
Anneal	*	0:30	29
Extension	72	*	
Final extension	72	10:00	1

**parameters are listed in Table 6.3 specific for each of the constructs*



Figure 6.1. Constructs used to generate mutant strains. (a) the Δ*str* deletion strain was generated by placing an erythromycin resistance cassette in lieu of the *str* gene (b) the Δ*pspE* deletion strain was generated by interrupting and partially replacing the *pspE* gene with a kanamycin resistance cassette without the terminator (c) the Δ*cjaA* deletion strain was generated by placing a kanamycin resistance cassette in lieu of the *cjaA* gene (d) the Δ*tcyA* deletion strain was generated by placing a erythromycin resistance cassette in lieu of the *tcyA* gene.

6.4 Natural log plots of the growth curves

All growth curves were plotted using the natural log of OD_{550} to visualise the exponential phase, which was used to define the time window for calculating doubling times under each condition. For most datasets, a 0 – 3 hr interval was applied (Figure 6.2 a); however, for the varying cysteine concentration experiments (Section 3.1.1), a 0 – 2 hr window was most appropriate, as this best captured the exponential phase across all concentrations tested (Figure 6.2 b).

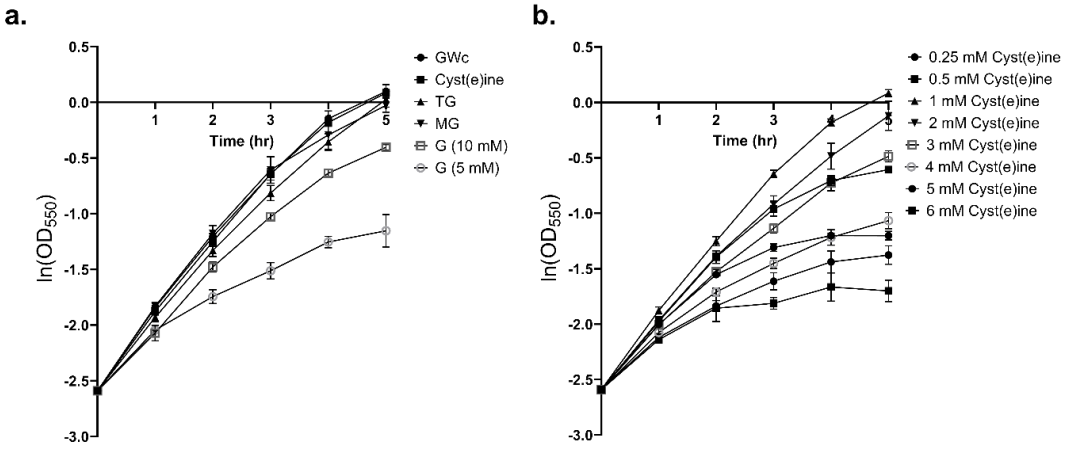


Figure 6.2. *N. gonorrhoeae* WT growth curves under with different sulfur sources. The natural log OD_{550} plots of *N. gonorrhoeae* grown under the following conditions: (a) GW, 1 mM cyst(e)ine, 2 mM thiosulfate and 5 mM glutathione (TG), 2 mM methionine and 5 mM glutathione (MG), 5 and 10 mM glutathione only (G) and (b) 0.25 – 6 mM cyst(e)ine. Data represents the mean ±SEM of ≥3 biological replicates.

6.5 Str and PspE protein information

6.5.1 PspE (VT05RS00565)

PspE protein sequence:

MNIKQLITAALIASAAFATQAAPQKPVSAAQTAQHSAVWIDVRSEQEFSE
GHLHNAVNIPVDQIVRRIYEAAPDKDTPVNLYCRSGRRAEAALQELKKAG
YTNVANHGGYEDLLKKGMK*

PspE with the N-terminal his-tag and excluding the signal peptide:

MGSSHHHHHHSSGLVPRGSHMTAALIASAAFATQAAPQKPVSAAQTAQH
SAVWIDVRSEQEFSEGHLHNAVNIPVDQIVRRIYEAAPDKDTPVNLYCRS
GRRAEAALQELKKAGYTNVANHGGYEDLLKKGMK*

6.5.2 Str (VT05RS10770)

Str protein sequence:

MDIVQLPSAALKAWMDEGRMFCLLDVRTDEEAAVCSLPNALHIPMNLIP
LRQNELPDDVPLVVYCHHGIRSLHTAMYLAEAGFENLYNLQGGIDAWAV
EV DAEMARY*

Str with the N-terminal his-tag:

MGSSHHHHHHSSGLVPRGSHMMDIVQLPSAALKAWMDEGRMFCLLDVR
TDEEAAVCSLPNALHIPMNLIPLRQNELPDDVPLVVYCHHGIRSLHTAMY
LAEAGFENLYNLQGGIDAWAVEVDAEMARY*

6.6 Growth of *N. gonorrhoeae* in the presence of inorganic sulfur sources, sulfate and sulfite

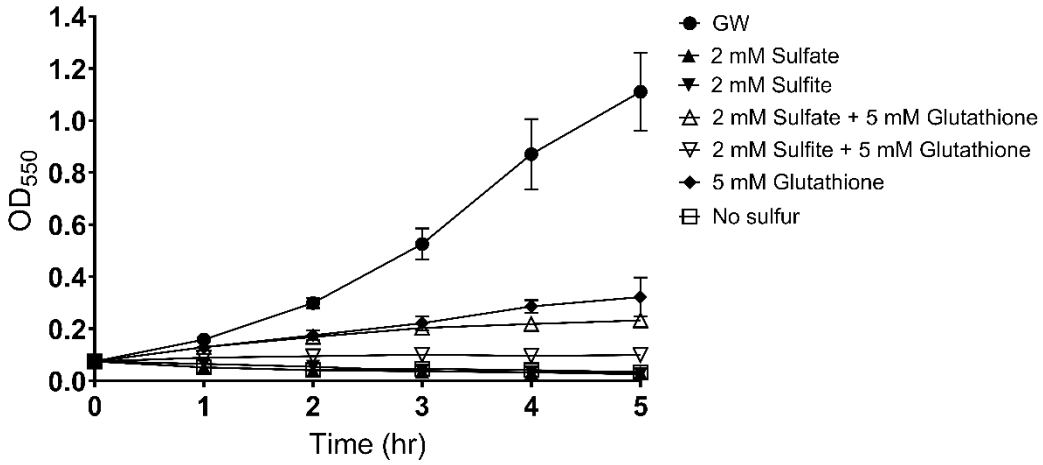


Figure 6.3. Growth of *N. gonorrhoeae* with sulfate and sulfite with and without glutathione supplementation. Growth curves of *N. gonorrhoeae* WT grown under sulfate and sulfite (2 mM), with and without glutathione (5 mM) compared to growth under GW control conditions. Data represents the mean ± SEM from 1 - 3 biological replicates per condition.

6.7 Activity of Str and PspE recombinant proteins with cysteine as the thiol acceptor substrate

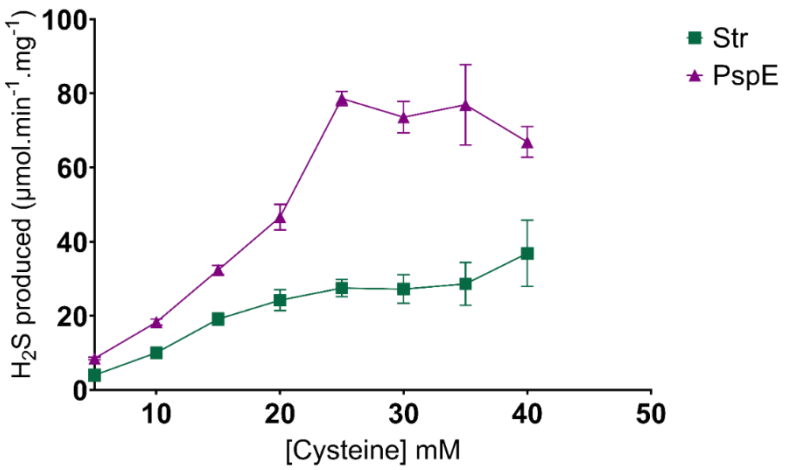


Figure 6.4. Str and PspE thiosulfate reductase activity with cysteine as the thiol acceptor substrate. Thiosulfate reductase activity was determined by varying cysteine concentration (5 - 40 mM) at constant thiosulfate concentration (50 mM). All data represents the mean ± SEM from ≥ three replicates.

6.8 CFU confirmation of sulfurtransferase deletion strains grown in thiosulfate and glutathione

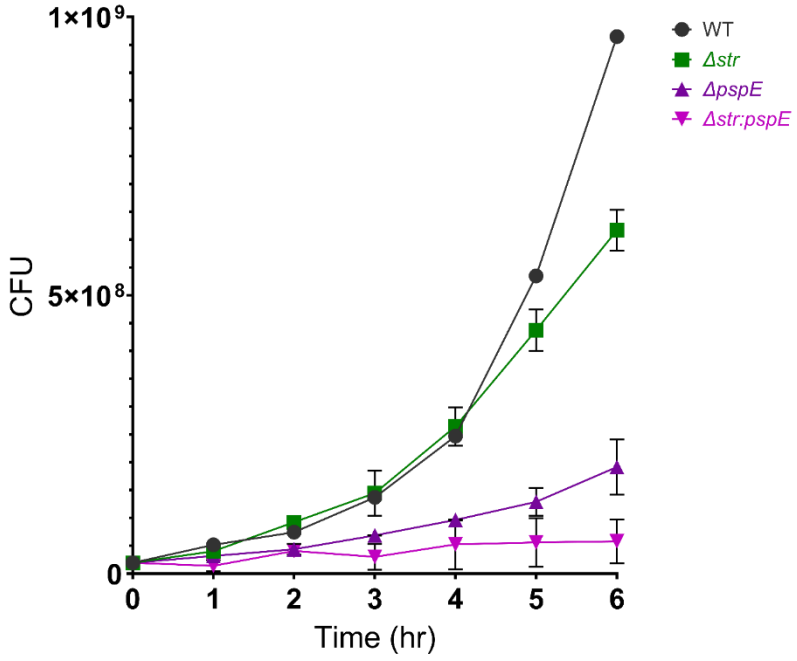


Figure 6.5. Growth of sulfurtransferase (Δ*str*, Δ*pspE*, and Δ*str:pspE*) deletion strains compared to WT in the presence of thiosulfate (2 mM) and glutathione (5 mM). Data represents the mean ± SEM from 2 biological replicates per condition.

6.9 Growth of *N. gonorrhoeae* cultures grown for metabolite extraction and RNA-sequencing sampling.

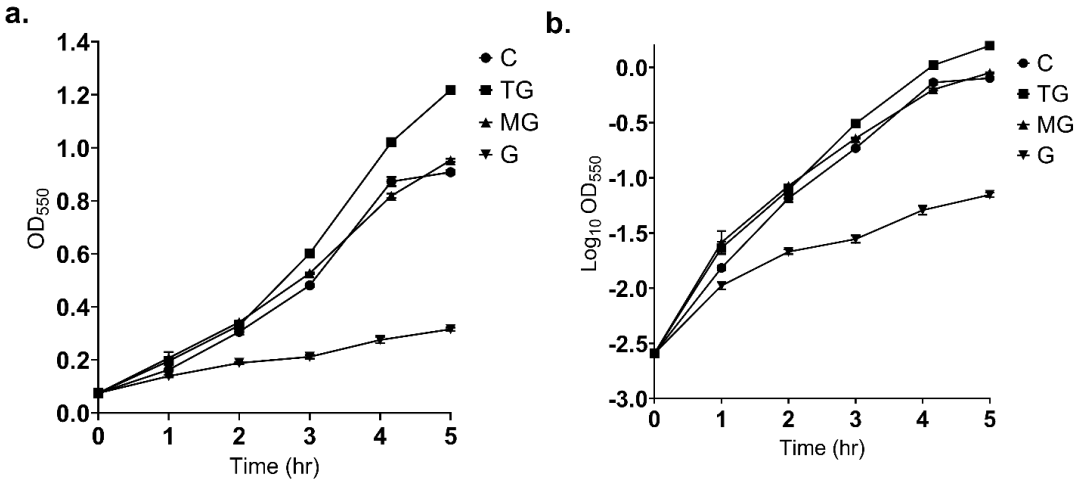


Figure 6.6. Growth of *N. gonorrhoeae* for extraction of metabolites and total RNA. WT was grown in 1 mM cysteine (C), 2 mM thiosulfate and 5 mM glutathione (TG), 2 mM methionine and 5 mM glutathione (MG), or 5 mM glutathione (G). Samples were extracted at 2.5 hr (mid-log). (a) OD_{550} plots and the corresponding (b) log_{10} plots to visualize exponential growth. Data represents means ± SEM of 3 biological replicates.

6.10 Summary sheet results of UoA-MaSH metabolite analysis pipeline

https://docs.google.com/spreadsheets/d/13I-iM5W_9D0R8_RmHW22qFfGuftfzFR6/edit?usp=sharing&ouid=116028177569790417014&rtpof=true&sd=true

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