The nitrous oxide reductase of Neisseria gonorrhoeae is a transcriptionally active yet translationally silent potogene

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1 Abstract

Many parasitic organisms undergo *reductive evolution*, the process of losing genes which are now redundant since nutrients can be acquired from the host (Gómez-Valero et al., 2007). Such shortening of the genome may confer a selective advantage upon cell division (D'Souza et al., 2014). The bacterium *Neisseria gonorrhoeae* is an example of a niche specialist. This obligate human pathogen survives in the urogenital tract, a microaerobic environment, via denitrification. During denitrification, nitrogen oxides (NO_X) can be reduced from nitrate (NO_3^-) to dinitrogen (N_2) by a series of metalloenzymes as the terminal electron acceptors in oxidative phosphorylation instead of O_2 (Barth et al., 2009a).

N. gonorrhoeae acquires both NO_x and the metal ion cofactors needed for denitrification from the host. The key metal is copper (Cu), which is leached from intrauterine contraceptive devices used by a high percentage of women (Crandell and Mohler, 2021). In rare instances when an immune response to *N. gonorrhoeae* is induced, host phagosomal Cu level is elevated (Djoko et al., 2015), yet gonococci cells survive (Château and Seifert, 2016). Whilst expressing several respiratory cuproenzymes needed for growth and surviving high Cu level, *N. gonorrhoeae* lacks proteins from the Cue, Cso, Cus and Cop systems for Cu sensing and export (Andrei et al., 2020). Little is known generally about how bacteria acquire and insert Cu into cuproenzymes (Stewart et al., 2019).

N. gonorrhoeae possesses a truncated denitrification pathway; the nitrate reductase (*nar/nap*) operon is absent and the nitrous oxide reductase (*nos*) operon is nonsense-mutated. The nitrous oxide reductase enzyme, NosZ, catalyses reduction of nitrous oxide (N_2O) to dinitrogen (N_2). This reaction is the last in the denitrification pathway and is catalysed by a Cu_Z site. The *nos* operon consists of six genes (*nosRZDFYL*). There is a single nonsense substitution in each of the following gonococcal genes: *nosR* (the putative transcription regulator and electron donor to NosZ), *nosZ* (the reductase enzyme) and *nosD* (part of the Cu delivery system to NosZ). These mutations are unique to *N. gonorrhoeae* among *Neisseria*, suggesting they evolved after speciation from *N. meningitidis*. In NosZ, the nonsense-truncated protein would lack the C-terminal Cu_A site implicated in electron

transfer to Cu_Z. Hence, the gonococcal *nosZ* has thus long been assumed to be a non-functional "pseudo" gene (Barth et al., 2009a; Overton et al., 2006).

The premature stop codons in gonococcal *nos* are conserved across different strains. It is unclear how *N. gonorrhoeae* may benefit from inactivation of NosZ, particularly in a microaerobic Cu-rich environment (such as that generated by a Cu contraceptive). Hence, we were interested in the possibility that stop codon suppression may be occurring in the gonococcal *nos* mRNA. In recent decades, much work has been done on how premature stop codons can be readthrough (Feng et al., 2022), and how supposed pseudogenes possess functionality (Li et al., 2013). This has led to authors coining the terms *pseudo-pseudogene* for putative pseudogenes which are actually functional (Prieto-Godino et al., 2016) and *potogene* for DNA sequences which have potential to evolve into novel genes (Brosius and Gould, 1992).

Under low Cu levels, the *Paracoccus denitrificans* NosZ polypeptide is subject to degradation such that a truncated NosZ lacking the C-terminal Cu_A domain accumulates in the periplasm which still retains activity (Felgate et al., 2012). While the product of gonococcal denitrification has been determined to be N_2O, not N_2 (Lissenden et al., 2000), the presence of a NosZ protein in *N. gonorrhoeae* has yet to be experimentally tested.

In this study, we investigated the expression of the gonococcal *nos* operon and NosZ protein. Our research shows the gonococcal *nos* operon is fully transcribed and upregulated by NO_2^-, but a *ΔnosZ* strain has no distinct phenotype versus the WT. This corroborates other studies which found transcriptionally active but phenotypically silent gonococcal genes (Takahashi and Watanabe, 2005). Expression of the gonococcal *nos* operon was not found to be influenced by Cu, contradicting findings from other bacterial species that Cu upregulates *nos* expression (Felgate et al., 2012; Sullivan et al., 2013; Woolfenden et al., 2013). Despite transcription, we have determined the NosZ protein is not stably expressed. Supplementation of 1 and 10 µM Cu in *N. gonorrhoeae* cultures does not generate a detectable NosZ protein. We suggest this lack of a stable gonococcal NosZ protein

may be due to nonsense mutations in the *nosRZD* genes causing degradation of the nascent polypeptide, or inhibition of translation by mRNA secondary structures such as hairpins.

Other *Neisseria* thought to lack NosZ include *N. meningitidis*, another obligate human pathogen (Stephens, 2009) and *N. mucosa*, an emerging opportunistic human pathogen (Osses et al., 2017). *N. meningitidis* has a deletion from the middle of *nosR* to the middle of *nosD*, eliminating the catalytic subunit *nosZ*, in addition to premature stop codons in *nosY* and *nosX*, while *N. mucosa* contains a premature stop codon within *nosZ* and a deletion in *nosF* (Barth et al., 2009a). Commensal *Neisseria* species largely retain a functioning denitrification pathway. We propose the pathogenic *Neisseria* independently lost the requirement for N_2O reduction by either acquiring some yet unknown alternative respiratory mechanism, or compensation by the host minimising the need for the last step of denitrification. Alternatively, the *nos* mRNA transcripts in these species may have an unknown regulatory role which provides a selective advantage (Kamieniarz-Gdula and Proudfoot, 2019).

We hope these findings may contribute to an increased understanding of potogenes, as well as the evolution and pathogenicity of *Neisseria* species. Determining the evolutionary drivers behind this loss of protein expression in pathogenic *Neisseria* species is an objective for future research.

2 Project Aims

Since denitrification is implicated in *N. gonorrhoeae* survival in the host, NosZ may present a novel target for therapeutic treatment. This study aimed to determine expression of the *nos* operon and the effect of O_2 and Cu concentration on expression of the NosZ protein.

3 Table of Contents

4 List of Figures

5 List of Tables

6 List of Abbreviations

AccA	AniA copper chaperone A
AniA	Anaerobically induced outer membrane protein A (formerly Pan1)
BLAST	Basic local alignment search tool
Cu	Copper
Cox	Cytochrome cbb_3 oxidase; Cco
Fe	Iron
FNR	Fumarate/Nitrate reductase regulator
GCBL	Gonococcal base liquid medium

GSH	Glutathione
LB	Luria-Bertani medium
Met	Methionine
Mo	Molybdenum
Nar/Nap/Nas	Nitrate reductase (membrane, periplasmic, cytoplasmic)
NCBI	National Center for Biotechnology Information
NEB	New England Biolabs
Nd	Not detected
Nt	Nucleotides
Nor	Nitric oxide reductase
Nos	Nitrous oxide reductase
NO_3^-	Nitrate
NO_2^-	Nitrite
$^{\bullet}NO$	Nitric oxide
N_2O	Nitrous oxide
OD_{600}	Optical density at 600nm wavelength
ORF	Open reading frame
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PTM	Post-transcriptional modification
qPCR	Quantitative polymerase chain reaction
ROS	Reactive oxygen species
specR	Spectinomycin resistance cassette
SNPs	Single nucleotide polymorphisms
SD	Shine-Dalgarno sequence

WGS	Whole-genome sequencing
Zn	Zinc

7 Declaration

I confirm that this thesis was composed by myself, that the work contained herein is my own except except where explicitly stated otherwise in the text, and that this work has not been submitted for any other degree or professional qualification except as specified.

8 Statement of copyright

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10 Chapter 1: Introduction

10.1 Copper in biology

10.1.1 Metal ions in biology

Metal ions are fundamental to life, required for the structure and function of 50% of proteins and 40-50% of enzymes (Andreini et al., 2008). Metal ions found in enzymes are often d-block metal ions such as iron (Fe), manganese (Mn) and zinc (Zn), although magnesium (Mg) is also common (Waldron et al., 2009).

Metal ions confer enzymes various unique properties: 1) positive charge causes metal ions to act as Lewis acids (electron acceptors) for binding and activating substrates, 2) stability in different oxidation states enables metal ions to participate in various redox reactions, and 3) ability to bind four or more ligands causes metal ions to crosslink multiple protein side chains, inducing conformational changes which modulate function (Berg, 1987; Ermler et al., 1998; Riordan, 1977).

The binding of metal ions to proteins relies on interactions with sulphur, nitrogen or oxygen atoms of amino acids (Gooding et al., 2001). Roughly 40% of amino acids are capable of binding metal ions (Lu, 2010), most commonly cysteine, methionine and histidine (Yamashita et al., 1990). The affinity of amino acids for metal ions follows the Irving-Williams series (Figure 1) as a consequence of their ionisation potentials and atomic radii, with Cu ions always forming the most stable complexes inside proteins (Irving and Williams, 1953, 1948).

$$Mg^{2+} < Mn^{2+} < Fe^{2+} < Co^{2+} < Ni^{2+} < Cu^{2+} > Zn^{2+}$$

Figure 1: The Irving-Williams series of the stability of complexes formed by metal ions.

However, the Irving-Williams series can be overcome in order to metalate proteins with the correct metal. The cell has various mechanisms by which to achieve this, including metallochaperones, metal concentrations being buffered to the inverse of the Irving-Williams series, cooperativity (steric selection) favouring insertion of the correct metal, and changes in oxidation state of the correct metal (Grāve et al., 2020; McGuire et al., 2013; Robinson and Glasfeld, 2020).

10.1.2 The bacterial requirement for nutrient copper

Cu is an essential nutrient found in all living organisms (Djoko et al., 2015). Cu is found in various bacterial enzymes such as the *cbb*$_3$-type cytochrome *c* oxidase, NADH dehydrogenase 2, and tyrosinases - all of which reside in the periplasm where they are loaded with Cu, thus reducing risk of Cu toxicity to cytoplasmic components (Festa and Thiele, 2011). Within the reducing bacterial cytoplasm, Cu exists as the more toxic Cu^{+} ion (Davis and O'Halloran, 2008), and is maintained at a concentration of less than one free ion per cell by intracellular Cu scavengers (Rae et al., 1999). Inside the oxidising bacterial periplasm, Cu is found in significantly higher concentrations and readily cycles between Cu^{+} and the less toxic Cu^{2+} under aerobic conditions (Kim et al., 2010).

10.1.3 Copper in host-bacteria interactions

Metal ions are vital for both bacterial and host survival, yet their high reactivity causes toxicity in high concentrations. It is therefore not surprising that the acquisition and utilization of metal ions is key for both host defence systems and pathogen virulence (Becker and Skaar, 2014). During infection, the host initially deprives the pathogen of nutrient metal ions, before utilizing metal ion toxicity to elicit a number of killing mechanisms, a phenomenon termed *nutritional immunity* (Hennigar and McClung, 2016).

Metals such as Cu and Zn are essential for optimal innate immune function (the "brass dagger") (Djoko et al., 2015; Festa and Thiele, 2012). Host Cu ion levels are known to be elevated in response to infection (Besold et al., 2016) and it is thought that the host primarily utilises Cu by importing Cu ions via ATP7A into the phagosomal compartment (German et al., 2013; Hood and Skaar, 2012; Ladomersky et al., 2017; White et al., 2009). The Cu level within the phagosome can increase 10-fold up to ~400 µM (Wagner et al., 2005). This Cu is then thought to be oxidised via the Fenton reaction to produce the extremely reactive hydroxyl radical, $^{\bullet}OH$, which causes damage to pathogens, part of the *oxidative burst* - the rapid, transient, production of large amounts of reactive oxygen species (ROS) (Figure 2) (Hood and Skaar, 2012; Leary and Winge, 2007). An opportunity arises, therefore, to develop clinical Cu ionophores which will utilize this host-derived Cu targeted to the site of infection

and boost Cu delivery into invading bacteria. This has been demonstrated using the Cu ionophore 8-hydroxyquinoline, which is activated by innate immune cells to intensify the hostile antimicrobial host environment (Festa et al., 2014).

It is known that pathogens such as *M. tuberculosis* and *E. coli* upregulate Cu resistance genes during phagocytosis (Rowland and Niederweis, 2013; White et al., 2009), and bacterial strains lacking Cu homeostasis proteins often experience reduced intracellular survival relative to the wildtype (Djoko et al., 2015). *N. gonorrhoeae* is known to be able to survive inside phagosomes, potentially via the cupredoxin Laz which reduces Ccp, which in turn H_2O_2 to H_2O (Château and Seifert, 2016; Quillin and Seifert, 2018; Russell et al., 2020).

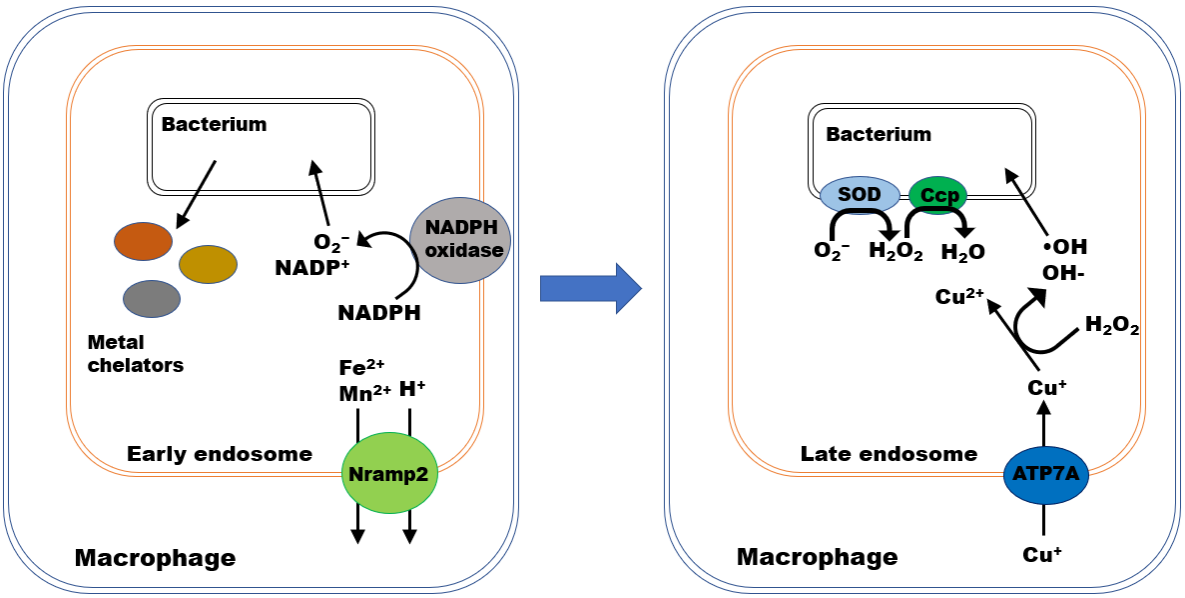


Figure 2: The role of Cu in nutritional immunity.
Divalent metal ions are first removed from the endosome by the Nramp1 transporter, to deprive the bacterium of key nutrients. Later, the ATP7A transporter floods the endosome with reduced Cu, a potent Fenton reagent which produces the extremely damaging hydroxyl radical (Becker and Skaar, 2014).

10.1.4 Copper ion toxicity

Whilst metal ions are an important nutrient within cells, they are often toxic in high concentration, due to their ability to participate as catalysts in redox cycling or the mismetallation of metalloproteins with the incorrect metal (Becker and Skaar, 2014; Guengerich, 2016). Since metal ions cannot be broken down, unlike other (more complex) metabolites, their concentrations are

under strict control in microbes to prevent toxicity. The antimicrobial mechanisms of Cu are varied but can generalised as: 1) production of ROS, 2) membrane damage, and 3) protein mismetallation.

Cu ions form ROS such as hydroxyl radicals via Fenton chemistry (Figure 3), which go on to cause oxidation of lipids, proteins, nucleic acids and iron-sulphur clusters (Besold et al., 2016; Macomber and Imlay, 2009; Meyer et al., 2015).

The peroxidation of double bonds in unsaturated fatty acids, an essential component of some phospholipids, causes distortions of the plasma membrane. This results in a loss of membrane integrity, impeding its role as a selectively permeable barrier and leading to cell lysis, and ultimately, cell death (Meyer et al., 2015). Such oxidation also leads to cleavage of protein side chains and the protein or DNA backbone (Cadet et al., 2003; Reznick and Packer, 1994).

$$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + {}^{\bullet}OH + OH^{-}$$

Figure 3: The Fenton reaction of Cu^{+} with H_2O_2 to form the hydroxide radical, $^{\bullet}OH$.

Due to the position of Cu at the top of the Irving-Williams series, excess Cu ions can displace metals lower in the series from proteins, a process termed *mismetallation* (Foster et al., 2014; Tottey et al., 2012). Such replacement of metal cofactors in proteins renders them inactive (Imlay, 2014; Linder and Hazegh-Azam, 1996). For example, excess Cu causes displacement of Fe from bacterial Fe-S cluster-containing proteins when Cu coordinates sulphur ligands (Reeder et al., 2011). This leads to a loss of activity of essential Fe-S proteins and causes subsequent Fe-mediated Fenton chemistry to create ROS, ultimately causing bacterial death (Chillappagari et al., 2010; Halliwell, 2006; Macomber and Imlay, 2009).

There is high variability of different bacterial species to Cu (Trevors and Cotter, 1990). *Pseudomonas* tolerate copper up to mM concentrations but growth of *Klebsiella aerogenes* is inhibited at as little as 0.01-1 µM Cu (Zevenhuizen et al., 1979). *E. coli* has been shown to tolerate Cu concentrations up

to 4 mM on LB agar (Grey and Steck, 2001), several orders of magnitude greater than *N. gonorrhoeae* can tolerate (Fiscina et al., 1973).

10.2 *Neisseria gonorrhoeae* physiology and pathogenesis

Neisseria is a genus of Gram-negative aerobic coccal β-proteobacteria comprised of at least twenty-eight species, most of which colonize mucosal surfaces (Elias et al., 2015). Most *Neisseria* are part of the normal flora and are only rare opportunistic pathogens in immunocompromised individuals. However, two *Neisseria* species frequently cause disease in healthy individuals: *N. gonorrhoeae* and *N. meningitidis*, which cause gonorrhoea and meningitidis respectively (Herbert and Ruskin, 1981; Rotman and Seifert, 2014). *N. gonorrhoeae* and *N. meningitidis* are thought to have evolved from a common non-pathogenic ancestor, but now occupy different niches, the urogenital tract mucosa and nasopharyngeal mucosa (Jarvis et al., 1999; Quillin and Seifert, 2018). Generally, *N. gonorrhoeae* infections have a high prevalence and low mortality, *whereas N. meningitidis* infections have a low prevalence and high mortality (Morse, 1996). In both *N. gonorrhoeae* and *meningitidis*, denitrification is implicated in pathogenicity (Barth et al., 2009a).

The natural competence of *N. gonorrhoeae* means it has developed resistance to every major class of antibiotic and is increasingly recognised as a "superbug" (Unemo and Shafer, 2014), contributing to a rise in cases worldwide (Rice et al., 2017). *N. gonorrhoeae* is an obligate human pathogen which is often asymptomatic in both sexes (Lovett and Duncan, 2019). Efforts to produce an effective vaccine for *N.* gonorrhoeae are frustrated by the bacteria's ability to evade the immune system via considerable phase and antigenic variation of surface proteins (Baarda et al., 2021).
Due to this high level of host adaptation, gonococcal infection often induces a limited immune response mainly involving recruitment of polymorphonuclear leukocytes (Johnson and Criss, 2011) and protective immunity is not often observed in humans (Belcher et al., 2023; Lovett and Duncan, 2019).

N. gonorrhoeae is thought to actively recruit macrophages and neutrophils but survive the host oxidative burst inside phagocytes (Palmer and Criss, 2018), allowing the bacteria to spread to other

tissues (Escobar et al., 2018). The generation of ROS during inflammation by neutrophils is thought to generate a low O_2 concentration in affected tissues (Williams and Chambers, 2016), suggesting *N. gonorrhoeae* may encounter low O_2. The gonococcal nitrite reductase, AniA, has been proposed as a vaccine candidate since it is essential for anaerobic growth (Mellies et al., 1997), is important for biofilm formation (Phillips et al., 2012) and is highly expressed on the outer membrane (Shewell et al., 2017).

It is estimated that there are ~87 million new cases of gonorrhoea annually (Rowley et al., 2019). *N. gonorrhoeae* is a sexually transmitted obligate human pathogen usually infecting the urogenital tract (Costa-Lourenço et al., 2017), although the infection may spread to a variety of tissues (disseminated gonorrhoea) (Rice, 2005), including the urogenital tract, oropharynx (Cornelisse et al., 2019), rectum (Assi et al., 2014), and conjunctiva (McAnena et al., 2015). Localized gonorrhoea symptoms include urethritis, cervicitis, proctitis, pharyngitis, and conjunctivitis, while disseminated symptoms include pelvic inflammatory disease (PID), epididymitis, skin lesions, tenosynovitis, septic arthritis, and endocarditis (Morse, 1996). Infection can lead to infertility (Costa-Lourenço et al., 2017) and, during pregnancy, ectopic pregnancy (Lenz and Dillard, 2018), premature birth, low birth weight (Donders et al., 1993), and neonatal conjunctivitis (Donders et al., 1993).

10.2.1 The microaerobic gonococcal niche

N. gonorrhoeae and *N. meningitidis* are both thought to exist in an O_2-limited niche (Clark et al., 2010; Isabella and Clark, 2011a; Rock et al., 2005). Upon transmission between humans, *N. gonorrhoeae* colonises the human genital mucosa. Surface adherence and biofilm formation is followed by invasion into the epithelia and transcytosis. Inflammation at the site of infection recruits macrophages and neutrophils to the site of infection, which engulf *N. gonorrhoeae*. Survival of *N. gonorrhoeae* within these immune cells further facilitates transmission (Quillin and Seifert, 2018).

N. gonorrhoeae is a facultative anaerobe, meaning it primarily respires aerobically via cytochrome cbb_3 oxidase (Cco; Cox), the terminal enzyme of the respiratory chain, if O_2 is present. However, it is

capable of switching to anaerobic respiration (a less efficient form of respiration) using NO_2^- as a terminal electron acceptor in denitrification (Clark et al., 1987; Isabella and Clark, 2011b).

Regions of the human genital mucosa are thought to be microaerobic, but different subcellular locations vary in O_2 level (Quillin and Seifert, 2018). The vaginal flora is comprised predominantly of anaerobes and facultative anaerobes (Paavonen, 1983). The finding that gonococci are often clinically isolated with obligate anaerobes (Smith, 1975) and the finding of AniA in Western blots of sera from patients with gonorrhea (Clark et al., 1988) suggest that the gonococcus encounters an anaerobic environment during infection. Furthermore, *N. gonorrhoeae* is known to form biofilms, which are associated with anaerobic respiration in other organisms (Apicella et al., 2011). Gonococcal biofilms are thought to use a combination of aerobic and anaerobic respiration (Falsetta et al., 2010).

Mounting evidence suggests the anaerobic lifestyle is an important state during gonococcal infection (Sikora et al., 2017). Inside neutrophils, it is suggested that *N. gonorrhoeae* encounters a microaerobic environment (Cohen and Sparling, 1992). During infection, environmental O_2 concentration decreases and can drop to 1% due to invading pathogens and translocating immune cells (Branitzki-Heinemann et al., 2016).

The different niches of *N. gonorrhoeae* and *N. meningitidis* may reflect their denitrification pathways. *N. meningitidis* occupies a more oxygenated niche than *N. gonorrhoeae* (Neugent et al., 2020) and appears to be in the process of evolving to lose the ability to reduce NO_2^-, since a significant proportion of *N. meningitidis* strains lack a functional NO_2^- reductase (Moir, 2011a).

Neisseria species only possess a single electrogenic cytochrome oxidase, the Cu-dependent cytochrome *cbb*$_3$ oxidase (Falsetta et al., 2010), which has a very high affinity for O_2 (low nanomolar) (Kaminski et al., 1996) and is associated with O_2 respiration in microaerobic conditions (e.g. in nitrogen-fixing endosymbionts and human pathogens) (Moir, 2011b; Xie et al., 2014). As such, the only alternative respiratory pathways available to *N. gonorrhoeae* other than Cox is denitrification. It

should be noted that *N. gonorrhoeae* can still generate energy via metabolism of glucose (Morse et al., 1974). *N. gonorrhoeae* ferment glucose, but not maltose, sucrose or lactose (Ng and Martin, 2005). However, glucose fermentation does not require terminal electrons, and the level of gonococcal glucose fermentation is insufficient to promote growth in static gonococcal cultures without the supplementation of NO_2^- (Figure 26B). NO_2^- is known to be the critical terminal electron acceptor supporting gonococcal growth in O_2-limited conditions (Knapp and Clark, 1984).

10.2.2 Cu homeostasis in *Neisseria gonorrhoeae*

Cu is required for various important bacterial enzymes, most notably respiratory enzymes such as Cox, AniA and NosZ. In the reducing cytoplasm, copper will be in the more toxic Cu^+ oxidation state, while in the oxidising periplasm copper will be in the less toxic Cu^{2+} oxidation state (Davis and O'Halloran, 2008). Due to the potential toxicity of Cu, it is vital that free Cu is kept to a minimum inside the cell. Hence, various Cu homeostasis systems have evolved in bacteria to sequester and safely transport exogenous Cu to cuproenzymes (Figure 4A).

Homeostatic pathways include: 1) Cu sensing by transcriptional regulators (Rademacher and Masepohl, 2012), 2) import/export across membranes (Argüello et al., 2012), detoxification, storage and trafficking between cellular compartments (Inesi, 2017; Osman and Robinson, 2023; Robinson and Winge, 2010). Generally, opportunistic bacteria possess a range of Cu homeostasis systems, while obligate symbiotes such as *Neisseria gonorrhoeae* possess limited Cu homeostasis systems (Figure 4B) evolved to function in the relatively stable host environment (Rivera-Millot et al., 2023). We will discuss here the current knowledge of Gram-negative Cu homeostasis systems which comes to us from a variety of species within the clade, primarily *E. coli*.

Various Cu importers have been identified in Gram-negative bacteria – including *E. coli* PcoB (Li et al., 2022), *Pseudomonas stutzeri* NosA (Lee et al., 1991), *Pseudomonas aeruginosa* OprC (Bhamidimarri et al., 2021) and *Rhodobacter capsulatus* CcoA (Khalfaoui-Hassani et al., 2018). However, it is thought that Cu can also enter *E. coli* via transport through Omp porins or passive diffusion across the outer membrane (Rensing and Grass, 2003).

Once inside *E.coli*, oxidase enzymes such as CueO and PcoA detoxify Cu^{+} to Cu^{2+} under aerobic conditions (Djoko et al., 2008; Grass and Rensing, 2001a). Copper storage proteins such as periplasmic *Methylosinus trichosporium* Csp1 and cytosolic Csp3 bind excess Cu^{+} ions to prevent off-target binding before insertion into metalloproteins (Dennison et al., 2018; Vita et al., 2016, 2015). PcoE is a putative periplasmic storage protein which sequesters free Cu^{2+} ions (Lee et al., 2002).

While some specific Cu buffering molecules (e.g. GSH) have been identified (Stewart et al., 2020), a variety of non-specific cellular moieties (thiols, methionines, imidazoles, histidines or glutamates) may bind Cu to act as a buffer. A single atom of Cu will travel down a thermodynamic gradient to find the lowest energy, highest affinity, site. Adding more Cu will populate the high affinity buffers and Cu will flow down to lower affinity sites (Stewart et al., 2019).

Chaperone proteins direct intracellular metal to the correct metalloprotein, reducing off-target metal binding (Foster et al., 2014) and lowering the energy barrier for the insertion of the metal into the target protein (Stewart et al., 2019). For instance, gonococcal AccA inserts Cu into AniA (Firth, 2023) and the *Pseudomonas stutzeri* NosDFYL complex inserts Cu into NosZ (Müller et al., 2022). CueP is periplasmic chaperone which acquires Cu^{+} directly from the CopA inner membrane transporter and delivers it to superoxide dismutase (SOD) (Osman et al., 2010). CopA also delivers cytoplasmic Cu^{+} to CueO, where it is oxidised to the less toxic Cu^{2+} ion, which helps protect periplasmic proteins (Djoko et al., 2010; Roberts et al., 2002). CopZ is a cytoplasmic Cu chaperone responsible for loading Cu^{+} into CopA and CueR (Argüello et al., 2013).

A well-characterised *E. coli* Cu sensor is CueR, a DNA-binding transcriptional activator (Brown et al., 2003). CueR binding Cu induces a conformational change in the protein which bends the DNA, allowing RNA polymerase to bind the promoter region (Outten et al., 2000; Sameach et al., 2017). This activates expression of *copA* (Fan and Rosen, 2002) and *cueO* (Grass and Rensing, 2001b). *E. coli* CusRS (Outten et al., 2001) and PcoRS (Munson et al., 2000) are additional two-component Cu^{+}-sensing systems. The CusRS complex helps regulate periplasmic Cu+ conditions in anaerobic

conditions. Cu^+ binding activates the transmembrane kinase CuS, which phosphorylates the transcription factor CusR, initiating the expression of the cusCFBA operon (Munson et al., 2000). The plasmid-encoded Pco system is only found bacterial strains with high Cu tolerance (Bondarczuk and Piotrowska-Seget, 2013). PcoRS is a two-component transcriptional regulator system similar to CusRS , which activates the expression of the *pcoABCD* operon upon Cu^+ binding (Rouch and Brown, 1997). PcoA is a periplasmic oxidase, similar to CueO. PcoC is a periplasmic chaperone which provides Cu^+ for oxidation by PcoA (Djoko et al., 2008). PcoD is an inner membrane transporter which exports Cu^+ from the periplasm to the cytoplasm (Cha and Cooksey, 1993).

E. coli CopA1 is a key component of Cu homeostasis and exports Cu^+ from the cytosol to the periplasm (Wijekoon et al., 2017). *Rhodobacter capsulatus* CopA2 is a related transporter protein thought to deliver Cu to Cox via the SenC chaperone (Andrei et al., 2021). *E. coli* CusABC is an additional Cu exporter which transports Cu from the cytosol (Long et al., 2012) and periplasm (via the CusF chaperone) (Franke et al., 2003) to the extracellular milieu.

Interestingly, bacteria do not seem to require Cu in the cytosol, with all known cuproenzymes being periplasmic or membrane-associated (Argüello et al., 2013; Festa and Thiele, 2012) (Figure 4A), yet they acquire Cu which has been first routed via the cytoplasm (e.g. via CopA) (Stewart et al., 2019). This highlights the need for greater research in this area.

Various questions remain surrounding gonococcal Cu homeostasis (Stewart et al., 2019). Primarily, many of the Cu homeostasis proteins found in other Gram-negative bacteria are lacking in *N. gonorrhoeae*. The CopA exporter is present (Djoko et al., 2012), however the CueR transcriptional regulator is missing, along with the CopZ Cu chaperone to CopA, the cuprousoxidase CueO, the CusRS Cu tolerance system, and the OprC Cu importer (Bhamidimarri et al., 2021). Gonococcal CopA does not respond to Cu, and is instead regulated by a redox-responsive MerR-like regulator, NmlR, which lacks Cu-binding residues and is thought to respond to oxidative and/or nitrosative stress (Djoko et al., 2012; Kidd et al., 2005). Homology searches have suggested *N. gonorrhoeae* contains a

periplasmic Csp1 protein, but this has yet to be characterised (Vita et al., 2015) and the findings of this study reveal expression of the *csp* gene to be highly repressed. Additionally, *N. gonorrhoeae* is thought to lack the Cu-dependent NO_3^- reductase and N_2O reductase (Barth et al., 2009a; Overton et al., 2006). Despite this apparent truncation of the Cu homeostasis pathway, the AniA enzyme involved in anaerobic respiration of *N. gonorrhoeae* is known to have a high requirement for Cu. Since both major gonococcal respiratory enzymes (Cox and AniA) require Cu, the lack of a Cu sensor or Cu importer in *N. gonorrhoeae* is particularly puzzling, raising the question of how *N. gonorrhoeae* acquires Cu from the host.

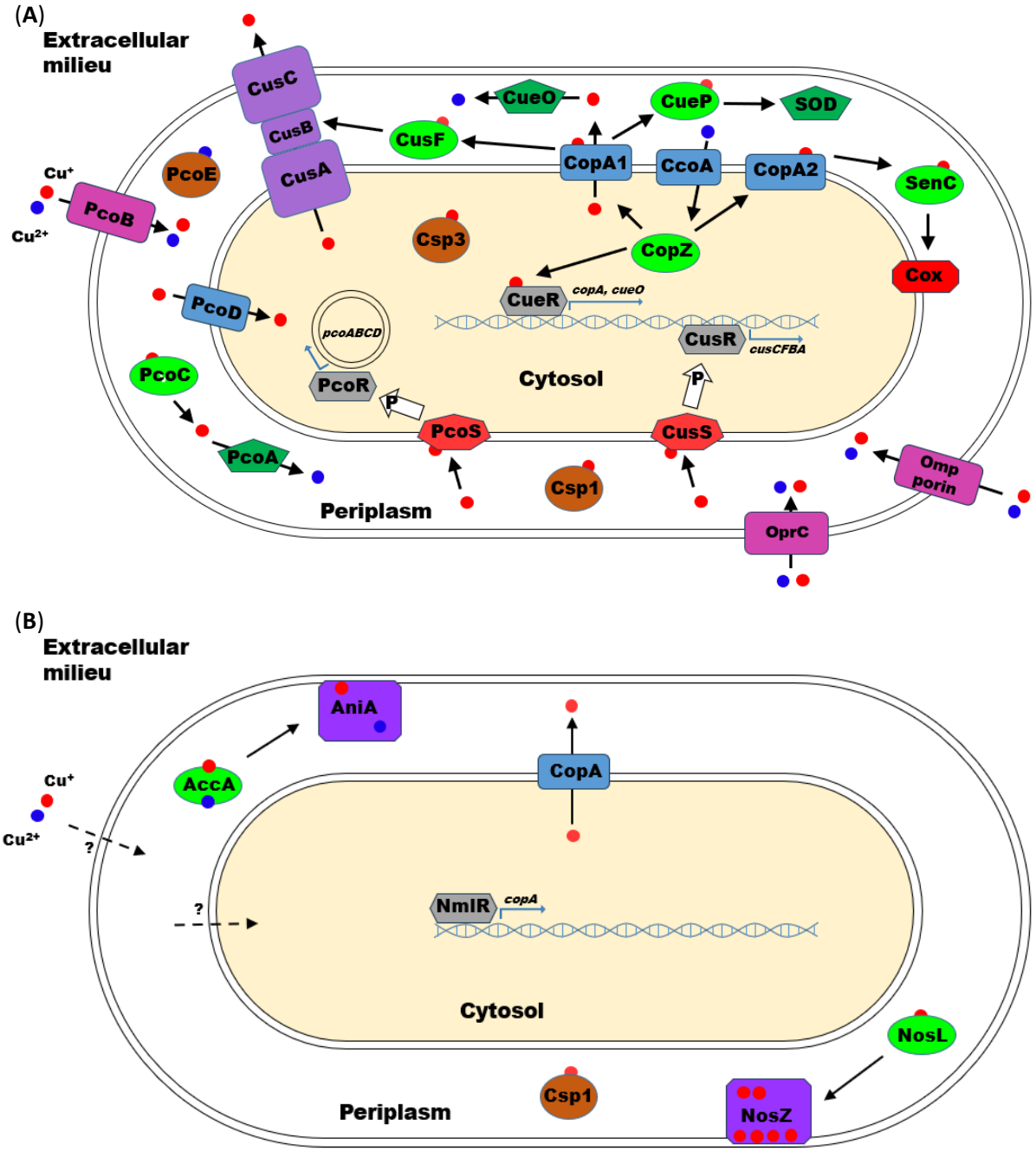


Figure 4: Cu homeostasis pathways in Gram-negative bacteria (A) and *Neisseria gonorrhoeae* (B).
Figure 4A illustrates the major homeostasis systems (not all cuproproteins depicted); various bacterial species contain different subsets of proteins. Membrane transporters (rectangles), oxidoreductases (pentagons), chaperones (ovals), storage proteins (circles), sensors (heptagons), transcriptional regulators (hexagons), enzymes (octagons), Cu^{+} (red), Cu^{2+} (blue).

10.2.3 Availability of Cu in the gonococcal niche

Cu availability in the genital mucosa is difficult to determine. Normal serum concentrations are 10–25 µM (Institute of Medicine, 1998), however, more than 95% of the total Cu in healthy human plasma is bound to ceruloplasmin (Hellman and Gitlin, 2002). We will now discuss the influence of copper contraceptive use and the host innate immune system.

Surveys find intrauterine devices (IUDs) are the third most common contraceptive method, after female sterilization and the male condom (United Nations, 2019). IUDs primarily use Cu as opposed to hormones (Kaneshiro and Aeby, 2010). These devices slowly erode over time, releasing Cu into the uterus, and need replacing after a few years (Gosden et al., 1979). Hence, it may be expected that the Cu content of the female genital tract is increased with use of a Cu IUD. Despite multiple studies, there is little consensus as to the effect of Cu IUDs on blood Cu levels. Various studies indicate no increase in blood Cu levels while others show significant increases. A recent literature review points out that all previous studies measured total Cu rather than free Cu (the form of Cu which is toxic), which raises questions about the clinical significance of this research (Crandell and Mohler, 2021). Free metals are scarce in humans and other animals, nearly always bound instead by proteins. However, new methods for determining the concentration of free Cu in serum may raise hope for better studies in the future (Catalani et al., 2018). The Cu released from an IUD is likely complexed with proteins, avoiding the toxicity of Cu in its free form (Arancibia et al., 2003). A previous study measured the Cu concentration of Cu IUDs before insertion and after 1 year of use (Hagenfeldt, 1972). The devices release an average of 10 mg of Cu per year, which is equivalent to 28.7 mcg per day, only 1- 2% of what is considered to be a healthy Cu intake per day (Crandell and Mohler, 2021).

Of interest to this study are findings that the endometrium tissue experiences increased levels of leukocytes (Zhao et al., 2017), which may provide *N. gonorrhoeae* with Cu from immune cells. Gonococcal infection is associated with inflamed male urethral mucosa, but is often asymptomatic in the female cervix with a lack of inflammation (Edwards and Apicella, 2004). Incidentally, Cu IUDs have been linked to nonspecific inflammation (Serfaty and Yaneva, 1988).

Studies suggest that the macrophage phagosome accumulates Cu during bacterial infection (Hodgkinson and Petris, 2012), and that *N. gonorrhoeae* experiences Cu overload within cervical epithelial cells, which it is able to resist via the cytoplasmic exporter CopA (Djoko et al., 2012). Cu

bound to ceruloplasmin is thought to be mobilised by the host to limit the growth of uropathogenic *Escherichia coli* within the urinary tract (Hyre et al., 2017). On the other hand, cervical secretions contain and neutrophils release the Cu-binding protein calprotectin which is thought to limit Cu availability to *N. gonorrhoeae* (Liyayi et al., 2023).

As such, it is thought that the human host initially restricts and then overloads bacteria in the phagosome with toxic concentrations of Cu to limit pathogenesis, meaning that *N. gonorrhoeae* will experience varied metal availability depending on the stage of infection and level of immune response. Given the Cu homeostasis mechanisms available to it, it is likely that *N. gonorrhoeae* acquires nutrient copper from the host while resisting toxicity (Branch et al., 2022).

10.2.4 A link between Cu-IUD use and *N. gonorrhoeae*

Having established the limited increase in free Cu available to *N. gonorrhoeae* resulting from an IUD, we were interested to research if there was any link described in the literature between Cu IUD use and incidence of gonorrhoea. While Cu IUD use is known to influence the abundance of certain bacterial clades (Brown et al., 2023), data is sparse and undecided on *N. gonorrhoeae*. Some studies suggest there is no effect of a Cu-IUD on gonorrhoea incidence vs oral contraceptives (Johannisson et al., 1976), while others suggest there is an increased risk of gonorrhoea using a Cu-IUD vs a hormonal IUD (Deese et al., 2021), and that bacterial vaginosis prevalence increases in women initiating a Cu-IUD compared to a hormonal IUD (Achilles et al., 2018). However, in the case of bacterial vaginosis this is hypothesised to be due to increased volume and duration of menstruation, a common side effect of Cu-IUD use (Peebles et al., 2021).

10.3 Reductive evolution

Reductive evolution is where genes and their corresponding functions are lost, resulting in a shortening of the genome. The genes of species with reduced genomes can be grouped into three categories: retained, absent, or pseudogenized (Gómez-Valero et al., 2007). Absent genes have either been totally deleted or have diverged so much they cannot be recognised, while pseudogenes

may contain small indels or nonsense SNPs yet have sufficient levels of nucleotide similarity to a related homolog as to be recognisable.

Various causes have been suggested to explain the phenomenon of reductive evolution, including 1) transition from a varied environment to a restricted niche, 2) specialization from multiple hosts to a specific host, and 3) specialization from multiple tissues to a specific tissue. These lifestyle changes cause a relaxation of natural selection pressure, favouring loss-of-function mutations and gene loss (Gómez-Valero et al., 2007). An example of this is eukaryotic mitochondria, which are thought to be an example of an ancestral bacteria that lost its ability to survive outside the host and shortened its genome (Gray et al., 2001). One of the best examples of bacterial reductive evolution is in *Mycobacterium leprae*. It has a smaller genome than other mycobacterial species, and the highest number of pseudogenes known, with 26% of the genome of *M. leprae* thought to consist of pseudogenes (Dagan et al., 2006). One of the genes lost from *M. leprae* is NO_3^- reductase (Cole et al., 2001). Obligate intracellular bacteria (such as *N. gonorrhoeae*) often have a reduced genome size due to their nutritional dependence on and adaptation to a host environment (de Korne-Elenbaas et al., 2022).

10.4 Denitrification

Since *N. gonorrhoeae* colonises the microaerobic urogenital tract (Miller, 2006; Roos and Klemm, 2006), it must have mechanisms to survive microaerobic conditions (low O_2 but not totally anaerobic). In such O_2-limiting conditions, *N. gonorrhoeae* uses NO_x as a terminal electron acceptor to respire (Knapp and Clark, 1984), a process termed *denitrification*. Anaerobic respiration is less efficient than aerobic respiration - the energy yield of *Paracoccus denitrificans* (formerly known as *Thiosphaera pantotropha*) denitrification is approximately half that of aerobic respiration (Chen and Strous, 2013; Koike and Hattori, 1975a). After O_2, NO_x is the next most favourable electron acceptor, followed by Mn/Fe oxides, sulfates and CO_2 (Chen and Strous, 2013)

Denitrification typically involves the reduction of NO_3^- to dinitrogen gas N_2 via a four-step process (Figure 5). The enzymes involved in this process are NO_3^- reductase (Nar, Nap or Nas), NO_2^- reductase (Nir), nitric oxide reductase (qNor or cNor), and N_2O reductase (Nos) (Durand and Guillier, 2021). Denitrifying microbes include bacterial, archaeal and fungal species (Graf et al., 2014).

All sequenced *Neisseria* species contain Nor and Nir, while only two species (*N. mucosa* and *N. sp. oral taxon 014 str. F0314*) contains Nar, and eight species (not including *N. gonorrhoeae, N. meningitidis, N. mucosa*, *N. bacilliformis* and *N. elongata*) contain Nos (Figure 5) (Barth et al., 2009a; Moir, 2011a).

E. coli NarGHI is a cytosolic heterotrimerdimer protein complex attached to the inner membrane, containing one Mo atom and 11 Fe atoms per heterotrimer (Bertero et al., 2003; Blasco et al., 2001; Chen and Strous, 2013). NO_3^-/NO_2^- is delivered across the inner membrane to NarG by the NarK antiporter (Goddard et al., 2017; Rowe et al., 1994). NO_3^- reductases can be grouped into three types based on location, operon organisation and active site structure: **p**eriplasmic NO_3^- reductase (Nap), membrane-associated cytoplasmic **r**espiratory NO_3^- reductase (Nar) and cytoplasmic assimilatory **s**oluble NO_3^- reductase (Nas) (Sparacino-Watkins et al., 2014). *N. gonorrhoeae* and *N. meningitidis* are both thought to lack any kind of NO_3^- reductase; absence of NO_3^- reduction is used as a test for *N. gonorrhoeae* colonies (Ng and Martin, 2005). Homology BLASTn and BLASTp searches of the *N. mucosa narGHIJ* genes and NarGHIJ proteins during this study found no homology in FA1090.

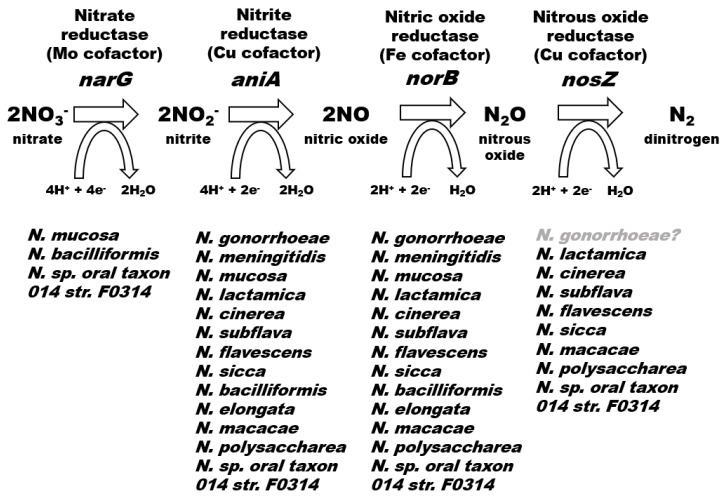


Figure 5: The denitrification pathway of the reduction of NO_3^- to N_2 in *Neisseria*.

N. gonorrhoeae is known to encode a functional NO_2^- reductase, AniA (previously known as Pan1; also known as NirK), and a functional NO reductase, NorB. The gonococcal *aniA* and *norB* genes are back-to-back adjacent in the genome and are flanked by unannotated hypothetical proteins.

Denitrifying bacteria contain two classes of NO_2^- reductase: NirK, a homotrimeric copper-containing enzyme which has been found in both Gram-negative and -positive bacteria, and NirS, a homodimeric cytochrome *cd1* NO_2^- reductase that has been characterized in Gram-negatives (Durand and Guillier, 2021). While most Gram-negative bacteria express a periplasmic NO_2^- reductase NirK, the NO_2^- reductase of pathogenic *Neisseria* AniA is anchored to the outer membrane (Jen et al., 2015). AniA is a homotrimer containing 6 Cu per trimer (Boulanger and Murphy, 2002; Sikora et al., 2017). This enzyme is strongly induced when *N. gonorrhoeae* is grown anaerobically in the presence of NO_2^- (Mellies et al., 1997). Each AniA monomer binds two Cu ions at a Type I and Type II binding site. NO_2^- is bound and reduced at the Type II Cu, while the Type I Cu acts as an electron donor (Boulanger and Murphy, 2002). The inner transmembrane protein cytochrome *cbb*$_3$ oxidase delivers electrons to AniA and is essential for microaerobic NO_2^- reduction (Hopper et al., 2009). AniA is not in an operon, but requires the periplasmic AccA Cu chaperone for metalation during Cu starvation (Firth, 2023). AccA binds one Cu^+ ion and one Cu^{2+} ion (Jen et al., 2015).

There are two types of NO reductase in Gram-negative bacteria: cNOR, a cytochrome *bc* complex which uses a *c*-type cytochrome as an electron donor, and qNOR which lacks a cytochrome *c* component and instead accepts electrons from quinols (Hendriks et al., 2000). Gonococcal NO reduction occurs via a single protein, NorB, which is predicted to be a homodimer containing two catalytic Fe per functional enzyme (Jamali et al., 2020). *N. gonorrhoeae* possesses a qNOR-type NorB (Barreiro et al., 2023). Accessory proteins for gonococcal NorB have not been characterised (Householder et al., 2000).

Unlike AniA and NorB, the N_2O reductase NosZ requires a large operon of multiple auxiliary proteins for function, although the necessity of all Nos proteins for N_2O reduction is debated. NosZ is thought to be a homodimer containing 12 Cu per functional enzyme (Zhang et al., 2019). A diagram of the gonococcal denitrification pathway is given in Figure 6.

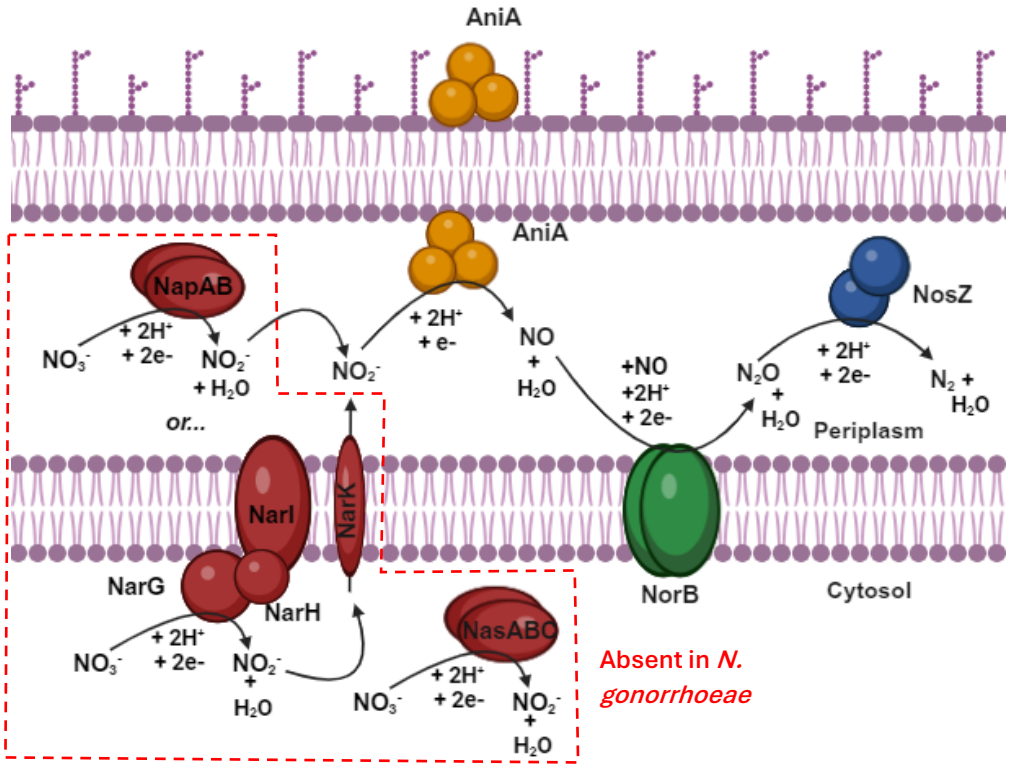


Figure 6: The gonococcal denitrification pathway for anaerobic respiration.
Respiratory terminal electrons are transferred from NADH to NOx reductases via complex I, ubiquinone/ubiquinol, complex III and cytochrome *c* (Chen and Strous, 2013).

10.4.1 Denitrification and pathogen virulence

Denitrification is implicated in the virulence of various bacterial pathogens (Baek et al., 2004; Van Alst et al., 2007). *Pseudomonas aeruginosa* denitrify using NO_3^- and NO_2^- in cystic fibrosis sputum (Barraud et al., 2006). *P. aeruginosa nirS* has been shown to be important for virulence by maintaining a steady state of nitric oxide ($^{\bullet}NO$) within the host (Graf et al., 2014). $^{\bullet}NO$ contributes to the regulation of expression of *P. aeruginosa* virulence factors involved in the type III secretion system (Alst et al., 2009) and has been demonstrated to be a signal involved in *P. aeruginosa* biofilm dispersal (Barraud et al., 2006). NnrA, which is involved in the expression of the last three reductases of the *Brucella melitensis* denitrification pathway (*nirK*, *norB*, and *nosZ*), is required for virulence in mice and for intracellular resistance to $^{\bullet}NO$ (Haine et al., 2006).

N. gonorrhoeae has been isolated with obligate anaerobes in patients (Clark et al., 1987) and anaerobically grown gonococci show increased serum resistance compared to when aerobically grown, suggesting denitrification plays a key role in virulence (Cardinale and Clark, 2000). It is known that pathogenesis of *N. gonorrhoeae* relies upon AniA and NorB (Overton et al., 2006) since their inactivation hinders biofilm formation and prevents establishment of anti-inflammatory $^{\bullet}NO$ steady-state levels (Barth & Clark, 2008; Cardinale & Clark, 2005; Falsetta et al., 2008).

Denitrification has also been implicated in the pathogenicity of *N. meningitidis*, where active denitrification has been linked to modulation of host cytokine responses, enhanced intracellular survival, and inhibition of apoptosis in a macrophage model (Stevanin et al., 2005, 2007; Tunbridge et al., 2006).

10.4.2 Denitrification disruption and pathogenicity in *Neisseria*

The denitrification pathway exhibits considerable variation in *Neisseria* (Figure 5). A previous study comparing denitrification genes found the greatest difference between *Neisseria* species was the lack of a functional Nos system in the pathogenic species *N. gonorrhoeae* and *N. meningitidis* (Barth et al., 2009a). In addition to *N. gonorrhoeae* and *N. meningitidis*, the species *N. mucosa*, *N. bacilliformis* and *N. elongata* all lack NosZ. All these species have reports of opportunistic infection in humans.

N. mucosa is the most commonly reported opportunistic human pathogen in *Neisseria*, and can infect a wide range of anatomical locations (Mechergui et al., 2014; Osses et al., 2017; Ren et al., 2023; Stotka et al., 1991; Tronel et al., 2001; Walsh et al., 2023; Yadav et al., 2024). *N. bacilliformis* and *N. elongata* are two related rod-shaped bacteria. *N. bacilliformis* is known to cause or contribute to infections of the oral cavity-related, respiratory tract, and endocardium (Abandeh et al., 2012; Han et al., 2006), while *N. elongata* is associated with endocarditis, bacteraemia, and osteomyelitis (Haddow et al., 2003; Wong and Janda, 1992).

The rare *N. meningitidis* strains isolated from the urogenital tract are often found to have increased survival in a microaerobic environment due to acquisition of gonococcal *aniA* and *norB* (Ladhani et al., 2020; Tzeng et al., 2017). However, additional evidence suggests that *N. meningitidis* cervical isolates sometimes lose the whole denitrification pathway (T. Mortimer, personal communication). Past studies have suggested that loss of a full denitrification pathway may be minimised by abundance of one particular NO_x substrate in the host (Felgate et al., 2012), or abundance of O_2 in a certain niche (Moir, 2011a).

10.4.2.1 NarG

N. mucosa, N. bacilliformis and *N. sp. oral taxon 014 str. F0314* are the only *Neisseria* species known to possess a NO_3^- reductase. It is unclear why these are the only Neisseria species to have a NO_3^- reductase. The *N. mucosa narG* gene is separated from the *narHJI* operon by 11,950 nucleotides, with genes in the spanning region including a NO_3^-/NO_2^- transporter and genes for molybdenum cofactor biosynthesis. The genes flanking *N. mucosa narG* have no homologs in *N. gonorrhoeae* FA1090.

10.4.2.2 AniA

All gonococcal isolates are thought to express AniA, implying a crucial role for AniA in gonococcal biology (Ku et al., 2009). Gonococcal *aniA* is highly conserved, and gonococci display a higher level of AniA activity than all other *Neisseria*, with the exception of *N. mucosa* (Barth et al., 2009a).

Meningococcal isolates, however, have a wide range of sequence variations within *aniA*, with some isolates containing frameshift nonsense mutations in *aniA* and one isolate (053442) lacking *aniA* in its entirety (Barth et al., 2009a; Ku et al., 2009; Stefanelli et al., 2008). Another study found a nonsense SNP in the meningococcal Cox subunit *ccoP* codon 366 (**C**AG→**T**AG) which results in a loss of a heme group in cytochrome cbb_3 oxidase, inhibiting AniA-mediated NO_2^- reduction (Aspholm et al., 2010). Previous authors have suggested this apparent pseudogenization of meningococcal *aniA* reflects how this species is in the process of losing metabolic versatility to adapt to a more aerobic niche in the human nasopharynx than its non-pathogenic ancestors (Moir, 2011b). While the finding of denitrification loss makes sense for the niche of *N. meningitidis*, it does not fit with our understanding regarding low O_2 level in the gonococcal niche, the urogenital tract.

10.4.2.3 NorB

Compared to other denitrification enzymes, the NorB protein is more conserved across *Neisseria* species (Barth et al., 2009a). However, it has been shown that 23.3% of cervical gonococcal isolates have premature stop codons in *norB*, suggesting an unknown fitness cost to NorB expression in the cervix (Mortimer et al., 2020). Meningococci and most commensal *Neisseria* species have a higher $^{\bullet}NO$ reductase activity than *N. gonorrhoeae* (Barth et al., 2009a).

10.4.2.4 NosZ

The *nos* operon is generally highly conserved across *Neisseria* (Chapter 3, Figure 15). Interestingly the pathogenic species *N. gonorrhoeae*, *N. meningitidis* and *N. mucosa* have inactivated NosZ via independent mechanisms. *N. gonorrhoeae* possesses all *nos* genes and has single nonsense substitutions in *nosRZDX*. *N. meningitidis* has far more severe mutations; there is a total loss of *nosZ* caused by a 4 kb deletion extending from the middle of *nosR* into the middle of *nosD* (Barth et al., 2009a). Furthermore, meningococcal *nosF* and *nosX* each contain premature stop codons (Barth et al., 2009a), as well as a large 5'-end deletion in *nosF* (Chapter 3, Figure 15D). *N. mucosa* contains a premature stop codon within *nosZ* and a deletion in *nosF*. Also, unlike other *Neisseria* (in which the

organisation of the *nos* operon is generally well conserved), *N. mucosa* has *nosRZ* and *nosDFYL* at different loci (Barth et al., 2009a).

10.5 Nitrous oxide reductase

10.6 Pseudogenes

Pseudogenes are considered to be mutated non-functional segments of DNA descended from functional genes. In addition to transposition and duplication, a common mechanism by which a gene becomes a pseudogene is via a nonsense mutation creating a premature stop codon which will terminate mRNA translation and produce a truncated protein.

The term pseudogene is controversial, with some authors calling to replace the term *pseudogene* with *potogene*, i.e. a gene which has potential to become a new gene (Brosius and Gould, 1992). Brosius and Gould (1992) further delineate and *xapto-potogenes* as false pseudogenes found to have function (often variant or novel).

Growing numbers of genes previously classified as pseudogenes are now being found to be transcribed and translated (Goodhead and Darby, 2015; Schrimpe-Rutledge et al., 2012) and to have biological roles (Cheetham et al., 2020; Pink et al., 2011). This has led some to coin the term *pseudo-pseudogene*, i.e. a gene previously thought to be a pseudogene which is now known to have function (Feng et al., 2022; Prieto-Godino et al., 2016).

An example of a true gonococcal pseudogene is *porA* (Feavers and Maiden, 1998). The gonococcal *porA* gene contains mutations in the promoter region which prevent transcription, and frameshift mutations in the coding region of the *porA* gene. The authors suggest that *porA* was acquired by a common ancestor of gonococci and meningococci but was then inactivated in gonococci on speciation. This implies that, while advantageous during colonization of the upper respiratory tract, this protein has no function in, or hinders, colonization of the urogenital tract.

In the case of the gonococcal *nos* operon, we see three genes with high potential to be pseudo-pseudogenes: *nosR*, *nosZ* and *nosD*. These are three genes with single-base nonsense substitutions.

These nonsense mutations are less likely to be deleterious than nonsense mutations arising from indels or frameshifts, which will likely create true pseudogenes.

Pseudogenes such as *porA* have been used to map the evolution of *Neisseria* (Feavers and Maiden, 1998). Nonsense substitutions can be a molecular marker in *Neisseria* species, as is evidenced by CcoP in *N. meningitidis*, whereby a CAT → TAG mutation results in the loss of a metal centre (heme group) from the meningococcal CcoP protein (Aspholm et al., 2010).

10.7 Stop codon readthrough

Stop codon readthrough occurs when a stop codon is misread as a normal sense codon and an amino acid added to the nascent polypeptide instead of translation termination. This "recoding" is part of a larger emerging field in genetics whereby canonical translation is subverted such that a variety of proteins are synthesised which would not be predicted based on their nucleotide sequences (Riegger and Caliskan, 2022).

10.7.1 Mechanisms of stop codon readthrough

10.7.1.1 tRNA mismatches

Translation from mRNA to protein sequence is not always 100% accurate, compromising the fidelity of the ribosome. Non-Watson-Crick mismatches between tRNA and mRNA nucleotides (tRNA "wobbling") has been documented at all three codon positions and such errors can cause stop codon readthrough (Ou et al., 2019). Other mechanisms of mistranslation include tRNA misacylation and misdecoding (Ou et al., 2019). Such non-programmed basal readthrough, occurs without any *cis* and/or *trans* facilitator elements and at a very low rate, less than 0.1% (Tate et al., 1995). *Escherichia coli* strains display a range of ribosomal fidelity, suggesting a high rate of translational errors may be beneficial in conditions where there is a certain selection pressure (Mikkola and Kurland, 1992).

10.7.1.2 Programmed translational readthrough

Programmed translational readthrough involves *cis* and/or *trans* activator elements, and often reaches more than 20% efficiency. The nucleotide sequence surrounding the stop codon has been reported strongly to influence the stop codon readthrough rate (Palma and Lejeune, 2021).

10.7.1.3 Induced translational readthrough

Induced translational readthrough involves RNAs, proteins and small molecules which increase the recruitment of "near-cognate" tRNAs when the ribosome pauses at a stop codon (Palma and Lejeune, 2021). Antibiotics targeting the *E. coli* ribosome and ribosomal mutations have been found to cause near-cognate tRNAs to be translated in error (Kramer and Farabaugh, 2007).

10.7.1.4 Re-initiation of translation after premature termination

An example of re-initiation of translation occurs in the *E. coli* alkaline phosphatase gene at a downstream Met codon (Karamyshev et al., 2004). Furthermore, this re-initiation of translation can occur even in the absence of the Shine–Dalgarno (SD), and occurs inside a 7-codon “re-initiation window” regardless of ORF (Karamyshev et al., 2004).

10.7.1.5 Ribosome binding mid-transcript (leaderless initiation)

The initiation of protein synthesis in bacteria is thought to usually require more than an ATG codon. Additional elements (the SD sequence and a pyrimidine-rich region 5’ to the SD sequence), are thought to be necessary to promote correct initiation and avoid initiation from ATG codons encoding internal methionines of a protein (Laursen et al., 2005).

The *E. coli* SD sequence is thought to be GGAGG in the 7–12 nt region upstream of an ATG start codon (Wen et al., 2020), while the gonococcal SD sequence is putatively AAGGA (Callaghan et al., 2021), or AAGG (Goytia et al., 2015). The AAGGAA sequence is found 7–12 nt upstream of the FA1090 *aniA* ATG start codon, and the AAGGAG sequence is found 9-14 upstream of *norB*. While an AAGG sequence is not found upstream of *nosR*, the AAGGAG sequence is found 7–12 nt upstream of the FA1090 *nosZ* start codon, which would initiate *nosZ* translation (or *specR* translation in a *ΔnosZ::specR* mutant; see Methods Section 11.0). An AAGG sequence is also found 8-12 bp upstream of *nosY*, but not *nosR*, *nosD*, *nosF* and *nosL*.

However, there is evidence to suggest that translation initiation can occur mid-transcript, which may skip stop codons to synthesize a near-complete C-terminal protein. Bacterial translation can initiate via at least three different mechanisms: canonical (Shine-Dalgarno) initiation, readthrough (70S

scanning initiation), or “leaderless initiation”. In leaderless initiation, the ribosome binds to an ATG located at or near the 5’ end of mRNAs to initiate translation (Leiva and Katz, 2022). However, in our *nos* genes of interest, the premature stop codons occur far from the 5’ end of the gene. As such, we think it is unlikely that leaderless initiation will occur in the gonococcal *nos* operon.

10.8 The genetics of nitrous oxide reductase

10.8.1 Structure and function of the *Neisseria gonorrhoeae nos* operon

The NosZ N_2O reductase is the most complex denitrification enzyme, requiring multiple auxiliary proteins. In *N. gonorrhoeae*, the *nosZ* gene occurs in a large operon of auxiliary proteins (*nosRZDFYL*) while the *aniA* and *norB* genes do not appear to be part of a larger operon (*accA* is ~60 kb from *aniA*). However, other species possess large operons containing *aniA*, such as *narRKGHJI*, *napDABC* and *nirSMCFDLGHJEN* (Haine et al., 2006; Levy-Booth et al., 2014). We will use *nar*, *nir*, *nor* and *nos* as shorthand subsequently for these multi-gene operons.

The FA1090 *nos* operon genes are in a single operon, with the exception of *nosX* that is found 43,520 bp downstream of *nosL* (Figure 7). The additional ancillary *nosA* gene encoding a TonB-dependent Cu receptor is not present in *Neisseria gonorrhoeae* (Stewart et al., 2019) but is found in *Pseudomonas stutzeri* (Mokhele et al., 1987), *Pseudomonas aeruginosa* (Vollack et al., 1998), *Brucella melitensis* (Rodionov et al., 2005), and various other Gram-negative species, including: *Pseudomonas fluorescens* (NCBI: AEV60668.1), *Pectobacterium atrosepticum* (CAG75745.1), *Rubrivivax gelatinosus* (BAL96275.1), *Erwinia amylovora* (NCBI: E3DFZ0) and *Shewanella oneidensis* (AAN53708.1). Expression of *Pseudomonas putida nosA* is induced by low O_2 and limited Cu, suggesting NosA might be involved in Cu uptake rather than providing Cu insertase activity for NosZ (Wunsch et al., 2003). Studies in *Pseudomonas putida* show only NosD, NosF and NosY together with *nosRZ* in *trans* are necessary to produce catalytically active NosZ (Wunsch et al., 2003), suggesting *nosA* and *nosX* are dispensable. However, studies suggest *nosX* is essential for N_2O reduction in *Rhizobium meliloti* (Chan et al., 1997) and *Paracoccus denitrificans* (Bennett et al., 2020; Wunsch et al., 2005).

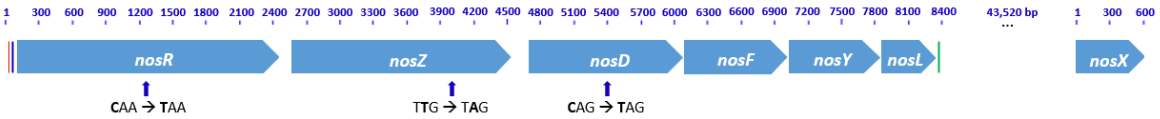


Figure 7: Structure of the *nos* operon in *Neisseria gonorrhoeae* FA1090.
FNR box in red, NsrR box in dark blue, terminator stem loop in green. Arrows note location of premature stop codons.

10.8.1.1 NosR

NosR is a polytopic inner-membrane-bound [4Fe-4S] flavoprotein which has been shown to regulate expression of *nosZ*, although the mechanism of DNA binding is unclear (Cuypers et al., 1992; Wunsch and Zumft, 2005). NosR is also thought to have roles in electron donation to NosZ and formation of the NosZ Cu_Z site, possibly accepting electrons from quinol (Wunsch and Zumft, 2005). In *Pseudomonas stutzeri*, NosR is essential for expression of *nosZ* (Cuypers et al., 1992). Mutant strains carrying either a *nosZ* or a *nosR* mutation accumulate N_2O when cultured microaerobically in the presence of NO_3^- (Velasco et al., 2004).

While the cluster *nosZDFYL* is found in every N_2O-reducing prokaryote, *nosR* and *nosX* are distributed mostly according to taxonomic patterns and are not ubiquitous (Simon et al., 2004; Zumft and Kroneck, 2007). This suggests *nosR* and *nosX* may not always be necessary for *nosZ* function.

10.8.1.2 NosZ

The *nosZ* gene in *N. gonorrhoeae* contains a premature stop codon and has been previously reported to be a pseudogene (Overton et al., 2006). However, this premature stop codon may only cause a sequence of nine nucleotides to go untranslated before an in-frame alternative start codon (TTG) may possibly resume translation (however this is unlikely that far from the 5' end of the gene, Leiva and Katz, 2022). Structural analysis reveals the three amino acid deletion occurs on a region of the NosZ distal to the catalytic centres of the enzyme (Figure 8). It is possible that each globular domain of the NosZ monomer could be separately translated to form a functional enzyme.

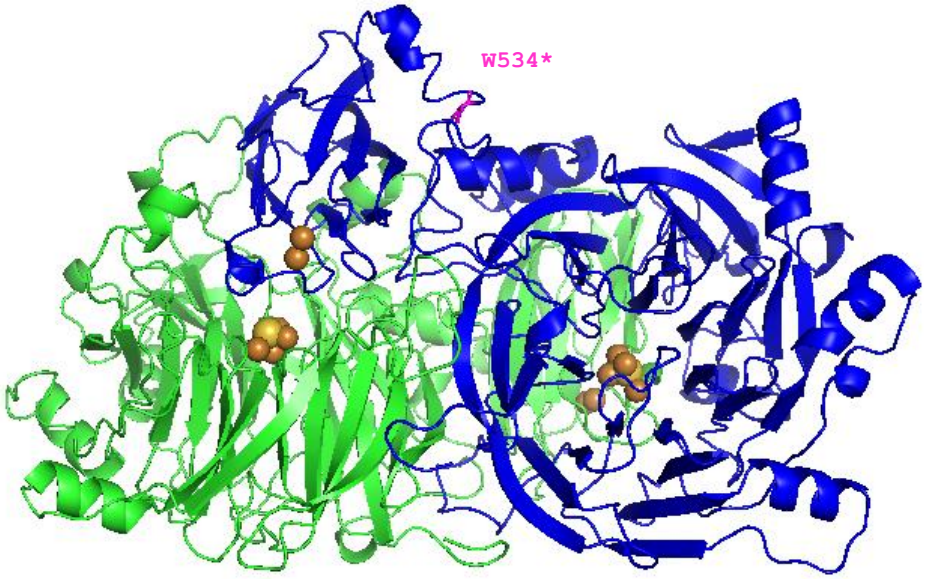


Figure 8: Structure of the NosZ homodimer.
The closest crystallised homolog of gonococcal NosZ is from *Achromobacter cycloclastes* (PDB: 2IWF). The residue corresponding to the gonococcal premature stop codon highlighted in pink.

NosZ is a Cu-containing water-soluble periplasmic homodimer which binds six Cu atoms per monomer at two Cu binding sites, Cu_A and Cu_Z (Prudêncio et al., 2000). The binuclear Cu_A site (also found in cytochrome *c* oxidase, Figure 9A) binds two Cu atoms which transfer electrons to the catalytic tetranuclear Cu_Z site, a metal coordination site unique to NosZ (Figure 9B) (Prudêncio et al., 2000; Wunsch et al., 2003). The Cu_Z site consists of four Cu atoms and binds N_2O (Rasmussen et al., 2000). Insertion of Cu into the Cu_Z site is thought to be facilitated by the outer-membrane associated chaperone NosL (Bennett et al., 2019; McGuirl et al., 2001), assisted by the NosDFY complex (Müller et al., 2022). The larger ~50 kDa N-terminal domain of NosZ includes the Cu_Z site, whereas the smaller ~15 kDa C-terminal domain of NosZ includes the Cu_A site (Figure 9C) (Felgate et al., 2012). In the Cu_A site, the two Cu atoms are coordinated by two histidine, one methionine, one tryptophan, and two bridging cysteine residues (Figure 9A). In the Cu_Z site, the four Cu atoms are coordinated by seven histidine residues and bridged by two inorganic sulphur atoms (Figure 9B) (Brown et al., 2000a, 2000b; Pomowski et al., 2011; Prudêncio et al., 2000). It has been hypothesised that the complexity of the Cu_Z site contributes to the large number of ancillary Nos proteins.

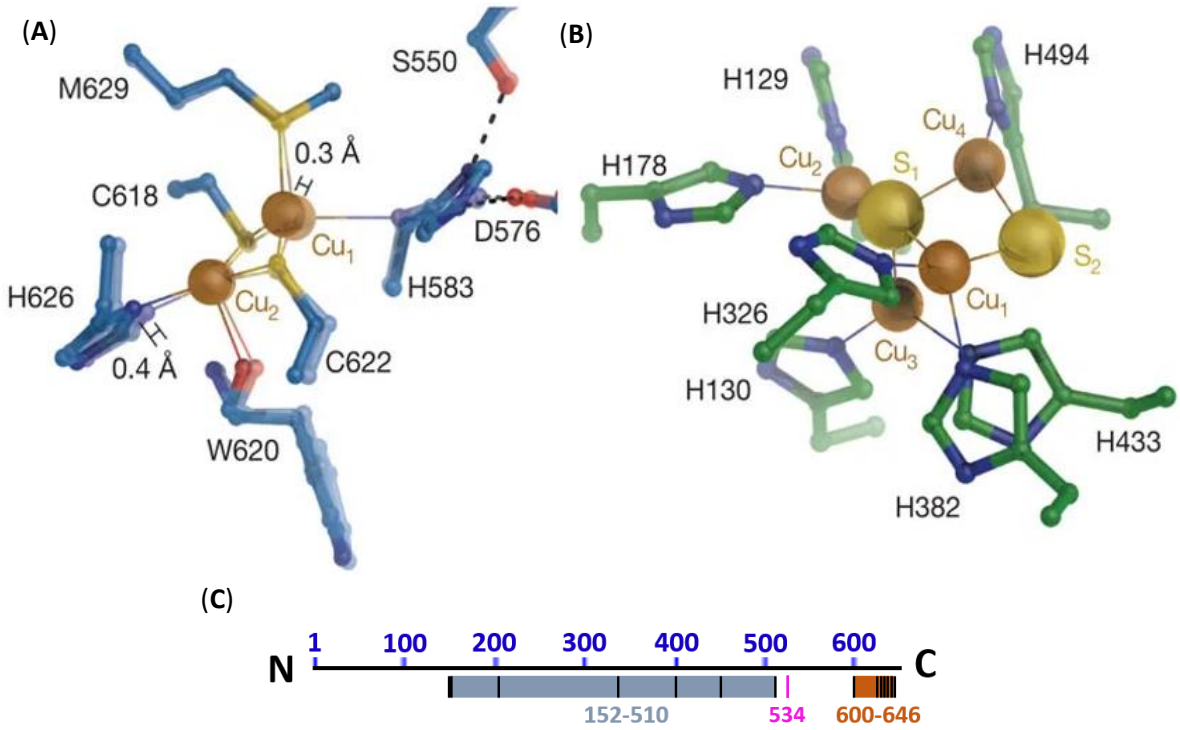


Figure 9: NosZ catalytic site structure.
(**A**) Cu_A site in *P. stutzeri* NosZ. (**B**) Cu_Z site in *P. stutzeri* NosZ. Structures taken from Pomowski et al. (2011). (**C**) *N. gonorrhoeae* NosZ protein structure. N: N-terminus, C: C-terminus, Cu_Z site in grey, premature stop codon in pink, Cu_A site in brown, Cu-binding residues as black bars.

While various enzymes generate N_2O, the bacterial NosZ is the only known enzyme which uses N_2O as a substrate (Spiro, 2012); there is no other enzyme capable of compensating for the loss of NosZ. Hence, NosZ is attracting attention from environmental researchers due to the greenhouse gas activity of N_2O, which is the third most important greenhouse gas after carbon dioxide and methane (Davidson and Kanter, 2014).

A previous study identified the product of NO_2^- reduction in microaerobic gonococcal cultures as N_2O via gas chromatography mass spectrometry, suggesting that if the gonococcal NosZ is present, it has minimal function (Lissenden et al., 2000). In their study, Lissenden et al (2000) estimated that conversion of as little as 0.1% of the NO_2^- to N_2 would have been detected.

In the absence of NosL, NosZ is suggested to be able to obtain Cu from alternative Cu chaperones or simply direct from the extracytoplasmic Cu buffer (Stewart et al., 2019). Cu^+ ions from the reducing cytoplasm are delivered to NosZ in the oxidising periplasm, where they are oxidised to Cu^{2+} ions along with the concurrent reduction of N_2O.

10.8.1.2.1 NosZ catalytic mechanism

NosZ catalyses the reduction of N_2O: $N_2O + 2H^+ + 2e^- \rightarrow N_2 + H_2O$, accepting two respiratory electrons. N_2O is a weak ligand with a low binding energy and has a linear gas phase geometry. However, the electronic structure of Cu_Z provides a strategy to overcome the reaction barrier of N_2O reduction by conformational change of the N_2O ligand.

The mature NosZ homodimer contains two catalytic centres shared between the two monomers. Each catalytic site is comprised of a Cu_A centre from one monomer and a neighbouring Cu_Z centre from the other monomer, separated by ~10 Å. The binuclear Cu_A centre is thought to accept electrons produced by respiration from NosR (via a *c*-type cytochrome or pseudoazurin) and then transfer them to the neighbouring Cu_Z centre. The tetranuclear Cu_Z centre is thought to be the catalytic site of N_2O reduction. This Cu_Z site contains a μ_4-sulfide bridged tetranuclear cluster coordinated by seven histidine residues and a weakly bound H_2O ligand at the Cu_1/Cu_4 edge, which is the substrate-binding site (Ghosh et al., 2003).

Various models have been proposed for the mechanism of N_2O reduction in NosZ (Ghosh et al., 2003; Gorelsky et al., 2006; Johnston et al., 2017). We will only provide a general summary here. Firstly, N_2O is coordinated by the fully reduced Cu_Z site in a linear end-on fashion with a coordinating H_2O molecule (Ghosh et al., 2003). The Cu_Z site has a terminal H_2O ligand coordinated to Cu_4, stabilized by a hydrogen bond to a nearby Lys residue (Johnston et al., 2017). The active Cu_Z cluster then turns off binding to the H_2O ligand and the O atom of N_2O in the Cu_Z-N_2O complex is subsequently activated for proton-assisted electrophilic N-O bond cleavage via a bent N_2O-Cu_Z bridging complex (Ghosh et al., 2003). In *Achromobacter cycloclastes*, the proton donor is thought to be the Lys412 residue near the edge of the Cu_Z cluster (Xie et al., 2015) while in *M. hydrocarbonoclasticus* the proton donor is thought to be Lys397 (Johnston et al., 2017). After the N−O bond cleavage, electron transfer from the neighbouring Cu_A return the Cu_Z cluster to the fully

reduced (Cu_4^+) state for future turnover (Gorelsky et al., 2006). Additional studies reveal a high degree of participation by a sulphur in Cu_Z site redox changes (Rathnayaka et al., 2020).

In the aerobic denitrifier *Paracoccus denitrificans*, either cytochrome c_{551} or the Cu electron-transfer protein pseudoazurin are possible physiological electron donors to NosZ (Berks et al., 1993). Electron donors to *Paracoccus denitrificans* NosZ are the heme-Fe cytochrome c_{550} and pseudoazurin (Felgate et al., 2012). Pseudoazurin has also shown to be capable of mediating electron transfer to the NosZ from *Achromobacter cycloclastes* (Fujita et al., 2012). While *N. gonorrhoeae* does possess cytochrome c_{551} and c_{550}, it is not thought to possess pseudoazurin, and no significant similarity to FA1090 was found in a BLASTn search of the pseudoazurin from *N. mucosa* C102.

10.8.1.2.2 NosZ classes

There are two main classes of NosZ: zNos (clade I, typical), and cNos (clade II, atypical). The common z-type NosZ is the homodimer found in *Neisseria gonorrhoeae*, containing Cu_A and Cu_Z sites. The less common c-type variant of NosZ is found in *Wolinella succinogenes* and related species. This c-type enzyme is characterized by an additional C-terminal mono-haem cytochrome *c* domain. Export to the periplasm is by the Sec secretory system rather than the Tat pathway used by the z-type Nos. Electron transfer to the c-type Nos involves membrane associated iron–sulphur proteins (NosG and NosH) which mediate electron transfer between the quinone pool and NosZ, perhaps via small soluble periplasmic *c*-type cytochromes (Spiro, 2012; Zumft and Kroneck, 2007).

10.8.1.3 NosZ truncation under Cu-limiting conditions.

There is evidence to suggest the periplasmic pool of Cu is filled by supply from the cytoplasm. *Rubrivivax gelatinosus* NosZ activity has been shown to depend on CtpA, a P-type ATPase that resembles known bacterial Cu-efflux transporters (Hassani et al., 2010). The closest homolog to CtpA in *N. gonorrhoeae* FA1090 is CopA, which exports Cu into the periplasm.

A truncated form of *Paracoccus denitrificans* NosZ comprising the 50 kDa N-terminal Cu_Z domain is synthesized in NO_3^--rich Cu-depleted cultures which can still reduce N_2O at ~40% the rate of the Cu-

rich cultures. Even under Cu-rich conditions, *P. denitrificans* NosZ is vulnerable to proteolysis since the Cu_Z 50 kDa band can also be detected, although at much lower levels than the 65 kDa full length polypeptide (Felgate et al., 2012). The authors hypothesised that the truncated 50 kDa Cu_Z-only domain has a continued activity in the periplasm in the absence of the 15 kDa Cu_A domain by receiving electrons from another periplasmic electron transport protein such as cytochrome c_{550}. This would represent the replacement of a Cu-dependent electron delivery system with an Fe-dependent system in response to Cu-depletion. The findings of Felgate et al (2012) informed the rationale of this project; we hypothesised that loss of the NosZ C-terminus by nonsense mutation will not necessarily result in a loss of protein function.

The entire *P. denitrificans* NosZ 595-amino acid sequence is predicted to be 66 kDa, the 470 aa N-terminal region up to the codon analogous to the FA1090 stop codon is 52 kDa, and the subsequent 124 aa C-terminal region after the codon analogous to the FA1090 stop codon is 14 kDa (Peptide Protein Research Ltd, 2020). This suggests the truncation point on NosZ described by Felgate et al., 2012, will be similar to the premature stop codon seen in gonococcal NosZ. The gonococcal NosZ N-terminus with signal peptide will be 58 kDa, 54.5 kDa N-terminus without, and the C-terminal domain 13.5 kDa.

10.8.1.4 NosDFY

Part of, NosF and NosY were once thought to form an ABC transporter where NosY is an inner membrane-spanning protein, NosF is a cytoplasmic ATPase, and NosD is a periplasmic protein which inserts sulphur into the NosZ apoenzyme (Holloway et al., 1996). However, more recent studies suggest *Pseudomonas stutzeri* NosDFY acts as a mechanical energy transducer, not a transporter. It is thought to link cytoplasmic ATP hydrolysis with a conformational transition of the periplasmic NosD subunit, which is required for NosDFY to switch its interaction partner such that Cu is delivered from the NosL chaperone to NosZ (Müller et al., 2022).

10.8.1.5 NosL

NosL is a lipoprotein which is proposed to be the Cu chaperone associated with Cu_Z assembly (Bennett et al., 2019; Zumft and Kroneck, 2007). As part of this study, the datasets of two previous gonococcal proteomics studies (El-Rami et al., 2019; Zielke et al., 2014)were searched for NosZ. Both studies showed the presence of gonococcal NosL but not NosZ, suggesting NosL may have some additional unknown role as a Cu chaperone in *N. gonorrhoeae*.

10.8.1.6 NosX

In *gonorrhoeae*, the *nosX* gene is distant to the *nos* operon. NosX is a member of the ApbE protein family and is suggested to be a periplasmic redox partner to NosR via flavin donation (Zhang et al., 2017) as well as being implicated in the formation of the Cu_A and Cu_Z centres in *P. denitrificans* (Saunders et al., 2000; Wunsch et al., 2005). Thus, it is thought NosX is a NosR maturation factor, so is only involved with N_2O reduction indirectly (Bennett et al., 2020).

In *N. gonorrhoeae*, *N. meningitidis*, *N. lactamica*, *N. cinerea* and *N. mucosa*, *nosX* is distant to the rest of the operon (Barth et al., 2009a). In FA1090, *nosX* is 43,000 bp downstream of *nosL*. *Paracoccus denitrificans nosX* gene is essential for N_2O reduction but not for assembly of Cu centres of NosZ (Bennett et al., 2020).

The roles of Nos proteins are summarised in Table 1 and Figure 10.

Table 1: The *nos* operon gene products of *Neisseria gonorrhoeae* FA1090.

Gene	Protein; function	Genome annotation ID
nosR	NO_2^-/•NO sensor; regulates denitrification genes/NosZ electron donor.	NGO_07420/NGO_07415
nosZ	N_2O reductase enzyme	NGO_07410/NGO_07405
nosD	Cu delivery complex subunit; periplasmic NosZ binding protein	NGO_07400/NGO_07395
nosF	Cu delivery complex subunit; cytoplasmic ATPase	NGO_1399
nosY	Cu delivery complex subunit; inner membrane spanning protein	NGO_1398
nosL	Chaperone; NosZ Cu insertion into Cu_Z site	NGO_1397
nosX	Periplasmic flavoprotein; NosR electron donor	NGO_07165
nosA	TonB-dependent Cu receptor/outer membrane Cu porin	(not found in *N. gonorrhoeae*)

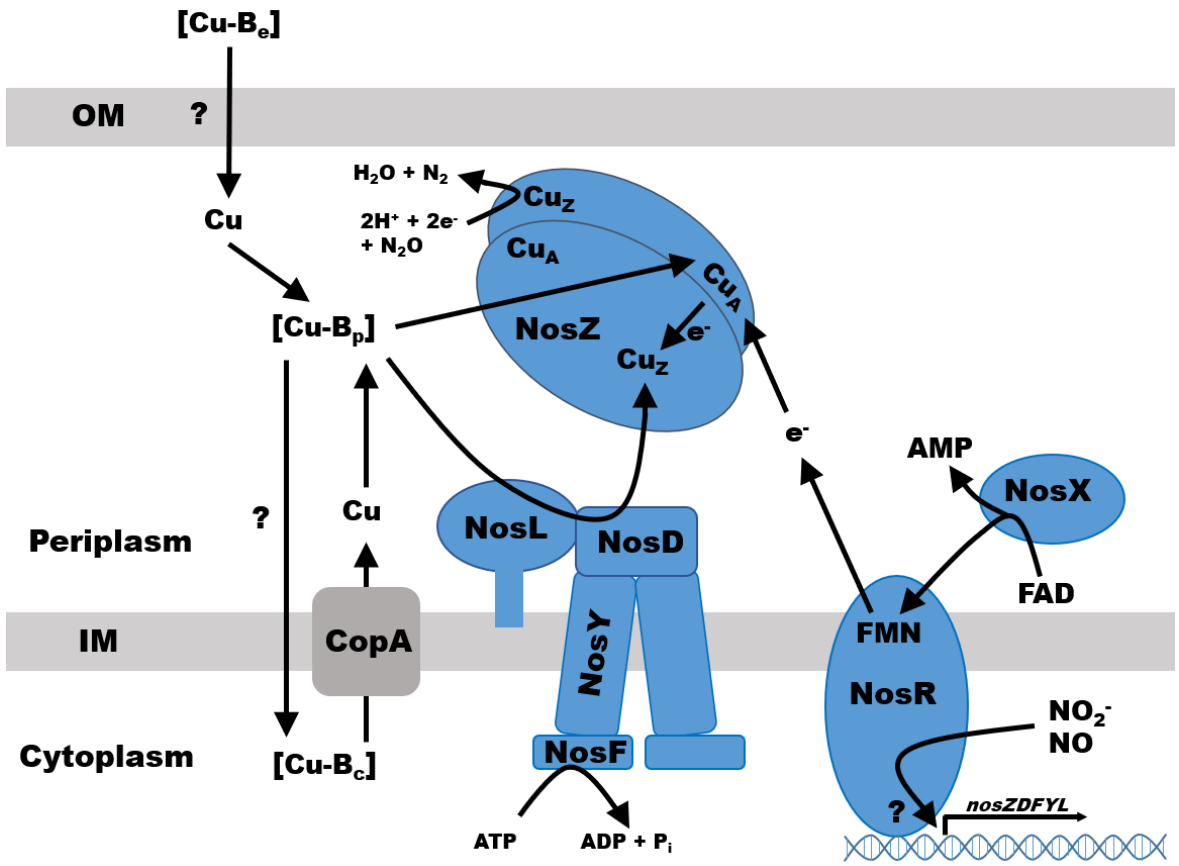


Figure 10: Auxiliary proteins involved in N_2O reduction.
Based on Müller et al (2022) and Zhang et al (2017).

10.8.2 Regulation of denitrification in *Neisseria gonorrhoeae*

Gene expression of denitrification is controlled by various environmental signals, with the key transcriptional activator being low O_2 (Bueno et al., 2017; Fernández et al., 2019; Torres et al., 2017). These signals are sensed by multiple transcriptional regulators, most notably FNR-like proteins (Djoko et al., 2012). Regulation of Neisserial denitrification is highly conserved, with the exception of the NO_2^- sensor NarQ, which is constitutively active in *N. gonorrhoeae* (Barth et al., 2009a).

We discuss the known denitrification regulators across all organisms (Figure 11A), regulators thought to be present in *N. gonorrhoeae* (Figure 11B), regulators which may yet be present in *N. gonorrhoeae* (Figure 11C), and regulators thought to be absent in *N. gonorrhoeae* (Figure 11D).

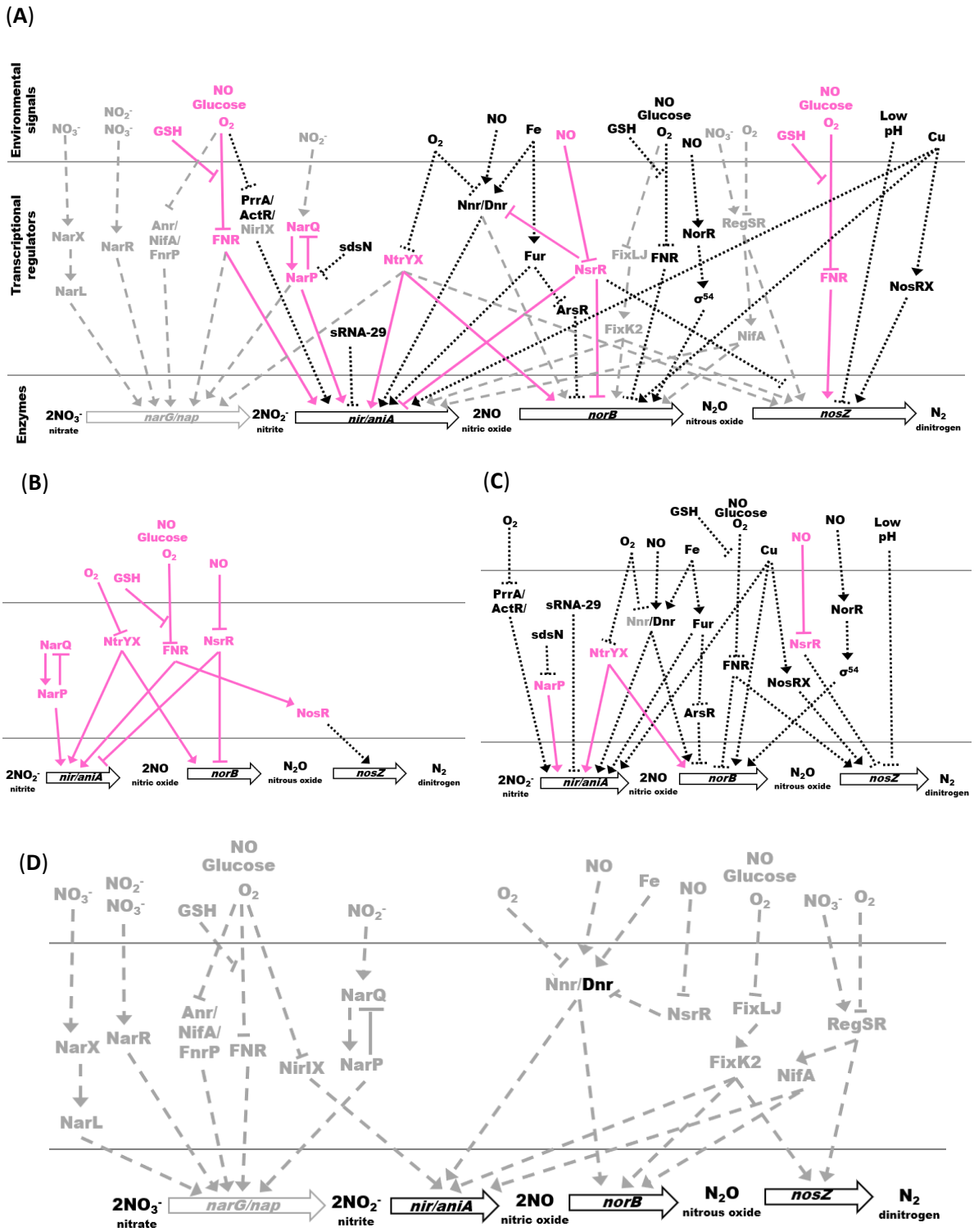


Figure 11: Regulation of bacterial denitrification.
(**A**) A compendium of regulatory denitrification pathways across all organisms. (NB: Not all interactions are present in all denitrifiers and no known organism has all pathways). Pink solid pathways are thought to be present in *N. gonorrhoeae*. Grey dashed pathways are thought to be missing in *N. gonorrhoeae*. Black dotted pathways may be present in *N. gonorrhoeae* but have yet to be investigated. (**B**) Pink solid pathways are thought to be present in *N. gonorrhoeae*. (**C**) Black dotted pathways may be present in *N. gonorrhoeae*. (**D**) Grey dashed pathways are thought to be absent in *N. gonorrhoeae*.

10.8.2.1 Oxygen sensors

While studies have shown that NosZ and the periplasmic NO_3^- reductase Nap can be expressed under oxygenated conditions in some bacterial species (Bueno et al., 2012; Torres et al., 2016), since

denitrification is less energy-efficient than aerobic respiration, most denitrifying bacteria (denitrifiers) will only express denitrification genes in the absence of O_2 and in the presence of NO_x (Torres et al., 2016). This study suggests, like a*niA*, gonococcal *nosZ* is induced in microaerobic cultures only in the presence of NO_2^- (Chapter 4, Figure 30). This is consistent with previous findings in other species (He and McMahon, 2011; Li et al., 2014, 2012; Park et al., 2017; Torres et al., 2016).

The most important type of O_2 sensor involved in denitrification are FNR (fumarate and nitrate reduction regulator) family sensors (Gaimster et al., 2018). The FNR family of transcriptional regulators include various proteins across different bacterial clades. There are various proteins related to FNR, including, NnrR, Fix, Anr, Dnr and FnrP all of which work in a similar fashion to activate denitrification genes. FNR is a transcriptional regulator inactivated by O_2, $^{\bullet}NO$ and glucose, and reactivated by glutathione (GSH) (Gaimster et al., 2018; Spiro and Guest, 1990; Unden and Schirawski, 1997). *B. subtilis* FNR activates *nar* (Reents et al., 2006). *P. denitrificans* FNR activates both *nar* and *nos* (Van Spanning et al., 1997). In *E. coli*, FNR activates *nar* and *nap* (Stewart and Bledsoe, 2005). Expression of gonococcal *aniA* (the major anaerobically induced outer membrane protein) is repressed by O_2 (Mellies et al., 1997), but is activated during O_2-limited growth by FNR (Householder et al., 1999; Whitehead et al., 2007). Of importance to this study, the gonococcal *nosR* gene is also thought to be upregulated by FNR (Whitehead et al., 2007).

FNR binds to a partially palindromic nucleotide sequence, the FNR box; anaerobox (Körner et al., 2003). The FA1090 FNR box is similar to that of *E. coli*, TTGATNNNNATCAA (Edwards et al., 2012; Whitehead et al., 2007), and while *N. gonorrhoeae* lacks *nar*, there is an FNR box upstream of FA1090 *aniA* (**TTGAC**TTAA**ATTAA**) and *nosR* (**TTGAT**TTGC**ATCAA**). There is a similar sequence upstream of FA1090 *norB* (**TGAAT**TTTA**AACAA**), but this sequence overlaps with the NsrR box. While some studies find gonococcal *norB* expression to not be regulated by FNR or NarP (Householder et al., 2000), others suggest *norB* is repressed by FNR (Whitehead et al., 2007). It should be noted that an FNR box is not found in the vicinity of *nosX*.

The *P. denitrificans* FNR homolog NnrR (NO_2^- reductase and $^{\bullet}NO$ reductase regulator) activates *nir* and *nor* in response to $^{\bullet}NO$ and is inactivated by O_2 (Bergaust et al., 2012; Lee et al., 2006b; Van Spanning et al., 1995). Nnr may also be dependent on Fe (Lee et al., 2006b). The *P. denitrificans* Nnr binding site is TTGACNNNNATCAA; very similar to the FNR box (Hutchings and Spiro, 2000). *Brucella melitensis* Nnr is involved in activation of *nos* in addition to *nir* and *nor* (Haine et al., 2006). *N. gonorrhoeae* is thought to lack Nnr (Householder et al., 2000).

Rhizobial species such as *Bradyrhizobium diazoefficiens* contain the two-component FixLJ-$FixK_2$ regulatory complex which senses low O_2 to induce expression of *nir*, *nor* and *nos* in a regulatory cascade which includes NnrR (Torres et al., 2017). *N. gonorrhoeae* is thought to lack FixLJ-$FixK_2$ – it is not listed in the FA1090 genome annotations. A BLASTp search reveals the closest gonococcal homolog to *B. japonicum* FixK2 is FNR. A BLASTn search finds no homology between *B. japonicum fixK2* and any gonococcal or meningococcal gene. Hence, we may assume *N. gonorrhoeae* lacks FixLJ-$FixK_2$. Anr is a *Pseudomonas aeruginosa* FNR analogue which activates *nar* and *dnr* in response to low O_2 (Arai et al., 1997; Schreiber et al., 2007).

While bacteria can sense O_2 directly via cytoplasmic transcription factors (e.g. Fnr) or membrane protein receptors (e.g. FixL), another type of O_2 sensing is via changes in redox state of molecules within the cell. Redox responsive regulatory systems respond to various redox-active molecules (e.g. quinones, cysteine) to regulate respiratory mechanisms (Bueno et al., 2012). Redox-sensitive two-component regulatory systems are present in various bacterial clades (RegBA, PrrBA, ActSR, RegSR, RoxSR) (Swem et al., 2001; Torres et al., 2016).

Bradyrhizobium japonicum RegSR activates *nor* and *nos* in response to NO_3^- and low O_2 via redox sensing (Torres et al., 2014). RegSR is thought to form a regulatory cascade with NifA to activate *nap*, *nir* and *nor* in response to low O_2 (Bueno et al., 2010; Sciotti et al., 2003). A BLASTp search reveals the closest gonococcal homolog to *B. japonicum* RegR is an unannotated regulator protein which appears disrupted (64% query cover, 43% identity). A BLASTp search of the *B. japonicum* RegS

against the gonococcal proteome finds no homologs. Hence, we may assume that *N. gonorrhoeae* lacks RegSR. NifA is not listed in the FA1090 genome annotations. A BLASTp search reveals no gonococcal homologs to *B. japonicum* NifA. A BLASTn search reveals no homology between *B. japonicum nifA* and any gonococcal or meningococcal gene. Hence, we may assume *N. gonorrhoeae* lacks NifA.

Rhodobacter sphaeroides PrrA and *Agrobacterium tumefaciens* ActR act as redox sensors to activate *nir* in response to low O_2 (Baek et al., 2008; Laratta et al., 2002). *Brucella* NtrYX acts as a redox sensor to activate expression of *nar*, *nir*, *nor* and *nos* in response to low O_2 (Roop and Caswell, 2012). In *N. gonorrhoeae* microarray analysis, NtrYX was found to activate *nir* and *nor*, but not *nos* (Atack et al., 2013). FA1090 *ntrX* is annotated NGO_1866 in the FA1090 genome; a BLASTp comparison reveals high homology to the *Brucella suis* NtrX. The *P. denitrificans* FnrP similarly activates *nar* in response to redox state (Van Spanning et al., 1997). *P. denitrificans* NirI-NirX are homologues of NosR-NosX**.** NirI activates *nir* in response to low O_2 and the presence of NO_x (Saunders et al., 1999).

10.8.2.2 NO_3^- and NO_2^- sensors

While NO_3^-, NO_2^-, and $^{\bullet}NO$ are all sensed by denitrifying bacteria to regulate denitrification, no N_2O sensor has yet been found, presumably because it is an inert and nontoxic gas. Instead, denitrifiers appear to induce *nos* expression in response to elevated NO_3^- and $^{\bullet}NO$. $^{\bullet}NO$ sensors are common, presumably as a mechanism to prevent accumulation of this toxic intermediate (Spiro, 2012).

Bacterial NO_3^-/NO_2^- sensors are various, including NarXL, NarQP, Nar and RegSR. Expression of *nar* in β- and γ-proteobacteria (including *Neisseria*) is activated by two homologous two-component regulatory systems, the membrane-bound sensors NarX and NarQ (which sense NO_3^- and NO_2^-) and DNA binding response regulators NarL and NarP (Egan and Stewart, 1991; Stewart and Rabin, 1995). Full activation of gonococcal *aniA* requires NarP-NarQ in addition to FNR (Whitehead et al., 2007). While studies suggest that *N. meningitidis* NarP is activated by NO_2^- (Rock et al., 2005), gonococcal NarPQ has been suggested to be constitutively active rather than a NO_2^- sensor (Overton et al., 2006). Gonococcal NarP represses *narQ* expression in an autoregulatory feedback (Overton et al.,

2006). The consensus *E. coli* binding site for NarP, TACYNMTNNAKNRGTA, found no matches in the gonococcal FA1090 genome. However, potential NarP-binding sites (at least a 12/14 match to the *E. coli* consensus NarP binding site) have reportedly been identified within 400 bp upstream of *aniA*, *accA*, *norB*, and *narQ* in the gonococcal F62 strain (Overton et al., 2006).

Interestingly, in *P. aeruginosa*, NarL represses the periplasmic NO_3^- reductase, Nap (Van Alst et al., 2009). NarR (NO_2^- reductase regulator) is another FNR-like protein in α-proteobacteria which activates *nar* in response to NO_3^- and NO_2^- in place of NarXL (Bueno et al., 2012; Wood et al., 2001).

10.8.2.3 •NO sensors

Multiple •NO sensors have been linked to denitrification, including NsrR, FNR, NorR , NnR and Dnr. Of these sensors, NsrR is the most important. NsrR boxes are found upstream of the gonococcal *nos* and *nor* operons in addition to FNR boxes (Edwards et al., 2012; Isabella et al., 2009; Rodionov et al., 2005). NsrR box sequence consensus varies; it has been described as a 29 bp inverted repeat sequence `CCATTAAGGTATTTTCCGTGAAACTTAAA` (Isabella et al., 2009), as `gATTCATNNNNNATrAATc` (Rodionov et al., 2005), and as `TTTAANATTCNANNNTNTGAATNNTAAA` (Edwards et al., 2012). In *N. gonorrhoeae*, NsrR is inhibited by •NO and is a repressor of *aniA* and *norB*, acting to prevent FNR-dependent gene expression under aerobic conditions, since FNR in *Neisseria* supposedly retains considerable activity aerobically (Heurlier et al., 2008; Overton et al., 2006; Rock et al., 2007). NsrR also represses *dnr* (Overton et al., 2006). A sequence similar to the Rodionov sequence (`vATTCATAhwwwrwsAAyh`) is found in the FA1090 genome between the FNR and Fur (ferric uptake regulator) boxes upstream of *aniA*, *norB*, *nosR* and *dnr*. Hence, we hypothesise NsrR may also regulate *nos*. No such binding site is found in the vicinity of gonococcal *nosX*, raising questions as to how gonococcal *nosX* transcription is initiated. Bacterial operons are polycistronic (wherein multiple proteins are encoded on a single molecule of mRNA), so *nosX* may be under the control of a different operon. There is a FNR box 1,392 bp upstream of *nosX*, separated from *nosX* by NGO_1357 (thought to encode a thymidylate synthase involved in nucleotide synthesis) and NGO_07165 (thought to encode an ApbE-family FMN transferase involved in protein PTM).

NorR activates *Ralstonia eutropha norB* in response to •NO (RpoN)(Pohlmann et al., 2000). This activation is mediated by the sigma factor σ^{54}. In *Pseudomonas stutzeri*, sigma factor σ^{54} (encoded by the *rpoN* gene) is suggested to play a role in post-translational processing of both AniA/NirK and NorB (Härtig and Zumft, 1998). The *P. aeruginosa* Dnr regulator (dissimilatory nitrate respiration regulator) activates transcription of *nir* and *nor* in response to •NO (Arai et al., 1997). DnrD, a Fnr family regulator, enhances *Pseudomonas stutzeri nos* operon expression under anaerobic conditions in response to •NO (Honisch and Zumft, 2003).

10.8.2.4 Other miscellaneous sensors: metals, pH

All four denitrification enzymes require a metal to function. NosZ is Cu-dependent, requiring 12 atoms per functional homodimer. Various studies show that Cu limitation represses *Paracoccus denitrificans* NosZ (Felgate et al., 2012; Sullivan et al., 2013; Woolfenden et al., 2013). Cu limitation also represses *B. diazoefficiens nir* and *nor* in addition to *nos* (Pacheco et al., 2023). In coastal wetland sediment, addition of natural chalcopyrite mitigates N_2O emissions (Yang et al., 2023). Addition of Cu increases N_2O reduction in wetland soils and stream sediments (Sharma et al., 2022). In *P. denitrificans*, Cu has been found to activate *nos* expression (Sullivan et al., 2013), while Zn depletion activates *nir* and *nor* (Neupane et al., 2017). The NosR (N_2O reductase regulator) is required for *Pseudomonas stutzeri nos* activation (Cuypers et al., 1992), and in *P. denitrificans* is Cu-dependent (Sullivan et al., 2013).

Fur is an Fe sensor involved in expression of gonococcal *aniA* and *norB* (Edwards et al., 2012; Jackson et al., 2010). The gonococcal Fur box is `TATTAGAAGCGTCATTTTA` (Edwards et al., 2012) and is found 116 bp upstream of FA1090 *aniA* but is not found directly upstream of *norB.* However, the two operons are back-to-back-adjacent so may share regulators. See Table 29 for list of putative regulatory box sequences affecting denitrification genes. Fur activates gonococcal *norB* by preventing the binding of a ArsR repressor homologue (Isabella et al., 2008).

Low pH inhibits N_2O reduction in soil bacteria (Šlmek and Cooper, 2002), which likely occurs by inhibiting NosZ enzyme assembly rather than inhibiting catalysis or repressing *nosZ* transcription (Bergaust et al., 2010; Fujita and Dooley, 2007; Liu et al., 2014).

Studies have suggest sRNAs may also play a role in regulating denitrification (Gaimster et al., 2018). The *E. coli* sRNA sdsN is induced in stationary phase when cells are grown with ammonium and represses NarP (Hao et al., 2016). The sRNA-29 (also known as DenR) is another sRNA which represses *P. denitrificans nir* (Gaimster et al., 2019).

10.8.2.5 Summary of denitrification regulation

In summary, gonococcal *nosZ* expression is possibly activated by FNR-NosR in response to low O_2 and $^{\bullet}NO$. Once translated, gonococcal NosZ likely requires Cu and a moderate pH to correctly assemble and function. Based on an upstream binding site, NsrR may also regulate gonococcal *nos*.

10.9 Concentrations of denitrification substrates in the host

The availability of respiratory electron acceptors is a major factor limiting the growth of human bacteria (Moir, 2011b). The nitrogen oxides NO_3^- and NO_2^- are present in the human diet and are reduced to the gases NO and N_2O. Concentrations of NO_x have been measured in different locations of the human body (Table 2). However, there is a lack of data for the urinary concentration of the gaseous $^{\bullet}NO$ and N_2O substrates.

Table 2: NOx denitrification substrate concentrations in the human host

NO_X/Location	**Concentration (μM)**	**References**
NO_3^-		
Urine (healthy)	250-2600	Chao et al., 2016; Green et al., 1982, Radomski et al., 1978; Xia et al., 2003
Urine (UTI)	360	Chao et al., 2016
Blood	30–60	Kelm, 1999
Seminal plasma	295-900	Arai and Iiyama, 2013
Saliva	60-1600	Mirvish et al., 2000; Xia et al., 2003
NO_2^-		
Urine (healthy)	0-1	Chao et al., 2016; Lundberg et al., 1997; Xia et al., 2003
Urine (UTI)	15	Chao et al., 2016
Blood	0.1–0.5	Kelm, 1999
Seminal plasma	10-25	Revelli et al., 2001
Saliva	100-1000	Mirvish et al., 2000; Xia et al., 2003
$^{\bullet}NO$		
Blood	0.003–1	Kelm, 1999
Seminal plasma	20-40	Aksoy et al., 2000; Mehraban et al., 2005
Vagina	0.3	Sioutas et al., 2008
N_2O		
Urine (healthy)	0.022	Brugnone et al., 1996
Blood	0.021	Brugnone et al., 1996

10.9.1 Nitrate

In humans, NO_3^- is ingested in food (mainly leafy vegetables) (Karwowska and Kononiuk, 2020), absorbed in the gastrointestinal tract, and is reduced to NO_2^- by oral normal microbes (Moir, 2011b; Olin et al., 2001). In the typical western diet, an individual will consume 1–2 mmols of NO_3^- per day,

with a long half-life of 5-8 hr (Gilchrist et al., 2011; Hord et al., 2009). Dietary NO_3^- is excreted in urine.

Total NO_3^- and NO_2^- concentration in vaginal secretions has been determined by treatment with NO_3^- reductase followed by the Griess reaction, and found to be in the region of 0-6 µM in healthy pregnant women (Nakatsuka et al., 2000).

10.9.2 Nitrite

NO_2^- is produced endogenously through the oxidation of •NO and reduction of NO_3^- by commensal bacteria in the mouth and gastrointestinal tract (Duncan et al., 1995; Moir, 2011b). Another major source of NO_2^- is in processed meats in the diet (Karwowska and Kononiuk, 2020). Both NO_2^- and •NO are bacteriostatic due to their ability to inhibit protein activity, although the antibacterial effect of NO_2^- is largely attributed to •NO formation (Guo and Gao, 2021). NO_2^- has a rapid half-life in animal models as it is soon oxidized to NO_3^-, meaning NO_2^- is present in lower concentrations than NO_3^- in the host (Gilchrist et al., 2011).

During infection with NO_3^--reducing bacteria is reduced to NO_2^- (Carlsson et al., 2003). Since urine contains sufficient NO_2^- to be used as a diagnostic for urinary tract infections, it has been suggested that *N. gonorrhoeae* may encounter NO_2^- in the genital mucosa intermittently through the flushing of urine (Barth et al., 2009a). However, NO_2^- and NO are usually not detectable in the urine of healthy individuals (Green et al., 1982) and it remains unknown as to the exact concentration of NO_2^- in the gonococcal niche. In some studies, urine NO_2^- was undetectable (Xia et al., 2003). As such, it is possible that the 2 mM NO_2^- utilised in this study was higher than the physiological concentration.

10.9.3 Nitric oxide

Both NO_3^- and NO_2^- are reduced to •NO in the gut (Moir, 2011b). •NO is a toxic, O_2-depleting free radical gas with diverse biological roles in eukaryotes and bacteria, including: vasodilation, blood clotting, immune regulation, and as an innate toxic defence molecule (Tripathi, 2007; Tucker et al., 2010; Wink et al., 2011). •NO toxicity results from binding to or reaction with a range of cellular components, with metalloproteins being especially vulnerable (Cole, 2021; Poole, 2005).

Due to the toxicity of •NO, the cell steady state concentration of free •NO during denitrification is maintained at a very low level (Thomas et al., 2008). The generation of •NO by AniA and reduction of •NO by NorB both occur in the periplasm, preventing •NO from reaching the cytoplasm. The physiological concentration of •NO inside human cells has been estimated to range from 100 pM to 5 nM (Hall and Garthwaite, 2009). However, macrophages generate •NO in response to infection as part of the innate immune response (Palmieri et al., 2020), and may be a source of •NO for gonococcal NorB. Both the nasopharynx and genital mucosa niches may provide host-derived •NO (Bogdan, 2001; Stefano et al., 2000).

While •NO is produced by the host immune system to kill invading microbes (Coleman, 2001), there is evidence to suggest that host-derived •NO does not exert a bactericidal effect on *N. gonorrhoeae*, and instead plays an important role in promoting gonococcal intracellular survival (Edwards, 2010). Hence, reduction of •NO may be less vital for virulence in *N. gonorrhoeae* compared to other bacteria, such as *P. aeruginosa* (Arai and Iiyama, 2013) *Brucella suis* NorD (Loisel-Meyer et al., 2006), and *R. solanacearum* (Dalsing et al., 2015). It has been suggested that *N. gonorrhoeae* maintains a steady state anti-inflammatory concentration of •NO (Barth and Clark, 2008; Barth, 2009; Barth et al., 2009b). As such, minimal reduction of •NO in *N. gonorrhoeae* may mean a low concentration of N_2O, reducing the concentration of the NosZ substrate.

•NO is freely diffusible and will spread from a producing cell to neighbouring cell within its biological half-life (Kelm, 1999; Lancaster, 1997). •NO levels have been measured in air incubated for five minutes in a vaginal catheter balloon, and were found to be 10 ppb (333 nM) in healthy individuals (Sioutas et al., 2008). Concentration of •NO in healthy seminal fluid was found to be ~20-40 µM (Aksoy et al., 2000; Mehraban et al., 2005).

10.9.4 Nitrous oxide

Most of the biological interest in N_2O stems from its role as a greenhouse gas. The only known studies measuring N_2O in humans are focused on its use as an anaesthetic, which find very low concentrations in the blood and urine of a control group (Brugnone et al., 1996)

N_2O is the third most important long-lived greenhouse gas, after CO_2 and CH_4 (IPCC, 2014), and is the most important of all ozone-depleting substances (Morgenstern et al., 2018). N_2O accounts for ~6% of the greenhouse effect, and is about 300 times more potent per molecule than CO_2 (IPCC, 2013; Oertel et al., 2016). Globally, 40% of total N_2O emissions come from human activities, and 60-73% of those emissions comes from agriculture (Masson-Delmotte et al., 2021; Syakila and Kroeze, 2011). With the need for greater food production in coming decades, N_2O emissions are likely to rise (Reay et al., 2012). N_2O emissions are currently accelerating beyond targets (Thompson et al., 2019), with some studies predicting an 83% estimated increase by 2050 (Davidson and Kanter, 2014).

The major producer of N_2O in many environments is $^{\bullet}NO$ reductase found in denitrifying bacteria and in some ammonia-oxidizing organisms (Park et al., 2017). Soils are the dominating source for atmospheric N_2O (Butterbach-Bahl et al., 2013), and denitrification is the primary source of N_2O in all soils (Stuchiner and von Fischer, 2022). Wetter soils tend to produce more N_2O due to the lack of dissolved O_2 (Wei et al., 2022). Denitrification in soil bacteria is generally not wanted, since it depletes the soil of NO_3^-, which plants need to grow, and produces N_2O (Aulakh et al., 1992). Since N_2O is largely inert, it can be tolerated by bacteria in high quantities.

Many agricultural soils are high in nitrogen thanks to modern fertilizer but in low Cu environments, NosZ fails to function, leading to a rise in N_2O emissions (Felgate et al., 2012). This had led some to suggest soil Cu supplementation as a bioremedial strategy for reducing N_2O emissions (Shen et al., 2020).

10.10 Project rationale summary: NosZ may be present in *N. gonorrhoeae*

Genome sequencing has revealed premature stop codons in the gonococcal *nosR*, *nosZ* and *nosD* genes, leading to them being classified as pseudogenes (Overton et al., 2006). In the absence of a bacterial nonsense-mediated mRNA decay pathway, as occurs in eukaryotes (Li and Lynch, 2020; Nickless et al., 2017), these premature stop codons will likely only exert their effect at translation, whereby they may generate a truncated NosZ with partial activity (Felgate et al., 2012) or be

readthrough to create a full-length protein (Palma and Lejeune, 2021). Hence, the main hypothesis of this study was that despite the presence of these premature stop codons a functional Nos protein may be present in *N. gonorrhoeae*.

Previous studies of the gonococcal proteome show that while the gonococcal AniA protein is found to be upregulated in microaerobic biofilms, NorB is sometimes not detected as a hit (Baarda and Sikora, 2015; Phillips et al., 2012). Since the NorB protein is known to function in biofilms, we hypothesised there may also be a NosZ protein which has been overlooked in past proteomics studies.

10.11 Discussion: Introduction

N. gonorrhoeae survives a microaerobic niche via denitrification. Gonococci contain genes for three denitrification enzymes: *aniA*, *norB* and *nosZ*. As an obligate human pathogen, *N. gonorrhoeae* acquires both the NO_x and metal ions needed for denitrification from the host.

The gonococcal N_2O reductase gene, *nosZ*, has been historically annotated as a pseudogene due to the presence of a premature stop codon in three *nos* operon genes: *nosR*, *nosZ* and *nosD*. In addition to *N. gonorrhoeae*, the *Neisseria* species *N. meningitidis* and *N. mucosa* are also thought to have inactivated *nos*, suggesting there may be a link between *nos* inactivation and pathogenicity. The gonococcal NosZ protein has only been experimentally investigated once previously, and is thought to have minimal catalytic function (Lissenden et al., 2000). However, the presence or absence of a gonococcal NosZ protein has not yet been determined.

Following recent findings surrounding the widespread readthrough of premature stop codons and the regulatory roles of apparent pseudogenes (Prieto-Godino et al., 2016), we hypothesised that the gonococcal *nos* operon may still have functionality. This study aimed to revisit the *nos* operon of *Neisseria gonorrhoeae* and assess its potential for stop codon readthrough.

11 Chapter 2: Materials and Methods

All reagents were analytical grade supplied by Sigma-Aldrich or Melford unless stated otherwise. All experiments involving bacterial cultures were repeated in three independent biological replicates (n = 3) unless stated otherwise (i.e. different overnight cultures, different daytime cultures, different media). Cultures were incubated in a Stuart Orbital Incubator S1600 at 37 ^{o}C at 0 or 200 RPM. Thermocycling was conducted in a Bio-Rad T100 Thermal Cycler. NEB Taq DNA polymerase (M0273) or Q5 DNA polymerase (M0491) manufacturers protocols were used for PCR. Annealing temperatures for PCR primers were determined using the NEB Tm Calculator (https://tmcalculator.neb.com/#!/main). DNA and RNA was quantified on a ThermoScientific Nanodrop ND-1000 Spectrophotometer for ng/µL concentration and purity (260 nm/280 nm protein contamination and 260 nm/230 nm salt contamination).

11.1 Culture media and supplements

All bacteria strains were stocked in 1X gonococcal base liquid (GCBL) + 20% glycerol (*N. gonorrhoeae*) or lysogeny broth (LB) + 20% glycerol *(E. coli*) and stored at -80 ^{o}C. The formulation of GC agar used was $^{1}/_{3}^{rd}$ standard agar content (see Table 6 for modified "soft" GC agar recipe). The Cu content of GCBL is thought to be ~1-2 µM (K. Djoko, personal communication). Reagent stocks used for bacterial growth media are given in Table 3 and Table 4. Recipes for bacterial growth media are given in Table 6.

Table 3: Reagent concentrations in routine GCBL

Media component	Stock concentration	Working culture/media concentration
Kellogg's I	100X	1X
Kellogg's II	1000X	1X
Sodium bicarbonate	100X; 4.2% w/v; 500 mM	1X; 0.042% w/v; 5 mM

Table 4: GCBL additives

Media component	Stock concentration	Working culture/media concentration
Sodium nitrite	1000X; 2M	1X; 2 mM
$CuSO_4$	10 mM	0, 1 and 10 µM

Table 5: Antibiotics

Media component	Stock concentration	Working culture/media concentration
Chloramphenicol (Cm)	30 mg/mL	30 µg/mL
Spectinomycin (Spc)	100 mg/mL	100 µg/mL

Antibiotics were added to media agar between autoclaving agar and pouring agar plates. Cm was prepared in 100% ethanol (molecular biology grade).

Table 6: Recipes for bacterial growth media

GCBL 20X powder		**Soft GC agar 1L**		**1000X 50 mL 10 mM $FeSO_4*7H_2O$ (Kellogg's II)**
300 g special peptone (Gibco bacto proteose peptone no 3)		24 g Oxoid GC agar base		139 mg $FeSO_4*7H_2O$
100 g NaCl		12 g GCBL 20X powder		
80 g K_2HPO_4				**LB broth 1L**
20 g KH_2PO_4		**100X 100 mL Kellogg's I**		20 g LB broth powder
		40 g glucose		
GCBL 1L (pH ~ 7.3)		1 g glutamine		**LB agar 1L**
26 g 20X GCBL powder		2 mg cocarboxylase		20 g LB both powder
				12 g agar powder

DNA Gel electrophoresis was conducted in 1X TAE buffer. DNA agarose gels were 1% unless otherwise stated and used Invitrogen SYBR Safe DNA Gel Stain.

11.2 Bacterial cultures

11.2.1 Overnight cultures

Unless otherwise stated, "overnight culture" refers to the inoculation of bacteria from frozen -80 °C glycerol stocks onto pre-warmed agar plates and incubation for 16-24 hr at 37 °C. *Neisseria gonorrhoeae* was incubated in an airtight Oxoid Anaerojar containing a wet paper towel and Oxoid CO_2 Gen sachet to create a 6% CO_2/15% O_2 atmosphere. *N. gonorrhoeae* is typically regarded as a capnophile, requiring 3-10% CO_2. This CO_2 requirement is not related to anaerobiosis, and is instead thought to be required for C_4 dicarboxylic acid replenishment (Spence et al., 2008). *Escherichia coli* was incubated in a lidded tupperware containing a wet paper towel.

11.2.2 *N. gonorrhoeae* broth culture conditions

Neisseria gonorrhoea strain FA1090 (W. Shafer, Emory) was used in this study. FA1090 was originally isolated in the 1970s from the endocervix of an Oklahoma woman with probable disseminated gonococcal infection (Hobbs et al., 2011; Nachamkin et al., 1981). *N. gonorrhoeae* was inoculated from frozen GCBL + 20% glycerol stocks onto GC agar supplemented with 1X Kellogg's I and II, as described previously (Dillard, 2011; Spence et al., 2008).

Both shaking and static liquid broth cultures were used in this study. From an overnight agar culture, *N. gonorrhoeae* static liquid cultures (30 mL GCBL in 50 mL Falcon tubes) were inoculated to an initial OD_{600} of 0.1 in GCBL as described previously (Dillard, 2011; Spence et al., 2008). GCBL was pre-warmed to 37 °C before use. Kellogg's I (100X), Kellogg's II (1000X) and 4.2% w/v 100X sodium bicarbonate were added to the GCBL immediately before inoculation such that all were 1X concentrated in the final culture media. Sodium nitrite was supplemented to a final concentration of 2 mM at 0, 5, 7, 8 and 9 hr to support microaerobic respiration without NO_2^- toxicity (Firth, 2023). Daytime cultures were then incubated for 8 or 10 hr at 37 °C. Culture tubes were briefly vortexed and OD_{600} values measured every hr using an Ultrospec 10 cell density meter. A Falcon tube containing uninoculated supplemented media was used as a blank. After 8 or 10 hr, cultures were centrifuged at 4800 RCF for 5 min at 4 °C in a Hettich Rotina 380 R and resuspended into 1 mL of GCBL + 20% glycerol. Cultures of 30 mL in 50 mL tubes are suggested to maintain a low O_2

concentration, which was confirmed experimentally using qPCR for low O_2-responsive FNR-induced genes (Chapter 4, Figure 34).

A no-cells negative control containing just media was incubated alongside all *N. gonorrhoeae* cultures. It was found that the Ultrospec OD_{600} reader used in this study often gave a slightly higher reading than was possible (for instance, blank media was measured to be 0.01 or even 0.02 Abs rather than 0.00). As such, for each OD_{600} reading, the value of the no-cells negative control was subtracted from the experimental culture values to give a better approximation of the actual culture OD_{600}.

From an overnight agar culture, *N. gonorrhoeae* shaking liquid cultures (50 mL in 250 mL conical flasks) were inoculated to an initial OD_{600} of 0.1 in GCBL as described above. Conical flasks were sealed with a tinfoil cap to keep cultures sterile. Shaking cultures were incubated for 8 hr at 37 ^{o}C with shaking at 200 rpm. Sodium nitrite was supplemented to a final concentration of 2 mM at 0, 5 and 7 hr.

11.2.3 F62 *nos* gene sequencing

The *Neisseria gonorrhoeae* F62 strain (Kellogg et al., 1963) was cultured overnight on GC agar. Genomic DNA (gDNA) was extracted by heating cell resuspensions at 95 ^{o}C for 10 min and diluting 20X in GCBL. The genes *nosR*, *nosZ* and *nosD* were amplified from the F62 gDNA extract using NEB Q5 PCR with FA1090 gDNA extract as a positive control and primers listed in Table 7.

Table 7: Primers used to amplify *N. gonorrhoeae nosR*, *nosZ* and *nosD* genes

Name	Primer code	Sequence 5' → 3'
nosR-full-Fv8	328	CGCGGATAACGGCGTTTTTTACC
nosR-full-Rv7	327	TTTCGGGTTTGACAAAACGGACGA
nosZ-full-F	211	ATGTCAGACGAAAAATTAGAACA
nosZ-full-R	212	TTATTTGGGATGAACAATCATC
nosD-full-F	219	TCCGTATATGGCTGAAGGC
nosD-full-R	220	CTAGTTCAAAGAACCGTTTT

PCR products were purified using the NEB Monarch PCR and DNA Cleanup Kit, quantified on a Nanodrop, and sent for Sanger sequencing using primers listed in Table 8.

Table 8: Primers used to confirm F62 strain with Sanger sequencing sequencing

Name	Primer code	Sequence 5' → 3'
nosR-int-F	613	GCTTCCGTTTTACCGATGC
nosR-int-Rv2	614	AGGCGCGCTGATTTCTAC
nosZ-int-F	615	GAAATGCGTCTGGTACACG
nosZ-int-Rv2	616	GGATACTGTAAGCAGGCGC
nosD-int-Fv2	617	TATGTACACCAACGACAGCG
nosD-int-R	618	CGGACAGTTTATCGTAGTTGGC

11.3 Bioinformatics of the *nos* operon

The *N. gonorrhoeae* FA1090 reference genome (NCBI assembly accession: ASM684v1) was used throughout this study. It should be noted that at the time of writing this genome has been superseded by the new *N. gonorrhoeae* reference genome assembly ASM1303007v1 from strain TUM19854.

PaperBLAST (https://papers.genomics.lbl.gov/cgi-bin/litSearch.cgi) was used to search the scientific literature for mentions of the *nosZ* gene (Price and Arkin, 2017).

The *N. gonorrhoeae* FA1090 *nos* operon and individual *nos* gene nucleotide sequences were searched for homology to other species using the NCBI BLASTn tool version 2.15.0+ (Altschul et al., 1990). Gene sequences were visualised and annotated using SnapGene Viewer version 5.2.4. Nucleotide sequences were translated into protein sequences using the ExPASY translate tool (https://web.expasy.org/translate/). Translated protein sequences were searched for homology using the NCBI BLASTp tool version 2.15.0+ (Altschul et al., 1990) and aligned using the ClustalW tool (Thompson et al., 1994) in MEGA X (Kumar et al., 2018). *Neisseria* reference genomes were acquired from NCBI (Table 9).

Table 9: List of *Neisseria* reference genomes used in bioinformatics analysis

Reference genome strain	**NCBI Assembly accession**
Neisseria gonorrhoeae FA1090	ASM684v1
Neisseria meningitidis MC58	ASM880v1
Neisseria mucosa C102	Neis_muco_C102_V1
Neisseria lactamica 020-06	ASM19629v1
Neisseria elongata subsp. glycolytica ATCC 29315	ASM81803v1
Neisseria polysaccharea ATCC 43768	ASM17673v1
Neisseria cinerea ATCC 14685	ASM17389v1
Neisseria subflava NJ9703	ASM17395v1
Neisseria wadsworthii 9715	ASM22776v1
Neisseria shayeganii 871	ASM22687v1
Neisseria macacae ATCC 33926	ASM22086v1
Neisseria bacilliformis ATCC BAA-1200	ASM19492v1

The location of the premature stop codons and alternative start codons on the three-dimensional *N. gonorrhoeae* NosZ protein structure were visualised using the X-ray crystal structure from the

closest protein sequence homolog, *Achromobacter* cycloclastes (PDB: 2IWF; 99% query cover, 3% gaps, 58% identity, 14% similarity). The structure was downloaded from the PDB: https://www.rcsb.org/structure/2IWF (Berman et al., 2003) and visualised using PyMOL (Schrodinger, LLC, 2010). NosZ crystal structures from additional species were superimposed using SWISS MODEL (Waterhouse et al., 2018).

11.4 Extraction of gDNA

N. gonorrhoeae strain FA1090 was grown statically for 8 hr in gonococcal base liquid medium (GCBL), then centrifuged at 4800 RCF for 5 min at 4°C. Then, gDNA was extracted from pellets using the Promega Wizard Genomic DNA Purification Kit with the following modifications made to the kit protocol: 6µL RNase (not 3 µl) and incubation at 55 °C in rehydration solution (not 65 °C). Extracted gDNA was quantified on a Nanodrop and confirmed by NEB Taq PCR using previously validated primers (Table 10, S. Firth). A previously validated gonococcal gDNA extract (S. Firth) was used as a positive control.

Table 10: Primers used to validate *N. gonorrhoeae* gDNA extracts

Name	Primer code	Sequence 5' → 3'
accA-goldengate-insert2-F	173	CAGTTGGGTCTCCAGATGAAAAAATTATTGGCAGCCG
accA-goldengate-insert2-R	174	CAGTTGGGTCTCCTTAGTGCTGATGCGCTTC

PCR products were confirmed using 1% agarose gel electrophoresis. A -20 °C freezer stock of 50 ng/µL gDNA was used subsequently to validate new primers, check for gDNA removal from RNA extracts, and as a positive control for *nos* transcription PCR and *nosZ* mutant transformant colony PCR.

11.5 Design of DNA primers

Primers against the *N. gonorrhoeae* FA1090 genome were visualised in SnapGene Viewer and tested *in silico* for secondary structure hairpins, primer dimers and self dimers using three online algorithms, including ThermoFisher Multiple Primer Analyzer (https://www.thermofisher.com/uk/en

/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html), Sigma-Aldrich OligoEvaluator (http://www.oligoevaluator.com/LoginServlet) and IDT OligoAnalyzer (https://eu.idtdna.com/calc/analyzer).

DNA primers were ordered from Integrated DNA Technologies. Primers were delivered in powdered form and were prepared to 100 µM -80 ^{o}C freezer stocks and 10 µM -20 ^{o}C freezer working stocks in TE buffer pH 8 (1 mM EDTA, 10 mM Tris-Cl). Newly ordered primers were validated against a gonococcal gDNA extract by NEB Taq PCR and gel electrophoresis. Newly ordered qPCR primers were validated against a gonococcal gDNA extract using the NEB LUNA Universal qPCR Master Mix protocol (M3003; contains Hot Start Taq DNA polymerase).

11.6 Sanger sequencing

Unless otherwise stated, DNA sequencing was ~1 kb read Sanger sequencing performed by DBS Genomics (Durham, https://www.durham.ac.uk/departments/academic/biosciences/research/services/dbsgenomics/dna-sequencing/). DNA template for DBS Genomics was supplied at 5 ng per 100 bp of product; 3 µl primers were supplied at 3.2 pmol/µl).

11.7 Gene expression qPCR studies

Static and shaking cultures of *N. gonorrhoeae* FA1090 were cultured as described above. Filter tips (Starlab) were used for all RNA steps. Molecular biology grade ethanol (Fisher Scientific) and UltraPure distilled water (dH_2O; Invitrogen) were used for preparation of samples for qPCR.

Cultures were centrifuged and pellets resuspended in ice-chilled 1 mL RNA Pro Solution (MP Biomedicals). Resuspensions were placed into 2 mL screw-cap tubes filled with 500 mg <106 µm glass beads (Sigma G4649) and frozen at -80^{o}C.

Cell resuspensions were thawed and chilled on ice for 10 min and lysed using a MP Biomedicals FastPrep-24 5G Bead beater with a QuickPrep adapter for 24 x 2 mL tubes at 9 m/s for 30 sec. Cell

lysates were incubated for 5 min at room temperature before being centrifuged at 12,500 RCF for 5 min.

RNA was extracted using chloroform/ethanol washes. Initially, 900 µL lysate supernatant was transferred into 300 µL 100% chloroform. The mixture was then briefly vortexed and incubated for 5 min at room temperature before being centrifuged at 12,500 RCF for 2 min. The aqueous phase (500 µL) was transferred into 200 µL 100% chloroform, briefly vortexed then centrifuged at 12,500 RCF for 1 min. The supernatant (300 µL) was transferred into 500 µL chilled (-20 ^{o}C) ethanol. Tubes were inverted five times to mix and mixtures incubated overnight at -20 ^{o}C.

The next day, mixtures were centrifuged at 12,500 RCF for 15 min at 4 ^{o}C and the supernatant discarded. Chilled (-20 ^{o}C) 500 µL 70% ethanol was added to the pellets. Tubes were centrifuged at 12,500 RCF for 15 min at 4 ^{o}C. The supernatant was discarded and tubes air dried for 10 min in a biosafety cabinet. Resuspension buffer (10 µL 10X NEB DNase I buffer + 90 µL UltraPure dH_2O) was added and the RNA extracts vortexed.

RNA extracts were quantified on a Nanodrop. Using these concentrations, 100 µL 200 ng/µL dilutions were prepared. gDNA was removed from RNA extracts via three rounds of 30 min 37 ^{o}C digestion with 2 µL DNase I (NEB) and 10 µL DNase I buffer. After these three incubations in DNase I, gDNA removal was confirmed using PCR. RNA dilutions (10 µL 50 ng/µL) were prepared and used in NEB Taq PCR with 173 and 174 primers against the *accA* gene mentioned above (S. Firth) to check for gDNA contamination. A 50 ng/µL gDNA extract was used as a positive control. To denature DNase I, PCR reactions were incubated at 95 ^{o}C for 10 min prior to primer addition. PCR products were analysed on a 1% agarose gel.

gDNA-free RNA samples were purified using the NEB Monarch RNA Cleanup Kit. Purified RNA was eluted in 50 µL UltraPure dH_2O. RNA quantification and purity was measured on a 1% agarose gel and Nanodrop.

Purified RNA samples (1 µg) were incubated with Invitrogen Random Hexamer and 1µL NEB 10 mM dNTP Mix in 14 µL UltraPure dH_2O for 5 min 65 ^oC then cooled for 5 min 4^oC. Invitrogen SuperScript IV Reverse Transcriptase (100 Units), 5X SuperScript IV Buffer (4 µL) and NEB RNase Inhibitor Murine (20 Units) were added to RNA samples then thermocycled: 10 min 23 ^oC, 10 min 55 ^oC, 10 min 80 ^oC. Synthesised complementary DNA (cDNA) samples (50 ng/µL) were stored at -20 ^oC until qPCR.

qPCR reactions were set up in opaque 96 well plates (Starlab E1403-5209) following the NEB protocol for LUNA Universal qPCR Master Mix. Template cDNA concentration was 10 ng per well. Reactions were prepared to 20 µL with UltraPure dH_2O. Primers used in qPCR are given in Table 11.

Table 11: Primers used in qPCR

Name	Target gene locus tag	Primer code	Sequence 5' → 3'	Amplicon size (bp)
gyrA*Ng*-qPCR-F	NGO_0629	189	TCCACCGATCCGAAGTTGC	90
gyrA*Ng*-qPCR-R		190	CGCAGTTTACGACACCATCG	
nosZ(N)-qPCR-F	NGO_07410	213	TGATCGGTCTGCCTTCTATGC	92
nosZ(N)-qPCR-R		214	CAAGCTCTCATTGGTGCGTC	
nosZ(C)-qPCR-F		215	CCCGCAGTTTGAAGTGAACC	90
nosZ(C)-qPCR-R		216	AAAGTGAAGCCGTGGGTCAG	
nosL-qPCR-F	NGO_1397	209	TATACCAAGCTGCCCGAAGAG	94
nosL-qPCR-R		210	TGTCGGCATTAGGATTCGTCC	
aniA-qPCR-F	NGO_1276	197	AAGTACCTCCCGCAATCGAC	106
aniA-qPCR-R		198	CAGTAGCGGTATTCCACCCC	
accA-qPCR-F	NGO_1215	203	CCTTTGACTTCGCGCATAC	97
accA-qPCR-R		204	ACTTTTTGCTCGTCGGAAGC	
laz-qPCR-F	NGO_0994	193	ATAGTCGGTATCGGCAGCAC	92
laz-qPCR-R		194	AAGCCAGCATGGGTCACAAC	
sco-qPCR-F	NGO_1237	207	TTACGCACTGTCCCGATGTC	99
sco-qPCR-R		208	CGAACACCACTTTCACGTCC	
copA*Ng*-qPCR-F	NGO_0579	199	AATCGTGCCGTGTCCAATTC	110
copA*Ng*-qPCR-R		200	AGAACACCATATCGGCTGGC	
copA2-qPCR-F	NGO_0685	413	GTGTTCCATAAAGCGTCCG	106
copA2-qPCR-Rv2		414	CATCGCCGGCATTTACAGC	
ompU-qPCR-F	NGO_1688	415	GCAGCTTATGGAGCAGGTCG	100
ompU-qPCR-R		416	GATATTGTGTTCGCGGGTAACG	
res-qPCR-F	NGO_0546	417	CTTCCTGAAACACGCGCACG	109
res-qPCR-R		418	CGCTACGGTGCAACATTTAACG	
aph-qPCR-F	NGO_1716	419	CAAACTCTTCTTCCCACTCG	88
aph-qPCR-R		420	GACTTCCAAGACGCGCTTTAC	
norB-qPCR-F	NGO_1275	421	GTGGGTGGAAGGCTTCTTTG	104
norB-qPCR-R		422	CCAAAGTGGAGGCAGTGG	
ccp-qPCR-F	NGO_1769	423	CTTTGTCGCGTTTCGGATCTTC	102
ccp-qPCR-R		424	CCTGCCACAACGGTGTCAAC	

Primers against the housekeeping gene *gyrA* (DNA gyrase topoisomerase) were included in every qPCR run as a positive control. UltraPure dH_2O template was used as a no-template control.

Expression of *gyrA* control gene was consistent during culture growth and was unaffected by addition of NO_2^- (data not shown).

qPCR plates were sealed using StarSeal Advanced Polyolefin Film and thermocycled according to Table 12 using a Bio-Rad CFX Connect Real Time System (SYBR scan mode, fast ramp speed).

Table 12: qPCR thermocycler protocol

Cycle Step	Temperature	Time	Cycles
	50 °C	2 min	1
Initial Denaturation	95 °C	2 min	1
Denaturation	95 °C	15 sec	40
Extension	60 °C	30 sec	
Read plate			
	95 °C	15 sec	1
	60 °C	1 min	1
Melt curve	65 → 95 °C	15 sec/0.5 °C	1
Read plate every 15 sec			1

Technical duplicate reactions were set up in every qPCR run and the duplicate data checked for consistency. Quantification Cq (threshold cycle) data of technical duplicates was always within ± 0.25 Cq. Technical duplicate values were averaged to give Cq_{gene}.

Rather than measuring total gene expression, expression relative to the housekeeping gene *gyrA* (ΔCq) was calculated by subtracting Cq_{gyrA} from every other Cq_{gene}. Expression of *gyrA* gene was determined to be stable and high throughout culture growth (data not shown). ΔCq values were multiplied by -1 to give -ΔCq. This meant a larger positive number represented greater upregulation. We plotted -ΔCq values (gene expression vs *gyrA*) for samples of cultures grown with and without NO_2^-.

For some analyses, $\Delta Cq_{-nitrite}$ was subtracted from $\Delta Cq_{+nitrite}$ to give ΔΔCq. ΔΔCq values were multiplied by -1 to give -ΔΔCq. The $2^{-\Delta\Delta Cq}$ values were the "fold change in gene expression with treatment relative to a housekeeping gene"(Livak and Schmittgen, 2001). We plotted -ΔΔCq (gene expression change with addition of NO_2^- relative to *gyrA*). These -ΔΔCq data are equivalent to $\log_2(2^{-\Delta\Delta Cq})$, also known as "$\log_2$(fold change in gene expression relative to *gyrA*)".

Quantification amplification curves of fluorescence against cycle were plotted. The efficiency of qPCR primers was validated using LinRegPCR (Ruijter et al., 2009). UltraPure dH_2O controls had no amplification (Cq = 0) or late amplification (Cq > 30). Melt curve derivatives were plotted against temperature and experimental reactions visually confirmed to have single melt peaks. UltraPure dH_2O controls had no peak or shorter peaks at a lower temperature.

The variations between qPCR technical duplicate Cq values were always within 0.5, meaning the sensitivity limit for ΔCq (subtract *gyrA* housekeeping Cq) was ± 0.5, annotated on qPCR graphs as dashed lines.

11.8 Checking transcription of the *nos* operon

Transcription of the WT *nos* operon was determined by NEB Q5 PCR using 10 ng RNA and cDNA from microaerobic cultures (see qPCR workflow above). Purified RNA extracts from WT strain were included as a negative control for gDNA contamination. gDNA extracts were used as a positive control. RNA and cDNA from a Δ*nosZ* strain was used as a negative control for WT *nosZ* transcription (Appendix 1). Reactions were prepared to 25 µL with UltraPure dH_2O. Primers used in transcription checking PCR are given in Table 13.

Table 13: Primers used in transcription checking PCR

Amplicon	Forward primer	Primer code	Reverse primer	Primer code	Region amplified
1	nosR-full-F-v8	328	nosR-full-R-v7	327	*nosR*
2	NosZTransAmp FWD	612	nosZ(N)-qPCR-R	214	*nosR* → *nosZ*
3	nosZ(N)-qPCR-F	213	nosZ-full-R	212	*nosZ*
4	nosZ(C)-qPCR-F	215	nosD-full-R	220	*nosZ* → *nosD*
5	nosD-full-F	219	nosF-full-Rv2	378	*nosD* → *nosF*
6	nosF-full-Fv2	377	nosY-full-Rv2	380	*nosF* → *nosY*
7	nosY-full-Fv2	379	nosL-full-Rv2	382	*nosY* → *nosL*

11.9 Cloning

NosZ knockout and NosZ-StrepII-tagged strains were generated during this study. The StrepII tag is recommended for purifying proteins under anaerobic conditions and for metal-containing enzymes (Terpe, 2003).

11.9.1 Plasmid extraction and purification

The *E. coli* DH5α strains K0027 (C. Steichen) and K0146 (S. Firth) were grown overnight at 37 °C on 100 µg/mL Spc and 30 µg/mL Cm LB-agar plates respectively. The K0027 strain contains a pCTS32 plasmid with a *specR* spectinomycin resistance gene, which was used as a positive control while selecting for successful NosZ transformants. The K0146 strain contains a Golden Gate Parent Construct plasmid (pGGA; NEB) with a chloramphenicol resistance gene. The pGGA plasmid was used as a positive control for pGGA transformation. Both plasmids were extracted from bacteria using the the NEB Monarch Plasmid MiniPrep Kit Protocol (NEB #T1010). Plasmid extract quantification and purity was measured on a 1% agarose gel and Nanodrop. The pCTS32 plasmid was linearised using BsaI-HFv2 (1 unit per 1 µg plasmid) at 1 hr 37 °C, followed by 1 hr 80 °C heat-inactivation of BsaI-HFv2 prior to transformation into *N. gonorrhoeae*.

11.9.2 Generation of *N. gonorrhoeae nosZ* mutant strains

The *nos* operon DNA sequence was downloaded from the NCBI genome ASM684v1. Synthetic DNA sequences of *nos* operon segments were acquired from GenScript (see Appendix 2) and used to create *nosZ* mutant strains using the Golden Gate assembly method (https://www.neb-online.de/en/cloning-synthetic-biology/dna-assembly/ golden-gate-assembly/). *N. gonorrhoeae* is naturally competent and readily uptakes DNA containing the 10-bp Neisseria DNA uptake sequence (DUS) 5' GCCGTCTGAA 3' (Goodman and Scocca, 1988). This sequence occurs twice in the region 1kb downstream of *nosZ*.

The six synthetic DNA sequences were: 1) 1 kb 5'-upstream of *nosZ*, 2) a spectinomycin resistance gene (*specR*) to replace *nosZ*, 3) a *specR* gene to insert downstream of *nosZ*, 4) a *nosZ* with a StrepII tag (`TGG AGC CAC CCG CAG TTC GAA AAA`) after the N-terminal signal peptide, 5) a *nosZ* with a StrepII tag before the final stop codon, and 6) 1kb 3'-downstream of *nosZ* (Figure 12, Appendix 2).

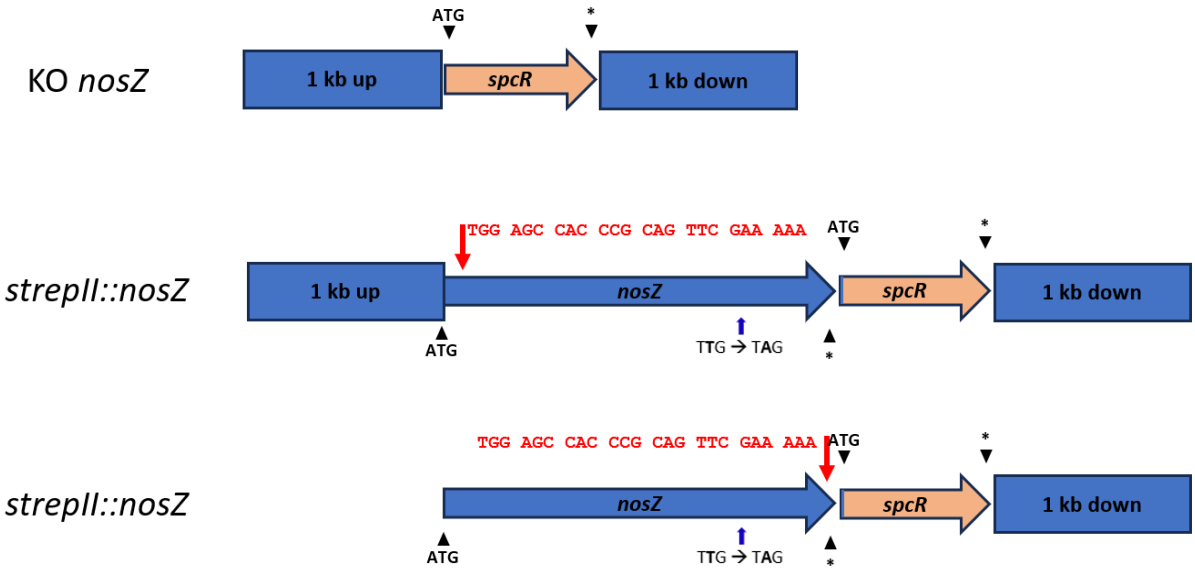


Figure 12: Golden Gate assemblies for homologous transformation into native *nosZ* gene site in *N. gonorrhoeae*.

These synthetic gene sequences were designed to contain flanking BsaI recognition sites which would be removed during the Golden Gate reaction. Hence, in these synthetic genes, a random 6-nuceotide sequence in the WT *nosZ* matching the BsaI recognition site was domesticated from

GG**T**CTC to GG**C**CTC (a synonymous mutation). A short linker sequence (GGCGGC) was inserted to separate s*trepII* and *nosZ*.

Powdered synthetic DNA from GenScript was dissolved to 200 ng/µL or 25 ng/µL with UltraPure dH_2O. Golden Gate assembly reactions were prepared according to the NEB Golden Gate Assembly protocol (Table 14).

Table 14: Golden Gate Assembly Reactions

Reagent	**KO *nosZ***	***strepII::nosZ***	***nosZ::strepII***	**No insert negative control**
pGGA (75 ng/µL)	1 µL	1 µL	1 µL	1 µL
1 kb up::pUC57 (200 ng/µL)	0.375 µL	0.375 µL	-	-
Mutant nosZ insert::pUC57 (25ng/µL)	-	3 µL	3 µL	-
specR::pUC57 (200 ng/µL)	0.375 µL	-	-	-
New specR::pUC57 (200 ng/µL)	-	0.375 µL	0.375 µL	-
1 kb down::pUC57 (200 ng/µL)	0.375 µL	0.375 µL	0.375 µL	-
T4 DNA Ligase Buffer (10X)	2 µL	2 µL	2 µL	2 µL
T4 DNA Ligase (400 U/µL)	1 µL	1 µL	1 µL	1 µL
BsaI-HFv2 (20 U/µL)	1 µL	1 µL	1 µL	1 µL
UltraPure dH_2O	13.875 µL	10.875 µL	11.25 µL	15 µL

Golden Gate assembly reactions were thermocycled (37 °C, 1 hr → 60 °C, 5 min). Then, pGGA::insert parent constructs were amplified using Q5 PCR (62 °C annealing temperature, 2 min 34 sec extension time) and the primers listed in Table 15.

Table 15: Primers used to amplify pGGA parent constructs

KO	Other mutants	Name	Primer code	Sequence 5' → 3'
Y		JJ_Pl_to_ins_1_FWD	342	ATTTTACACGACTCTCTTTACCAAT (binds pGGA)
	strepII::nosZ	JGG Insert 1 FWD	334	GGCTACGGTCTCTATCATGCGGTACGACCGCTT CCGCT
	Y	JGG 4 to Pl REV	351	GCATGATGCCCTAATCGC
Y		JJ_Ins_3_to_pl_REV	426	CGACTCACTATAGGGAGACG (binds pGGA)

The pGGA::insert products were confirmed on a 1% agarose gel and purified using the NEB Monarch PCR and DNA Cleanup Kit before being quantified on a Nanodrop and confirmed by Sanger sequencing using a range of forward and reverse primers (not shown) to amplify join regions between the genome and constructs to confirm correct assembly.

The *N. gonorrhoeae* strain FA1090 was grown overnight at 37 °C on pre-warmed GC agar plates. Colonies were resuspended in GCBL. A 10,000x 50 µL dilution of overnight cell resuspension was spread onto pre-warmed GC agar plates using a sterile L-shaped spreader and incubated overnight at 37 °C. (A previous efficiency of plating assay using a range of *N. gonorrhoeae* resuspension dilutions suggested a 10,000x dilution gave single isolated colonies suitable for transformation).

Transformation of naturally competent *N. gonorrhoeae* followed a protocol described previously (Dillard, 2011). Approximately eight single opaque (piliated) colonies were grabbed using a sterile loop and streaked through a 10 µL droplet containing 500 ng of each purified pGGA::insert PCR product on a pre-warmed GC agar plate. A droplet of purified solution of pCTS32 was used as a positive control to check for successful transformation, while a droplet of UltraPure dH_2O was used as a negative control. Plates were left open in a biosafety cabinet for the DNA solution to dry then incubated at 37 °C during the day for 6 hr. After 6 hr, the thin layer of cells which had developed was grabbed and streaked onto pre-warmed 100 µg/mL Spc GC agar plates before overnight incubation

at 37 °C for 16 hr. This selected for naturally transformed cells which had uptaken the pGGA::insert PCR products and incorporated them into the genome via homologous recombination.

Isolated colonies were picked once they had formed and restreaked onto pre-warmed 100 µg/mL Spc GC agar plates and incubated overnight at 37°C. Approximately 16 clones from each transformation were stocked in GC glycerol and frozen at -80 °C. Each transformant clone (50 µL) was saved for colony PCR to confirm transformation.

For colony PCR, transformant clones were diluted 2X in PBS, heated at 90 °C for 10 min, centrifuged at 12,500 RCF for 1 min, and diluted 100x in PBS if a pellet was present. Colony PCR was performed using Q5 DNA polymerase (65 °C annealing temperature, 1 min 15 sec extension time), a gDNA extract positive control template, and the primers listed in Table 16.

Table 16: Primers used in colony PCR

KO	All other mutants	Name	Primer code	Sequence 5' → 3'
Y		JJ_Ins_1_to_2_fwd	425	GTCTCTAAGCAGCCGCAG
	Y	nosZ-full-F	211	ATGTCAGACGAAAAATTAGAACA
Y	Y	JGG 3 to 4 REV	349	GCAGGCCCTTCAATCG

Colony PCR products were confirmed on a 1% agarose gel and by Sanger sequencing using a range of forward and reverse primers (not shown) to amplify join regions between the genome and constructs to confirm correct assembly.

11.9.3 Confirmation of *nosZ* mutant strains by whole-genome sequencing

The genome sequences of all *nosZ* mutant strains were confirmed with whole-genome sequencing by MicrobesNG (Birmingham). Strains were sent to MicrobesNG according to their Strain Submission protocol (https://microbesng.com/microbesng-faq/). Duplicate WT, KO, *strepII::nosZ* and *nosZ::strepII* clones were plated onto GC agar from frozen stocks and incubated overnight before being grown in shaking cultures to generate sufficient cell density for whole-genome sequencing.

Cell cultures (15 mL in 50 mL Falcon tubes) were pelleted at 4-5 hr (OD_{600} ~ 1), washed in 1X PBS, resuspended in 1X DNA/RNA Shield (Zymo Research) and shipped at room temperature. MicrobesNG compared the WT and mutant strain genome sequences to the FA1090 strain genome assembly ASM684v1 file from NCBI and provided a list of nucleotide variants.

11.9.4 Phenotypic assessment of *nosZ* mutant strain cultures

To confirm the growth of mutant *nosZ* transformants, triplicate microaerobic stocks of WT, KO *nosZ*, *strepII::nosZ* and *nosZ::strepII* strains were first grown overnight on soft GC agar plates as described previously then cultured in 1 mL cultures in capped sterile cuvettes (Merck Brand BR759200 cuvettes and BR759240 caps) for 10 hr at 37 ^{o}C. Cultures were measured at 0, 4, 5, 6, 7, 8, 9 and 10 hr and supplemented with 2 mM NO_2^- at 0, 5, 7, 8, and 9 hr. WT cultures were used as a positive growth control.

11.10 Western blotting *strepII::nosZ* and *nosZ::strepII* culture extracts

The following buffers were prepared for Western blots:

Table 17: Recipes of buffers used in Western blotting

Buffer	**Recipe**
4X Laemmli sample buffer (no thiol)	250 mM Tris base pH 7, 8% SDS, 4% glycerol, 0.001% w/v Orange G (5% β-mercaptoethanol added to buffer immediately prior to addition to sample and heating)
Tris-MOPS-SDS running buffer	50 mM Tris base, 50 mM MOPS, 0.1% SDS, 1 mM EDTA
Towbin transfer buffer (pH 8.4)	25 mM Tris base, 192 mM glycine, 10% methanol
PBS buffer	4 mM KH_2PO_4 pH 7.4, 16 mM Na_2HPO_4, 115 mM NaCl
PBS blocking buffer	PBS buffer, 3% BSA, 0.05% Tween 20
PBS-Tween buffer	PBS buffer, 0.1% Tween 20
Ponceau S stain	0.1% w/v Ponceau S, 5% glacial acetic acid

Both static and shaking cultures were grown to generate cell pellets for Western blot. Static 30 mL *N. gonorrhoeae* cultures supplemented with NO_2^- and 0, 1 and 10 µM Cu were grown in 50 mL Falcon

tubes for 8-10 hr until OD_{600} = 0.2-0.6. Shaking 50 mL *N. gonorrhoeae* cultures with and without NO_2^- were grown in 250 mL conical flasks for 4-7 hr until OD_{600} = 0.8-1.4. Cultures were pelleted and resuspended in GCBL + 20% glycerol for -80 °C storage.

The GFP-Strep-tagII (Iba Life Sciences 2-1006-005) was included as a positive control for StrepII detection. GFP-StrepII (2 µL) and culture samples (19 µL) were heated (94 °C, 10 min) in 4X Laemmli sample buffer (4X with 5% β-mercaptoethanol) to dissolve the cell membranes.

GFP-StrepII (3 µL) and culture lysates in buffer (25 µl) were loaded onto a GenScript SurePAGE Bis-Tris 4-20% gradient gel and separated by SDS-PAGE at 100 V alongside the NEB Broad Range Protein Standard (P7718) in Tris-MOPS-SDS running buffer in a Bio-Rad Mini-PROTEAN Tetra System tank at room temperature. The gel was stopped after ~1.5 hr once the Orange G dye front (~10 kDa band) reached the bottom of the gel.

The electrophoresed protein gel was washed in H_2O, rocked for 25 min in Towbin transfer buffer then transferred onto a BioRad 0.45 µm nitrocellulose membrane in Towbin buffer at 15 V overnight at 4 °C (~19 hr) in a Bio-Rad Mini-PROTEAN Tetra System tank.

After transfer, the membrane was washed in H_2O then immersed briefly in Ponceau S stain to check the transfer had worked across all lanes and that proteins of different sizes were sharply resolved (Appendix 3). Ponceau S stain was removed with 0.1% (200 µM) NaOH and the membrane rinsed in H_2O for 3 x 1 min at room temperature 70 RPM shaking. The membrane was blocked in PBS-BSA buffer for 1 hr at room temperature before washing in PBS-Tween for 3 x 5 min at room temperature 70 RPM shaking.

After the final wash, 10 mL PBS-Tween was added to the membrane and 1 µL Pierce High Sensitivity Streptavidin-HRP Conjugate Protein was added (ThermoScientific; 1:10,000 dilution). The membrane was incubated for 1 hr at room temperature 70 RPM shaking.

The membrane was then washed in PBS buffer 2 x 1 min room temperature 70 RPM shaking before addition of 1 mL ECL Prime Western Blotting Detection Reagent A and Reagent B (GE Healthcare Amersham; 500 µL:500 µL ratio). The membrane was then sealed with clingfilm and chemiluminescence was imaged on an Invitrogen iBright 1500 machine.

11.11 Statistical analyses and graphing

The GraphPad Prism version 10 software was used for calculating statistical tests for significance and graphing data.

12 Chapter 3: Bioinformatics of the *nos* operon

12.1 Previous genomics and proteomics studies of gonococcal NosZ

To determine if the gonococcal NosZ protein had already been identified in proteomics studies, the *N. gonorrhoeae* FA1090 amino acid sequence was searched against the text mining tool PaperBLAST (Price and Arkin, 2017). The only hit in *Neisseria* was Barth et al. (2009). Other hits were from genomics and proteomics studies which found NosZ to be differentially regulated in certain mutant strains of other bacterial species. Expression of the *nos* operon is often found to be co-regulated with the other denitrification operons *nar*, *nir* and *nor*, the genes encoding the cytochrome *c* complex (Marchesini et al., 2022; Tsujino et al., 2021), as well as the regulator *fixQ1/fixP1* (D'Alessio et al., 2017). The *nirK*, *norB*, *nosZ* and *nosR* (as well as cytochrome *c*) genes are all downregulated in stationary low C:N *Alcaligenes faecalis* cultures (Tsujino et al., 2021), suggesting these cultures have reduced denitrification. The *nos* and *nor* operons (as well as *fixQ1*/*fixP1*) are downregulated in phosphate-stressed *Sinorhizobium meliloti* strain Rm1021 (D'Alessio et al., 2017) but the *nos* operon is upregulated in a *Sinorhizobium meliloti tolC* mutant strain, which has higher sensitivity to osmotic and oxidative stresses (Santos et al., 2010). Differentially represented proteins in a *Brucella abortus rhomboid* knockout strain included NosZ and Cbb3-type cytochrome *c* oxidase. Overexpression of *rhomboid* had a negative impact on growth under static conditions, suggesting an effect on denitrification enzymes and/or the high O_2 affinity COX required for growth in low-O_2 conditions (Marchesini et al., 2022). NosZ is downregulated in a *Bradyrhizobium diazoefficiens nnrR* mutant compared to the WT (Jiménez-Leiva et al., 2019). The *Bradyrhizobium diazoefficiens* NapA, NosZ, FixO and FixP are upregulated in microoxic conditions at both transcriptional and protein levels. The *B. diazoefficiens* NosZ protein was confirmed to be 72 kDa on a Western blot (Fernández et al., 2019).

Past studies state that all *Neisseria* species contain a functional NO_2^- reductase (Nir) and $^{\bullet}NO$ reductase (Nor), but only *N. mucosa* contains a functional NO_3^- reductase (Nar), and only *N.*

lactamica, *N. cinerea*, *N. subflava*, *N. flavescens*, *N. sicca*, *N. macacae*, *N. polysaccharea* and *N. sp. oral. taxon* contain a functional N_2O reductase (Nos) (Figure 5) (Barth et al., 2009a).

The *nos* operon represents an example of extreme divergence among *Neisseria* species. *N. gonorrhoeae* contains premature stop codons within *nosR*, *nosZ*, and *nosD*, and as such was thought to be non-functional (Barth et al., 2009a; Cardinale and Clark, 2005; Isabella and Clark, 2011a; Seib et al., 2006). *N. meningitidis* has a 4 kb deletion that extends from the middle of *nosR* into the middle of *nosD*, completely eliminating *nosZ*, as well as premature stop codons in *nosY* and *nosX*.

Since the obligate human pathogens *N. gonorrhoeae* and *N. meningitidis* species have used divergent mechanisms to disrupt genes in the *nos* operon, some have suggested inactivation of N_2O reduction provides some benefit for pathogenic *Neisseria* (Barth et al., 2009a; Cardinale and Clark, 2005). Supporting this hypothesis is the case of *N. mucosa*. While *N. mucosa* is typically a commensal of the oral and nasal mucosa, it has been reported to cause bacterial meningitis, conjunctivitis, pneumonia, endocarditis, peritonitis and urethritis (Ren et al., 2023), as well as infections of the urinary tract (Osses et al., 2017). *N. mucosa* also contains a disrupted *nos* operon, with premature stop codon within *nosZ* and a deletion in *nosF* (Barth et al., 2009a).

12.2 Synteny of the Neisserial *nos* and *nir-nor* operons

While the Neisserial *nos* and *nir-nor* operons are always non-adjacent, this study found there to be a highly variable distance of 123,000-1,000,000 bp between the *nos* and *nir-nor* operons, suggesting that there has been a high level of genome evolution in *Neisseria* since the acquisition of these two loci (Table 18).

Table 18: Location of denitrification operons across *Neisseria* reference genomes

			Chromosome location				
Species	**Strain**	**Assembly**	**Nar**	**Nir**	**Nor**	**Nos**	**Genome size**
N. gonorrhoeae	FA1090	AE004969		924,020… 925,198	925,572… 927,827	785,473… 793,699	2.1 Mb
N. meningitidis	MC58	GCF_000008805.1		1,687,956… 1,689,128	1,685,330… 1,687,585	605,402… 608,373	2.3 Mb
N. subflava	ATCC 49275	CP039887		351,740… 352,921	353,322… 355,577	151,864… 153,831	2.2 Mb
N. lactamica	020-06	GCF_000196295.1		660,642… 661,814	662,186… 664,441	1,770,068… 1,772,035	2.2 Mb
N. polysaccharea	ATCC 43768	GCF_963392925.1		8,689… 9,867	6,052… 8,307	147,568… 149,529	2 Mb
N. mucosa	C102	GCF_900454435.1	745,418… 749,098	374,799… 375,983	372,069… 374,324	497,596… 499,566	1.9 Mb
N. cinerea	NCTC10294	GCF_900475315.1		1,090,680… 1,091,852	1,088,044… 1,090,299	229,171… 231,132	1.8 Mb
N. sicca	NS20201025	GCF_017753665.1		1,300,769… 1,301,959	1,298,047… 1,300,302	142,6126… 1428,096	2.6 Mb
N. flavescens	ATCC 13120	GCF_005221285.1		301,555… 302,736	303,136… 305,391	1,337,390… 1,339,357	2.2 Mb

12.3 Signal peptides in *nos* operon genes

SignalP 5.0 was used to predict signal peptides in *nos* operon genes (Almagro Armenteros et al., 2019; Teufel et al., 2022). NosR was predicted to have a 33 residue Sec signal peptide, NosZ was predicted to have a 37 residue TAT lipoprotein signal peptide (Figure 13), NosD was predicted to have a 26 residue Sec signal peptide, and NosL was predicted to have a 15 residue Sec lipoprotein signal peptide. Signal peptides were not predicted in NosF, NosY, and NosX.

FA1090_NosZ
Prediction: TAT Lipoprotein signal peptide (Tat/SPII)

Cleavage site between pos. 37 and 38.
Probability 0.762392

Protein type	Other	Signal Peptide (Sec/SPI)	Lipoprotein signal peptide (Sec/SPII)	TAT signal peptide (Tat/SPI)	TAT Lipoprotein signal peptide (Tat/SPII)	Pilin-like signal peptide (Sec/SPIII)
Likelihood	0	0	0	0	1	0

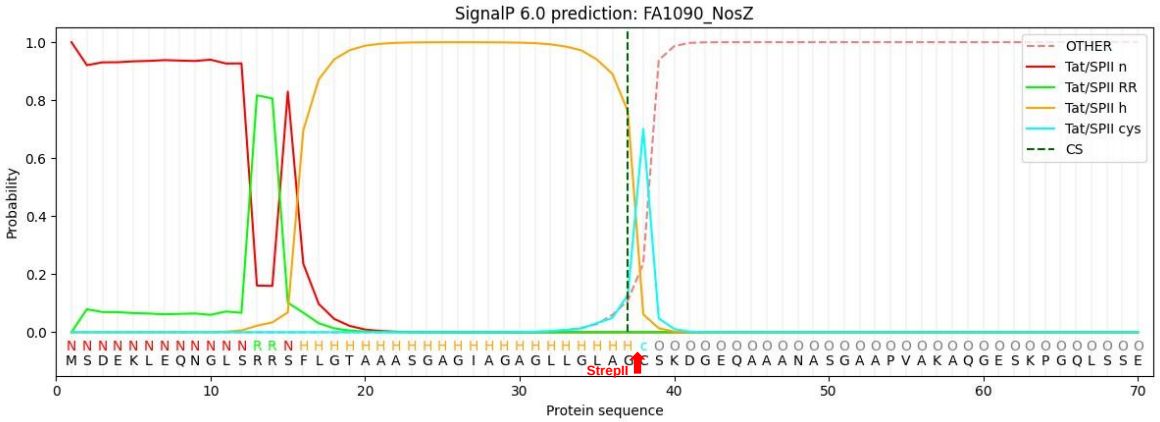


Figure 13: SignalP 6.0 predicts a TAT signal peptide at the NosZ N-terminus.

The finding that *nosZ* has a TAT signal peptide is curious given that the TAT pathway typically secretes fully assembled and folded proteins, yet it seems likely that some or all of the auxiliary Nos proteins have roles in assembly of the NosZ Cu sites in the periplasm. The 5'-end *strepII::nosZ* mutant designed in this study was engineered to contain the strepII tag sequence after the end of this predicted signal peptide.

12.4 Conservation of the Neisserial *nos* operon

12.5 Bioinformatics analysis methodology

Our bioinformatics analysis consisted of four sections: 1) non-specific homology searches of the *N. gonorrhoeae* FA1090 *nos* operon with all NCBI entries, 2) specific homology searches of the *N. gonorrhoeae* FA1090 *nos* operon with all other *Neisseria* reference genomes, 3) specific homology searches of the *N. gonorrhoeae* FA1090 *nos* amino acid sequence with those from PDB NosZ crystals, and 4) specific homology searches of the *nos* operon across disparate gonococcal isolates from different times and geographic locations.

The FA1090 *nos* gene nucleotide and translated amino acid sequences can be found in Appendices 4 and 5; sequences from species with a NosZ crystal structure can be found in Appendices 6 and 7.

12.6 Non-specific homology searches of the *N. gonorrhoeae* FA1090 *nos* operon with all NCBI entries

12.6.1.1 Non-specific BLASTn of gonococcal nos operon shows high homology to other Neisseria

The National Center for Biotechnology Information (NCBI) Nucleotide Basic Local Alignment Search Tool (BLASTn) program was used to find non-specific homologs from any species to the *N. gonorrhoeae* FA1090 *nos* operon. Search parameters included searching the non-redundant nucleotide database and optimising for highly similar sequences (megablast).

The FA1090 *nos* operon has highest homology (> 200 alignment scores) to other gonococcal isolates, and then other *Neisseria* species; most noticeably *polysaccharea* (95% identity, 99% query cover), *lactamica* (95% identity, 99% query cover), *cinerea* (91% identity, 98% query cover) and *flavescens* (96 % identity, 87% query cover). While exhibiting some regions of high homology (97% identity), *N. meningitidis* has substantially reduced query cover (37%) compared to the aforementioned species; particularly in the *nosR*, *nosZ*, *nosY* and *nosX* regions of the operon (Figure 14). This would suggest that *N. meningitidis* contains only truncated *nosR*, *nosD*, *nosF*, and *nosL* genes.

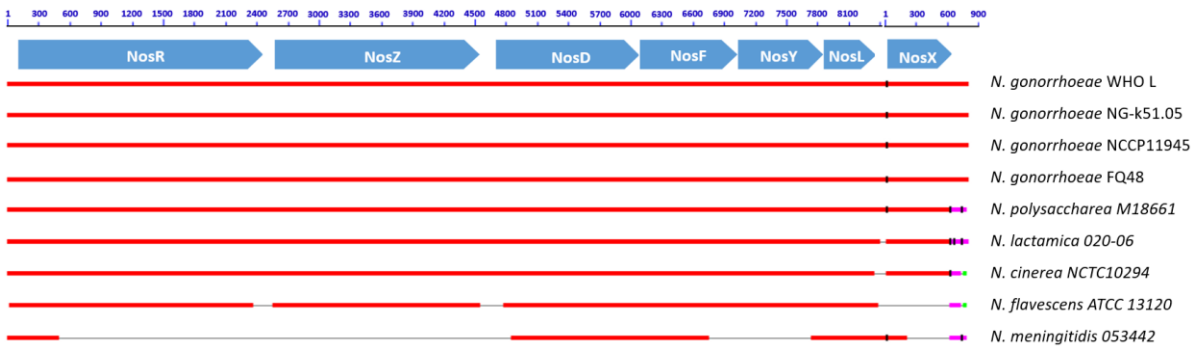


Figure 14: Neisserial *nos* operon homology.
BLASTn-generated alignments to the *nos* operon from *N. gonorrhoeae* FA1090. Alignments of the highest homology were selected for each species.

12.6.1.2 Non-specific BLASTn of individual gonococcal nos genes shows high homology to other Neisseria

BLASTn was then used to find alignments to individual genes from the *N. gonorrhoeae* FA1090 *nos* operon. Search parameters included searching the non-redundant nucleotide database and optimising for highly similar sequences (megablast).

In other *Neisseria*, FA1090 *nosR* has highest homology to *nosR* in *N. lactamica* (94% identity, 100% query cover), *N. polysaccharea* (94% identity, 100% query cover), *N. cinerea* (94% identity, 90% query cover), *N. mucosa* (82% identity, 92% query cover) and *N. subflava* (79% identity, 92% query cover). While exhibiting some regions of high homology to FA1090 *nosR* (97% identity), *N. meningitidis* only had homology at the start of the gene (16%), indicating an 5'-end deletion (Figure 15A). Analysis of the alignment with *N. lactamica* showed that the *nosR* gene in *N. gonorrhoeae* is prematurely stopped by a C→T single nucleotide polymorphism at base 1138, to encode a TAA stop codon (Figure 17).

In other *Neisseria*, FA1090 *nosZ* has highest homology to *nosZ* in *N. lactamica* (96% identity, 100% query cover), *N. flavescens* (96% identity, 100% query cover), *N. polysaccharea* (95% identity, 100% query cover), *N. cinerea* (100% identity, 93% query cover), *N. subflava* (87% identity, 100% query cover) and *N. mucosa* (86% identity, 100% query cover). No alignment with *N. meningitidis* was found for *nosZ*, indicating the gene is totally deleted (Figure 15B). Analysis of the alignment with *N. lactamica* showed that the *nosZ* gene in *N. gonorrhoeae* is prematurely stopped by a G→A single nucleotide polymorphism at base 1601 to encode a TAG stop codon (Figure 17).

In other *Neisseria*, FA1090 *nosD* has highest homology to *nosD* in *N. polysaccharea* (97% identity, 100% query cover), *N. lactamica* (96% identity, 100% query cover), *N. meningitidis* (97% identity, 90% query cover), *N. cinerea* (91% identity, 100% query cover), *N. mucosa* (84% identity, 87% query cover), *N. flavescens* (81% identity, 87% query cover), and *N. subflava* (80% identity, 87% query cover) (Figure 15C). Analysis of the alignment with *N. polysaccharea* showed that the *nosD* gene in *N. gonorrhoeae* is prematurely stopped by a C→T single nucleotide polymorphism at base 769 to encode a TAG stop codon (Figure 17).

FA1090 *nosF* has highest homology to *nosF* in *N. lactamica* (97% identity, 100% query cover), *N. polysaccharea* (96% identity, 100% query cover), *N. cinerea* (98% identity, 100% query cover), *N.*

meningitidis (98% identity, 70% query cover), and *N. mucosa* (84% identity, 98% query cover) (Figure 15D).

FA1090 *nosY* has highest homology to *nosY* in *N. lactamica* (96% identity, 100% query cover), *N. polysaccharea* (96% identity, 100% query cover), *N. cinerea* (90% identity, 100% query cover), *N. flavescens* (85% identity, 100% query cover), *N. subflava* (85% identity, 100% query cover), and *N. mucosa* (85% identity, 100% query cover). While exhibiting some regions of high homology to FA1090 *nosY* (98% identity), *N. meningitidis* was found to only have homology at the last 15% of the gene, indicating a large 5'-end deletion (Figure 15E).

FA1090 *nosL* has highest homology to *nosL* in *N. lactamica* (97% identity, 100% query cover), *N. meningitidis* (96% identity, 100% query cover), *N. polysaccharea* (95% identity, 100% query cover), *N. cinerea* (93% identity, 100% query cover), *N. subflava* (82% identity, 86% query cover), and *N. mucosa* (82% identity, 86% query cover) (Figure 15F).

In FA1090, the 600 nucleotide *nosX* coding sequence occurs 43,520 bp downstream of *nosL*. In other species, *nosX* is identified as being 996 and 915 nucleotides long (Chan et al., 1997; Saunders et al., 2000), suggesting in FA1090 the gene is truncated by at least 315 nucleotides. The FA1090 *nosX* gene has an alternative TTG start codon and is shown to be highly similar to sequences in *N. lactamica* (94% identity, 99% query cover), *N. meningitidis* (93% identity, 98% query cover), *N. polysaccharea* (90% identity, 98% query cover), and *N. cinerea* (90% identity, 93% query cover) (Figure 15G). The alignment to *N. meningitidis* is surprising, given the disrupted *nos* operon seen in that species, and suggests perhaps the *nosX* gene product has an unknown role in another aspect of virulence.

To date, the *nosA* gene has only been characterised in *Pseudomonas* species (Lee et al., 1991; Mokhele et al., 1987; Wunsch et al., 2003). BLASTn searches *nosA* from *Pseudomonas stutzeri* (GenBank: M60717), *Shewanella oneidensis* (NCBI ID: NP_716263.1), and *Burkholderia pseudomallei* (NCBI ID: YP_111748.1) against the FA1090 genome did not reveal any homology, suggesting FA1090 lacks *nosA*.

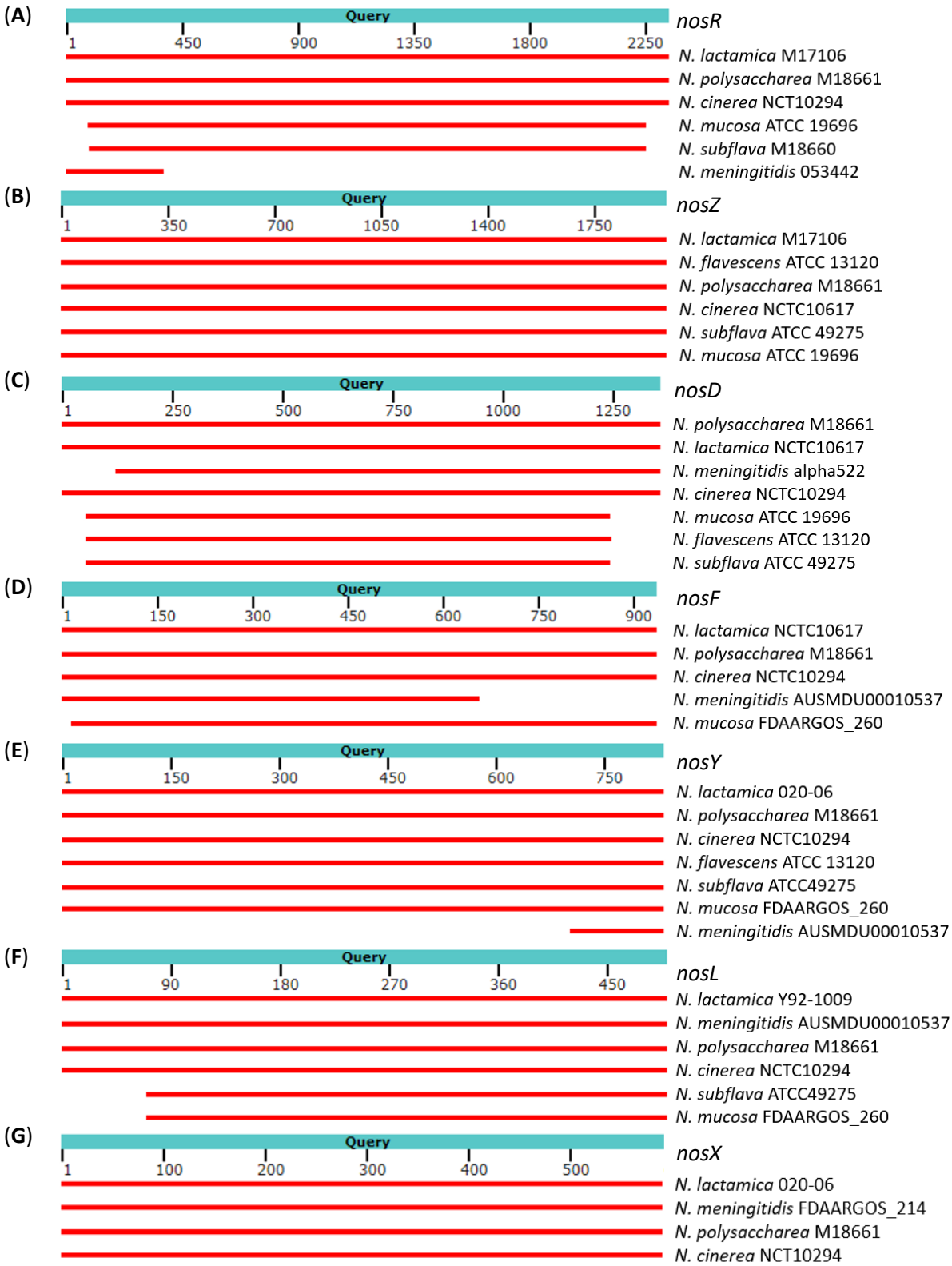


Figure 15: Homologous regions of individual *Neisseria gonorrhoeae* FA1090 *nos* genes to closest relatives. (**A**) FA1090 *nosR* alignment with other species. (**B**) FA1090 *nosZ* alignment with other species. (**C**) FA1090 *nosD* alignment with other select species. (**D**) FA1090 *nosF* alignment with other species. (**E**) FA1090 *nosY* alignment with other species. (**F**) FA1090 *nosL* alignment with other species. (**G**) FA1090 *nosX* alignment with other species.

12.7 Specific homology searches of the FA1090 *nos* operon with other *Neisseria* reference genomes

BLAST homology searches of FA1090 *nos* genes were repeated against *Neisseria* reference genome assemblies which were known to be complete.

12.7.1.1 Neisseria *reference genomes*

A search of NCBI revealed there to be twelve *Neisseria* reference genomes: *N. gonorrhoeae FA1090*, *N. meningitidis MC58*, *N. lactamica 020-06*, *N. elongata subsp. glycolytica ATCC 29315*, *N. polysaccharea ATCC 43768*, *N. mucosa C102*, *N. cinerea ATCC 14685*, *N. subflava NJ9703*, *N. wadsworthii 9715*, *N. shayeganii 871*, *N. macacae ATCC 33926*, and *N. bacilliformis ATCC BAA-1200.* Out of these species, only *N. lactamica*, *cinerea* and *subflava* were previously thought to contain a Nos complex (Barth et al., 2009a). Reference genome gene annotations were searched and BLASTn homology searches with the FA1090 *nos* genes were performed.

N. elongata subsp. glycolytica ATCC 29315, *N. shayeganii* 871, *N. wadsworthii* 9715 and *N. bacilliformis* ATCC BAA-1200 were found to lack the entire *nos* operon, while the remaining eight species have largely complete *nos* operons (Table 19). It is interesting to note that these remaining species all rely on an alternative *nosR* start codon. *N. meningitidis* had deletions in all *nos* genes except *nosL*.

The retention of *nosX* in *Neisseria* is debated. A previous study identified *nosX* in *N. meningitidis* MC58, *N. lactamica* ATCC 23970, *N. cinerea* ATCC 14685, *N. subflava* NJ9703, and *N. mucosa* ATCC 25996 based on homology to the gene from *Ralstonia eutropha* H16 (Barth et al., 2009a). However, while we detected a FA0190 *nosX* in non-specific homology searches in *N. meningitidis* FDAARGOS_214, *N. lactamica* 020-06, *N. polysaccharea* M18661 and *N. cinerea* NCT10294, no *nosX* homolog was detected in the reference genomes for *N. polysaccharea*, *N. cinerea* and *N. mucosa*. We did not detect any FA1090 *nosX* homolog in *N. subflava* or *N. macacae*.

Table 19: List of Neisserial *nos* genes

Reference genome species	***nosR***	***nosZ***	***nosD***	***nosF***	***nosY***	***nosL***	***nosX***	***nosA***
N. gonorrhoeae FA1090	+, * 777 cd	+, * 656 cd	+, * 452 cd	+ 309 cd	+ 277 cd	+ 165 cd	*, Δ, Alt 199 cd	-
N. meningitidis MC58	+, Δ 191 cd	-	+, Δ 345 cd	+, Δ 207 cd	+, Δ, * 43 cd	+ 165 cd	Δ, Alt, 60 cd	-
N. subflava NJ9703	+ 730 cd	+ 656 cd	+ 460 cd	+ 300 cd	+ 277 cd	+ 165 cd	-	-
N. lactamica 020-06	+, Alt 748 cd	+ 656 cd	+ 452 cd	+ 309 cd	+ 277 cd	+ 165 cd	+, Alt, 196 cd	-
N. polysaccharea ATCC 43768	+, Alt 748 cd	+ 656 cd	+ 452 cd	+ 309 cd	+ 277 cd	+ 165 cd	-	-
N. mucosa C102	+, Alt 753 cd	+ 656 cd	+ 462 cd	+ 300 cd	+ 277 cd	+ 165 cd	-	-
N. cinerea ATCC 14685	+, Alt, 745 cd	+ 654 cd	+ 452 cd	+ 309 cd	+ 277 cd	+ 165 cd	-	-
N. macacae ATCC 33926	+, Alt 758 cd	+ 657 cd	+ 452 cd	+ 299 cd	+ 277 cd	+ 165 cd	-	-
N. elongata subsp. glycolytica ATCC 29315	-	-	-	-	-	-	-	-
N. shayeganii 871	-	-	-	-	-	-	-	-
N. wadsworthii 9715	-	-	-	-	-	-	-	-
N. bacilliformis ATCC BAA-1200	-	-	-	-	-	-	-	-

+ indicates the gene is intact, * indicates the gene contains a premature stop codon, - indicates the gene is totally absent, Alt indicates the gene starts on a non-ATG start codon, Δ indicates the gene is partially deleted. Cd, codons.

12.7.1.2 Alignment of FA1090 nos genes to reference Neisseria *genomes*

BLASTn multiple alignments of individual FA1090 *nos* genes against all reference *Neisseria* genomes were conducted. This showed a high conservation in all *nos* genes across *Neisseria* species, with the exceptions of *N. meningitidis* and *nosX*.

FA1090 *nosR* showed wide conservation across six of the seven other reference *Neisseria* species which contained *nos* operons, and that the shortened *nosR* in *N. meningitidis* had strong homology to the 5'-end of FA1090 *nosR* (Figure 16A).

FA1090 *nosZ* showed high conservation across six of the seven other reference *Neisseria* species which contained *nos* operons. Optimization for more dissimilar and somewhat similar sequences did not reveal an *N. meningitidis* alignment to FA1090 *nosZ* in either the coding or complementary strands, confirming our suggestion that *nosZ* is absent in *N. meningitidis* (Figure 16B).

FA1090 *nosD* showed high conservation across the seven other reference *Neisseria* species which contained *nos* operons, and the shortened *N. meningitidis nosD* had high homology to the 3'-end of FA1090 *nosD*, suggesting an 5'-end deletion (Figure 16C).

FA1090 *nosF* showed that high conservation across the seven other reference *Neisseria* species which contained *nos* operons, and that the shortened *N. meningitidis nosF* had high homology to the 5'-end of FA1090 *nosF*, suggesting a 3'-end deletion (Figure 16D).

FA1090 *nosY* showed high conservation across the seven other reference *Neisseria* species which contained *nos* operons, and that the highly shortened *N. meningitidis nosY* had high homology to the 3'-end of FA1090 *nosY*, suggesting an 5'-end deletion (Figure 16E). It is worth noting that this *nosY* gene is not annotated as a coding sequence in the MC58 genome.

FA1090 *nosL* showed high conservation across the seven other reference *Neisseria* species which contained *nos* operons, with less conservation in the 3'-end of *N. mucosa* and *N. subflava*, suggesting an 5'-end deletion (Figure 16F).

A BLASTn search of the FA1090 *nosX* sequence from its TTG start codon till 100 bp after the premature stop codon show showed high conservation with *N. lactamica*, and with the 5'-end of *N. meningitidis*, but a little before the stop codon homology with *N. lactamica* decreased (Figure 16G), further suggesting a 3'-end deletion in *N. gonorrhoeae* has occurred which also introduced the

premature stop codon. The FA1090 *nosX* premature stop codon region aligns to a region of the *N. lactamica* genome distant to the *nosX* gene. This is a repeat region in a hypothetical protein (NLA_RS12840) containing a gonococcal DNA uptake sequence (GCCGTCTGAA), suggesting *N. gonorrhoeae* may have acquired this sequence via horizontal transformation (Spencer-Smith et al., 2016).

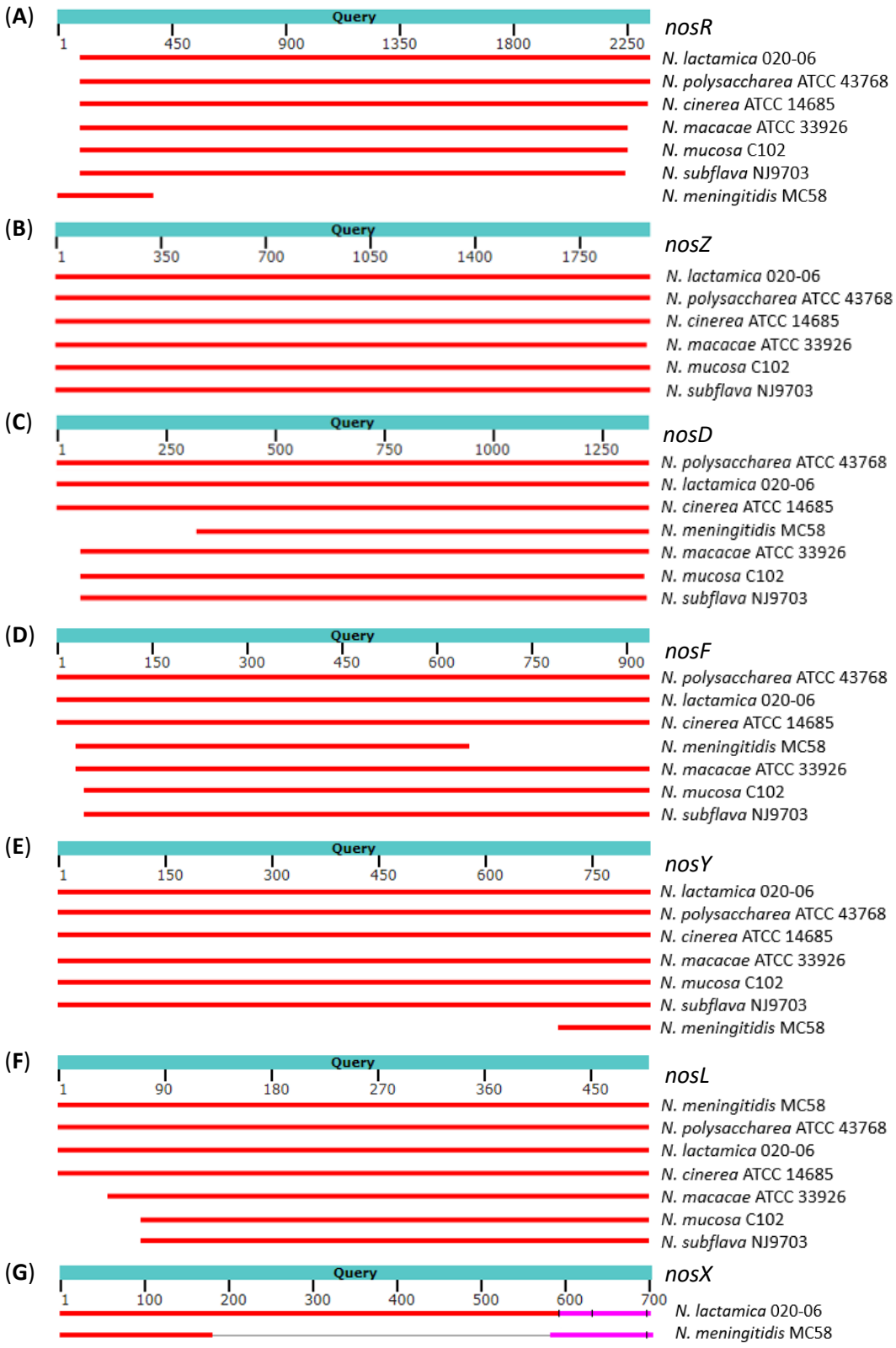


Figure 16: Homologous regions of individual Neisserial *nos* genes to reference genomes.
(**A**) NosR. (**B**) NosZ. (**C**) NosD. (**D**) NosF. (**E**) NosY. (**F**) NosL. (**G**) NosX and adjacent downstream region.

12.7.1.3 *The nonsense mutations in nos are unique to* N. gonorrhoeae

Codon MUSCLE alignment revealed that the TAA and TAG nonsense mutations encoding premature stop codons in the *nosR*, *nosZ* and *nosD* genes are unique to *N. gonorrhoeae* among the sequenced *Neisseria* reference genomes (Figure 17).

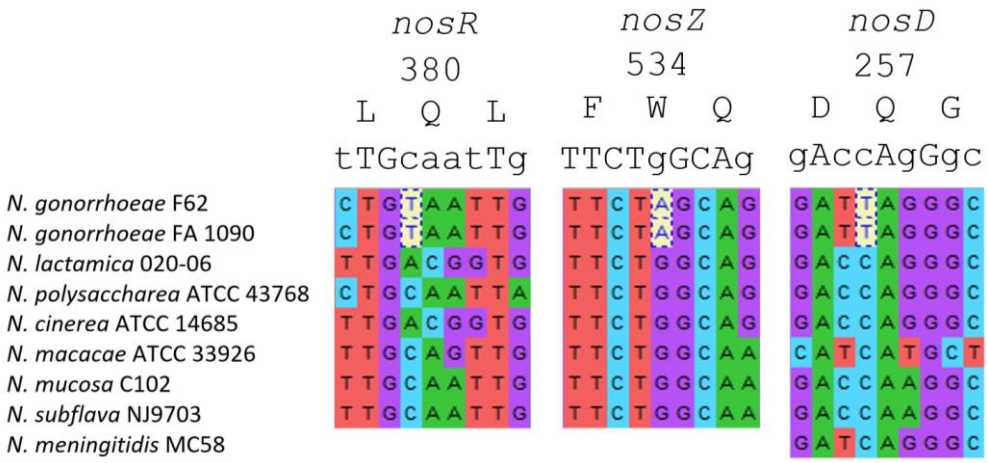


Figure 17: Alignment of gonococcal *nos* operon to *Neisseria* reference genomes.
MUSCLE codon alignment shows the gonococcal nos operon contains three novel nonsense SNPs (highlighted) compared to other *Neisseria* species.

12.8 Specific homology searches of the *N. gonorrhoeae* FA1090 *nos* amino acid sequence with those from PDB NosZ crystals

12.8.1.1 Copper site conservation in crystal NosZ structures

We aimed to investigate if nonsense truncations in NosR, NosZ, NosD and NosX proteins may affect binding sites for metals, DNA or other substrates. However, none of the *Neisseria* reference genomes have crystallised Nos proteins. So, we analysed the sequences of Nos proteins from other species which have been crystallised. To date, NosZ has been crystallised from five different organisms: *Shewanella denitrificans*, *Paracoccus denitrificans*, *Marinobacter hydrocarbonoclasticus* (previously *Pseudomonas nautica*), *Achromobacter cycloclastes*, and *Pseudomonas stutzeri*. The catalytic Cu binding sites are largely conserved, with varying N-terminal sequence lengths (summary in Table 20, sequences in Appendix 6). Cu_A and Cu_Z sites in FA1090 NosZ were determined based on homology to these other species.

Table 20: List of crystallised NosZ proteins and their Cu binding site residues

Species	Cu_A site residues	Cu_Z site residues	PDB codes
Shewanella denitrificans	H574, C609, W611, C613, C617, M620	H125, H126, H174, H318, H373, H424, H485	5I5M (with Ca^{2+}) , 5I5I (apo) & 5I5J (reduced apo)
*Paracoccus denitrificans**	H538, C573, W575, C577, H581, M762	H88, H89, H137, H280, H335, H386, H447	1FWX
Marinobacter hydrocarbonoclasticus	H526, C561, W563, C565, H569, M575	H79, H80, H128, H270, H325, H376, H437	1QNI
*Achromobacter cycloclastes**	H585, C620, W622, C624, H628, M631	H135, H136, H184, H327, H382, H433, H494	2IWF (resting form) & 2IWK (inhibitor-bound)
Pseudomonas stutzeri	H583, C618, W620, C622, H626, M629	H129, H130, H178, H326, H382, H433, H494	3SBQ (native 2), 3SBP (native 1) & 3SBR (N2O adduct)
Neisseria gonorrhoeae FA1090	H600, C635, W637, C639, H643, M646	H152, H153, H201, H343, H398, H449, H510	N/A

Predicted Cu binding sites in *Neisseria gonorrhoeae* FA1090 NosZ in grey. * indicates that residue count includes predicted signal peptide.

12.8.1.2 NosZ crystal structure sequence BLASTp

BLASTp reveals that the closest homolog to *N. gonorrhoeae* FA1090 is the NosZ from *Achromobacter cycloclastes* (PDB code 2IWF), with 58% identity and 99% cover (Figure 18). The NosZ crystal structure from this species was used in this study to model the structure of FA1090 NosZ.

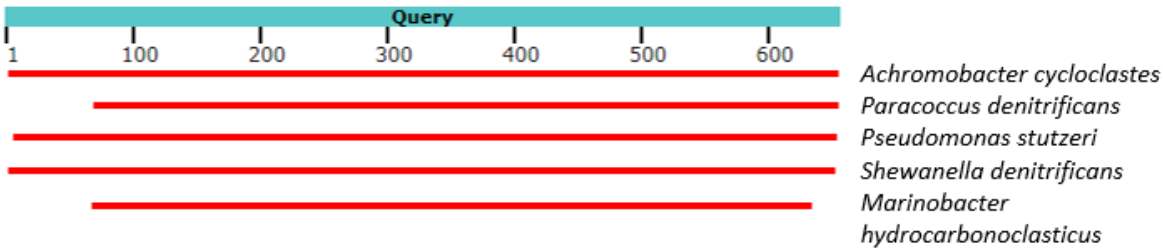


Figure 18: Homologous regions of the FA1090 NosZ amino acid sequence to sequences from crystallised NosZ proteins.

12.8.1.3 Crystal NosZ sequences codon MUSCLE alignment

MUSCLE codon alignment of the FA1090 NosZ amino acid sequence to amino acid sequences from species with a NosZ crystal suggests that the residue which has been nonsense-mutated in *N. gonorrhoeae* has been inserted in *Neisseria* (Figure 19). This is supported by a BLASTp alignment

showing that where *N. gonorrhoeae* has a stop codon, species with a NosZ crystal have a gap in the alignment (data not shown).

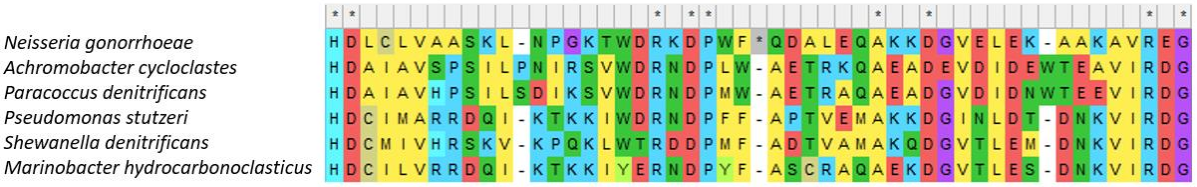


Figure 19: Alignment of FA1090 NosZ premature stop codon region to crystalised Nos proteins.
(**A**). MUSCLE alignment of FA1090 protein sequence to crystalised Nos proteins. Species are ranked in order of homology to FA1090. (**B**) BLASTp alignment of FA1090 protein sequence to crystalised Nos proteins. * indicates premature stop codon.

12.8.1.4 BLASTn of FA1090 nosZ *with species with a NosZ crystal*

The nucleotide sequences of the *nosZ* gene in the species which had produced crystallised NosZ proteins are available from NCBI (Appendix 7). BLASTn showed that these sequences lacked 5'-end homology to FA1090, but had some conservation at the C terminal (Figure 20A). Homology was moderate, with the best alignment (*Marinobacter hydrocarbonoclasticus*) having 67% identity to FA1090. BLASTp of the translated sequences found an increase in homology when comparing protein sequences, suggesting nucleotide differences between the species may not translate into amino acid differences (Figure 20B).

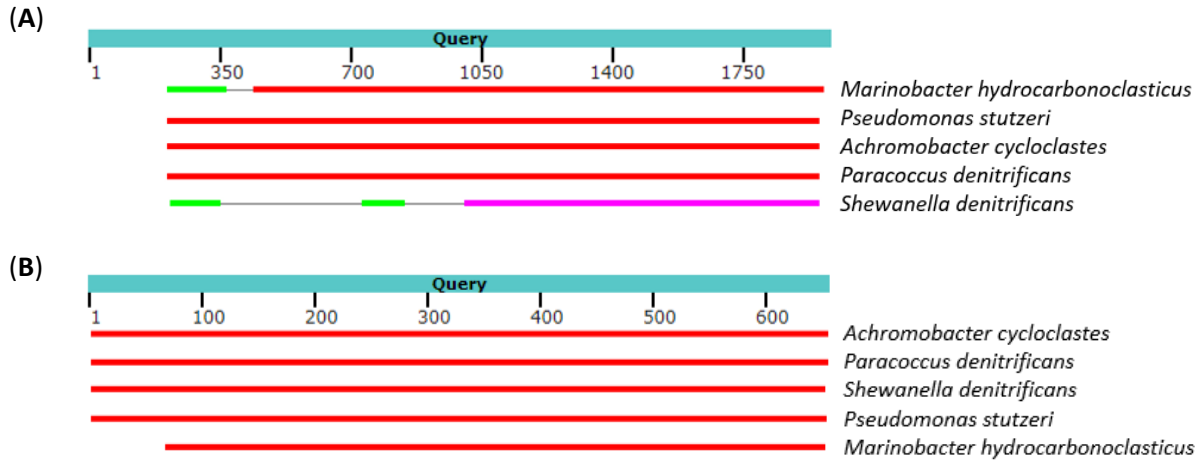


Figure 20: Alignment of FA1090 *nosZ* gene and NosZ protein to species with a crystallised NosZ.
(**A**) Homologous regions of FA1090 *nosZ* gene to species with crystallized NosZ. (**B**) Homologous regions of FA1090 NosZ protein residues to species with crystallised NosZ.

MUSCLE codon alignment suggests that a G/T → A mutation causes a premature stop codon in *N. gonorrhoeae* (Figure 21A). Interestingly, this is also seen in *Shewanella denitrificans*, but this does not translate into a stop codon because the nucleotide sequences from the two species are in different frames. BLAST alignments revealed that in some cases the FA1090-unique TAG premature stop codon in FA1090 has been caused by a TA insertion (*Marinobacter hydrocarbonoclasticus*, *Pseudomonas stutzeri*), but in some cases is a mutation of one nucleotide (*Paracoccus denitrificans*, *Shewanella denitrificans*) or two nucleotides (*Achromobacter cycloclastes*, Figure 21B). It is interesting to note that the nucleotide alignments (Figure 21) show the gonococcal premature stop codon is not inserted, compared to the amino acid alignments (Figure 19).

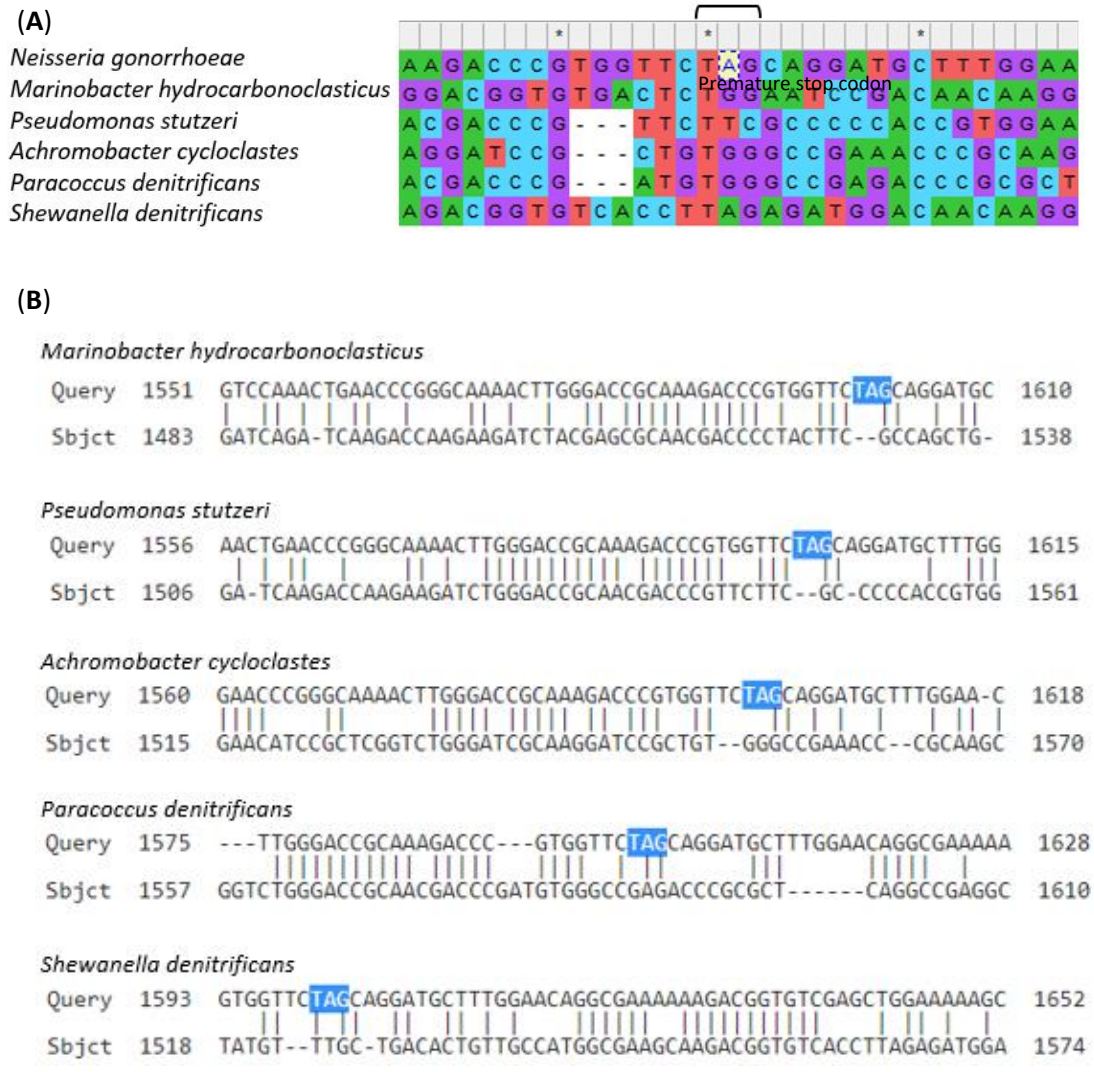


Figure 21: Nucleotide alignment of *nosZ* genes from species which have crystallized NosZ to FA1090 *nosZ*. (**A**) MUSCLE nucleotide alignment of *nosZ* genes from species which have crystallized NosZ to FA1090 *nosZ*. (**B**) BLASTn alignment of *nosZ* genes from species which have crystallized NosZ (Subjects) to FA1090 *nosZ* (Query).

12.8.1.5 Superimposition of crystal NosZ structures

To determine the conservation of the three-dimensional structure of the NosZ protein, the structures of five crystallised NosZ proteins (2IWF, 1FWX, 3SBQ, 5I5M, and 1QNI) were superimposed using SWISS-MODEL. This reveals high structural homology and conservation of Cu_Z site across all five species (Figure 22A).

The three-dimensional structure of the FA1090 NosZ N-terminus up to the premature stop codon was predicted by AlphaFold and superimposed onto the crystal structure of the closest protein sequence homolog with a crystal structure, *Achromobacter cycloclastes* 2IWF (Figure 22B). This revealed a high homology three-dimensional structural homology between the two species.

Felgate et al (2012) studied a ~65 kDa NosZ of *Paracoccus denitrificans*, finding that a ~50 kDa Cu_Z-only truncated form of NosZ was produced in low Cu. SignalP predicted the *Paracoccus denitrificans* PD1222 NosZ peptide to have a 57-residue signal peptide, leaving a 66 kDa peptide. We predicted a 50 kDa *Paracoccus denitrificans* PD1222 NosZ has 450 amino acids. The end of a 450-residue *Paracoccus denitrificans* PD1222 NosZ was within 19 aa (2.2 kDa) of the FA1090 premature stop codon.

The three-dimensional structure of the FA1090 NosZ N-terminus up to the premature stop codon was predicted by AlphaFold and superimposed onto the crystal structure of *Paracoccus denitrificans* 1FWX (Figure 22C). This revealed a high homology between the two species.

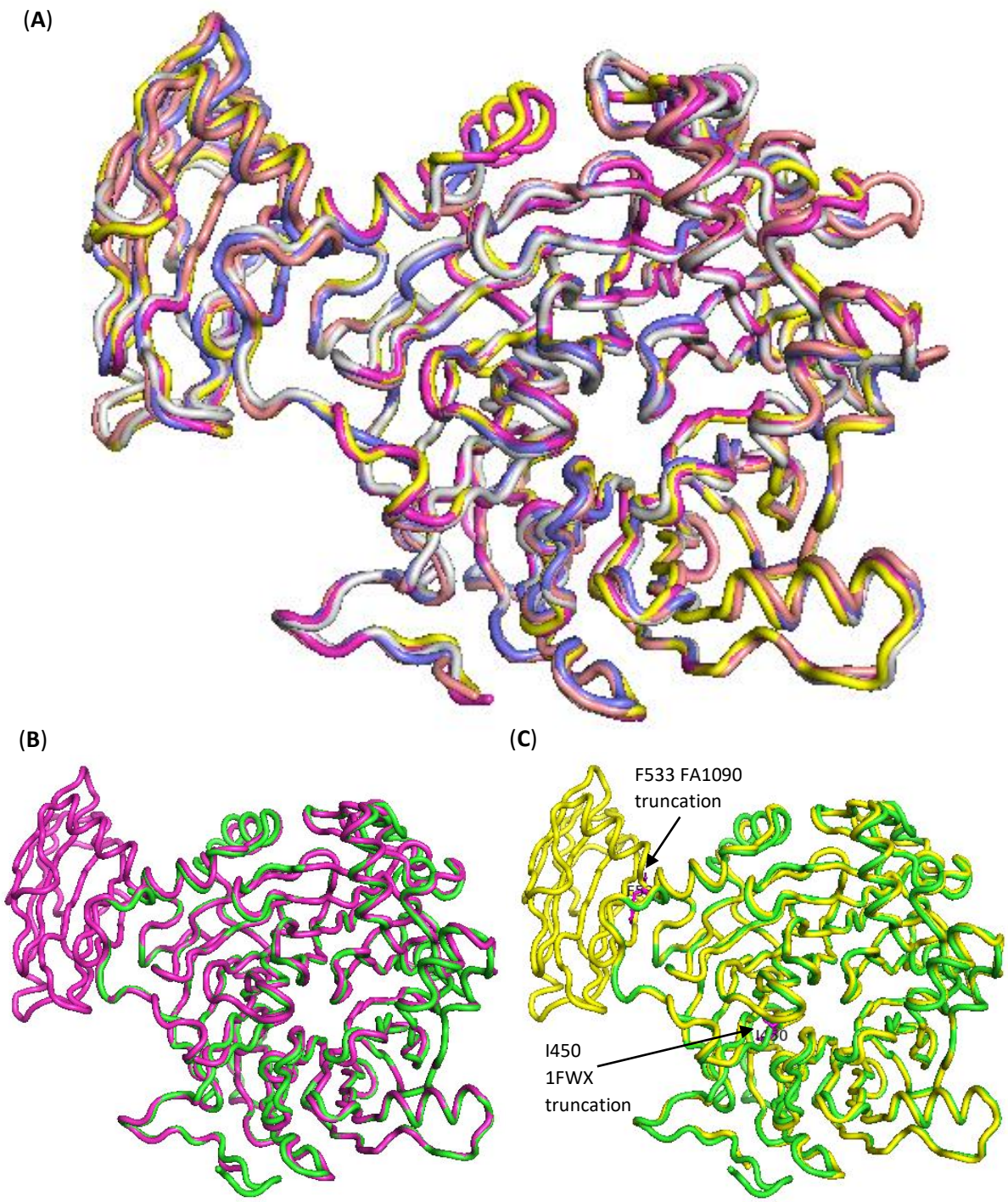


Figure 22: Superimposed crystal structures of various NosZ proteins.
(**A**) Species with a NosZ crystal structure homodimer: *Achromobacter cycloclastes* 2IWF (purple), *Paracoccus denitrificans* 1FWX (yellow), *Pseudomonas stutzeri* 3SBQ (pink), *Shewanella denitrificans* 5I5M (grey), and *Marinobacter hydrocarbonoclasticus* 1QNI (blue). (**B**) FA1090 NosZ AlphaFold monomer structure (green) superimposed onto the closest protein sequence homolog with a crystal structure*, Achromobacter cycloclastes* 2IWF (purple). (**C**) FA1090 NosZ AlphaFold monomer structure (green) superimposed onto *Paracoccus denitrificans* 1FWX (yellow); truncation residue estimated based on Felgate et al., 2012.

12.9 Specific homology searches of the *nos* operon to disparate gonococcal isolates

The 8439 bp *nos* operon was found to be highly conserved across *N. gonorrhoeae* isolates. A NCBI BLASTn search of the FA1090 *nos* operon reveals the top 100 gonococcal isolates all possess at least 99.83% homology.

The FA1090 *nosR*, *nosZ*, *nosD* and *nosX* genes were searched in NCBI BLASTn and the top 100 most homologous gonococcal isolates searched for the 9-nucleotide sequences ending in the premature stop codon. This found each premature stop codon was always conserved in the top 100 most homologous gonococcal isolates.

Subsequently, we were interested to consider the effect of rescuing the premature stop codons in an attempt to find gonococcal isolates which may not be nonsense mutated. We altered the sequences of the FA1090 *nosRZD* genes to rescue the sense codons seen in close homologs (Table 21 and Figure 23).

Table 21: FA1090 native and rescued *nos* premature stop codon sequences

Gene	FA1090 sequence	Rescued sequence
nosR	5′ ...TGGCGGCTG**t**AA...3′	5′ ...TGGCGGCTG**c**AA...3′
nosZ	5′ ...CCGTGGTTCT**a**G...3′	5′ ...CCGTGGTTCT**g**G...3′
nosD	5′ ...AGCCGCGAT**t**AG...3′	5′ ...AGCCGCGAT**c**AG...3′

When these rescued sequences were searched in BLASTn for homology across gonococcal isolates, the *nosR*, *nosZ* and *nosD* rescue codon were not found in the top 100 most homologous gonococcal isolates; the premature stop codon was always present. This gives further evidence to suggest that the gonococcal *nos* operon premature stop codons are always conserved in *N. gonorrhoeae* isolates.

However, a limitation of these analyses was that they selected for isolates which were similar to FA1090. It may be the case that the premature stop codons in the FA1090 *nos* operon may have only emerged in more recent strains, due to the impact of antibiotics or other selection pressures. Hence, we then thought to test the conservation of the *nos* operon premature stop codons in two

gonococcal isolates spanning 100 years which were expected to be disparate to FA1090 (isolated 1980 United States); DO371 (isolated 1928 Denmark, PubMLST id: 78659) and G97687 (isolated 2018, PubMLST id: 12039 England/Thailand). FA1090 is a 1970s United States endocervix isolate (Nachamkin et al., 1981), DO371 is a 1920s Danish isolate of unknown anatomical location (Golparian et al., 2020) and G97687 is a 2010s English/Thai male urethral isolate (Eyre et al., 2018; Liu et al., 2019). DO371 is the oldest known sequenced gonococcal isolate, and G97687 was the most recent antibiotic-resistant isolate to be sequenced at the time of writing.

Since pseudogenes may be expected to gradually accumulate mutations and deletions (Pink et al., 2011), particularly in the promoter region (Feavers and Maiden, 1998), we hypothesised the gonococcal *nos* operon should display highly variable deleterious mutations between different isolates. However, the number of variants between the DO371, FA1090 and G97687 Nos proteins (NosR 0.77%, NosZ 0.26%, NosD 0.89%, NosF 0.66%) were all within the same order of magnitude as GyrA (0.33%), which is thought to be an essential protein (Table 22). Of these amino acid variants in the *nos* operon between these three strains, 77% were conservative (i.e. an amino acid with similar biochemical properties), whereas only 33% were conservative in GyrA. There were no variants in the *nos* promotor FNR and NsrR box regions and terminator stem loop. Furthermore, BLASTn revealed the FA1090 and G97687 *nos* promoter regions (including the FNR and NsrR boxes) were identical. We hypothesise that homology of these regions across both homologous and disparate isolates from pre- and post-antibiotic eras may be indicative of some unknown function for the operon.

It has been estimated that the gonococcal genome mutates at a rate of only 3.6-6.4 SNPs per year (De Silva et al., 2016; Golparian et al., 2020). Therefore, the chance of any one nucleotide being mutated in 90 years (from isolate DO371 to isolate G97687) is only 0.015-0.0276%. Hence, the approximate likelihood of one SNP occurring within 90 years in *nosR*, *nosZ*, *nosD* and *gyrA* is 35-62%, 30-53%, 20-36% and 41-73% respectively. These estimates predict a lower level of amino acid

polymorphisms than is actually seen in our genes of interest (Table 22), suggesting they are mutating faster than the genome as a whole.

Table 22: Amino acid polymorphisms in the *nos* operons (*gyrA* as a control gene)

Gene	Protein size (aa)	Mismatch residue	DO371 (c.1928)	FA1090 (c.1980)	G97687 (c.2018)	Substitution	Query cover
NosR (2,331 bp/775 aa) – 0.77% of amino acids are mismatches							100%
		149	S	S	G	*	
		519	M	T	T		
		604	F	L	F	*	
		618	V	A	V	*	
		637	C	C	R		
		737	A	E	A		
NosZ (1,968 bp/654 aa) – 0.46% of amino acids are mismatches							99.54%
		141	G	S	G	*	
		361	S	P	P		
		491	D	G	D		
NosD (1,356 bp/450 aa) – 0.89% of amino acids are mismatches							99.11%
		126	P	A	A	*	
		128	V	V	I	*	
		229	S	S	C	*	
		303	M	M	I	*	
NosF (927 bp/305 aa) – 0.66% of amino acids are mismatches							99.35%
		196	V	I	V	*	
		288	L	F	F	*	
NosY (831 bp/276 aa)							100%
		-	-	-	-		
NosL (495 bp/164 aa)							100%
		-	-	-	-		
GyrA (2,751 bp/917 aa) – 0.33% of amino acids are mismatches							99.78%
		91	T	S	F		
		95	D	D	A		
		250	M	I	M	*	

Asterisks (*) indicate conservative amino acid substitutions.

12.10 Analysis of conservation of gonococcal isolates from PubMLST

SNPs in the *nos* operon premature stop codons of 19,134 *N. gonorrhoeae* isolate genomes from PubMLST (https://pubmlst.org/) were identified against FA1090 using BLASTn and MATLAB (Appendix 12, R. Thompson, personal communication). The *nos* premature stop codons showed a high degree of conservation across all gonococcal isolates analysed. Firstly, no isolates showed variation in the *nosR* premature stop codon; the TAA premature stop codon was always conserved. Secondly, only four isolates exhibited the *nosZ* TGG sense codon seen in other *Neisseria* species. This

variant comprised 0.02% of isolates and there was no other kind of variant at this codon. Finally, only three isolates exhibited the *nosD* CAA codon and four isolates the CAG codon seen in other *Neisseria* species. These variants comprised 0.04% of isolates and there was no other kind of variant at this codon. Metadata for the few isolates with variation at the *nos* premature stop codon regions is limited. Hence, we can currently draw no conclusions as to the effect of these mutations based on this analysis.

DO371 was an isolate from PubMLST and had no variants in the *nos* operon premature stop codons to FA1090.

12.11 Predicting NosR, NosD and NosX truncations

No crystal structures exist for NosR, NosD, NosX, but previous work has partially characterised their structure and function, allowing us to evaluate the effect of the deletions posed by premature stop codons.

In gonococcal *nosR*, the 380th codon is a premature stop codon which is unique to *N. gonorrhoeae* (Figure 23). However, the 381st codon is a TTG alternative start codon and the 382nd is an ATG start codon. So, there is a possibility FA1090 NosR may not truncated at all due to translation reinitiation. NosR is an integral membrane protein with six transmembrane helices and a C-terminal cytoplasmic domain containing sequence motifs for two [4Fe-4S] clusters (Wunsch and Zumft, 2005). The premature stop codon is predicted to occur after the periplasmic domain and before any transmembrane helices, distant to the Fe-S domain. Tn5 insertion mutagenesis of *nosR* in *P. stutzeri* resulted in failure to transcribe the *nosZ* gene (Cuypers et al., 1992) and both N and C-terminal sections of NosR are thought to be required for NosZ activity (Wunsch and Zumft, 2005).

In gonococcal *nosD*, the 257th codon is a premature stop codon which is unique to *N. gonorrhoeae* (Figure 23). The 260th codon is a start codon, meaning only three amino acids may be deleted should reinitiation occur. It is known that NosZ activity is abolished if any of the *R. meliloti nosDFY* genes are

mutated by Tn5 insertion (Zumft et al., 1990), but the effect of small deletions has not yet been characterized.

N. gonorrhoeae contains a *nosX* gene which is 43,520 bp downstream of the *nosRZDFYL* operon. In the FA1090 *nosX*, the 200th codon is a premature stop codon. Our analysis found FA1090 *nosX* homology to other species ends before this premature stop codon, suggesting there has been a deletion of the last 366 bp of the gene which has also introduced a premature stop codon (Barth et al., 2009a; Chan et al., 1997). NosX is suggested to be a periplasmic protein which has a known transmembrane helix at 270 -293 aa (Chan et al., 1997).

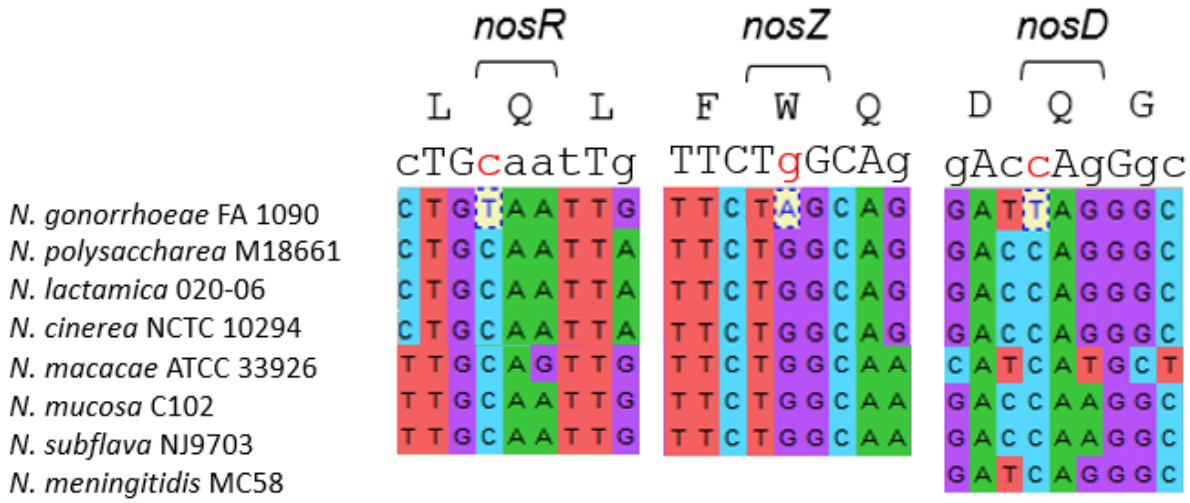


Figure 23: Alignment of gonococcal *nos* operon to other *Neisseria* with high homology.
MUSCLE codon alignment of FA1090 premature stop codon regions to those from other *Neisseria* species with high homology. Premature stop codon region indicated by bracket.

12.12 Predicting stop codon readthrough in the gonococcal *nos* operon

Three of the genes in the gonococcal *nos* operon (*nosRZD*) contain premature stop codons, suggesting inactivation of this denitrification step may confer an advantage. The effect of premature stop codons will occur during translation, whereby ribosomes would be expected to fall off the *nos* operon transcript prematurely, releasing truncated proteins. Another hypothesis is that the gonococcal *nos* premature stop codons are translationally readthrough such that a functional NosZ is present in *N. gonorrhoeae*.

Translational read-through (also known as *stop codon suppression*) occurs when a stop codon does not result in termination of translation. While pseudogene predictors are largely focused on eukaryotes (Karro et al., 2007), we will discuss the current knowledge regarding stop codon readthrough in bacteria. Readthrough events are thought to occur frequently in prokaryotic protein-coding genes, supporting the notion that nonsense substitutions in bacteria are common and do not always cause pseudogenization, rather, in-frame stop codons may provide a mechanism to down-regulate protein expression (Belinky et al., 2021).

Causes of stop codon suppression (or nonsense suppressors) can be tRNA mutations (Eggertsson and Söll, 1988; Ferré-D'Amaré, 2011), certain neighbouring nucleotide motifs (Chemla et al., 2018; Miller, 1992; Namy et al., 2004), ribosome mutations (Chernyaeva et al., 1999; Göringer et al., 1991), external stresses such as acid stress (Zhang et al., 2020) and aminoglycoside antibiotics (Diop et al., 2007; Wangen and Green, 2020), or reinitiation of translation at a downstream codon (Karamyshev et al., 2004).

The amino acid inserted at the stop codon depends on the identity of the stop codon itself. In yeast, glutamine, tyrosine, and lysine have been found to be translated instead of the TAA and TAG stop codons, while cysteine, tryptophan, and arginine have been found to be translated instead of the TGA stop codon (Blanchet et al., 2014).

12.12.1.1 Codon usage

The frequency of the TAG stop codon (the *nosZ* premature stop codon) is noticeably less than the frequency of TAA and TGA stop codons in bacteria (Belin and Puigbò, 2022; Wong et al., 2008). *E. coli* TAG codon is the least-used stop codon, terminating only ∼2% of essential genes (Johnson et al., 2012). TAG is also the least-used codon in *N. gonorrhoeae* (Table 23), and accounts for only ~16% of all stop codons (TAA and TGA make up the remaining ~56% and ~28%, respectively). The premature stop codon affecting the gonococcal *nosR* gene is TAA, the most commonly used stop codon.

Nearby start codons (ATG in the case of *nosD* and the alternative start codon TTG in the case of *nosR* and *nosZ*) may continue translation of the gonococcal *nos* mRNA and rescue protein function. Bacteria often use alternative non-ATG start codons to initiate translation, predominantly GTG (9-14%) and TTG (3-13%) as well as others to a lesser degree (Belinky et al., 2017; Blattner et al., 1997; Hecht et al., 2017; Panicker et al., 2015; Villegas and Kropinski, 2008). In some bacteria, the TTG alternative start codon is actually more prevalent and leads to higher levels of protein production than the GTG alternative start codon (Rocha et al., 1999). In addition to the NTG alternative initiations, there are rare instances of bacteria initiating translation from a ATT codon (Binns and Masters, 2002).

The 1st and 2nd codons downstream of the gonococcal *nosR* premature stop codon are TTG and ATG, and the 7th and 9th downstream codons are TTG and GTG (Figure 24). The 4th codon downstream of the gonococcal *nosZ* premature stop codon is TTG. The 3rd codon downstream of the gonococcal *nosD* premature stop codon is ATG. The 10th codon downstream of the *nosX* premature stop codon is TTG. These findings suggest that there are various likely re-initiation sites present in nonsense-mutated *nos* operon genes. However, there are no SD sequences immediately upstream of alternate start codons in *nosR*, *nosZ* & *nosD*.

Genes with an alternative start codons are less likely to possess a SD sequence, which may explain their decreased translation efficiency (Belinky et al., 2017) compared to genes with standard ATG start codons. The SD sequence in *Neisseria* is suggested to be 5' AAGG(AG) 3', beginning 10 to 12 nucleotides upstream of the start codon (Goytia et al., 2015; Moore and Sparling, 1995; Takahashi and Watanabe, 2005). In the FA1090 *nos* operon, there is no SD sequence preceding the first gene (*nosR*), only *nosZ* and *nosY* have SD sequences preceding the start codon, and no *nos* gene has a SD sequence such that it could begin translation to repair a break in the gene caused by a premature stop codon. However, there is evidence to suggest that bacteria have the capacity to initiate translation via a SD-independent mechanism (Fargo et al., 1998; Wu and Janssen, 1996).

Furthermore, there is no SD sequence preceding FA1090 genes with supposedly essential function such as *gyrA* (NGO_0629) and cbb_3-type cytochrome *c* oxidase subunit I (NGO_1374), suggesting that and SD sequence is not always needed for gonococcal translation.

We hypothesised that the gonococcal TTG codon has sufficient levels of cognate CAA 5'→3' Leucine anticodon tRNA to be readily available to reinitiate *nos* translation. Estimates of tRNA level in bacteria vary: in *E. coli*, it is estimated that the CAA anticodon makes up 3% of the total tRNA abundance (out of 46 anticodons, with an average of 2.17% of total tRNA for each anticodon) (Dong et al., 1996), while another study found CAA to be a relatively minor *E. coli* tRNA anticodon (Emilsson and Kurland, 1990). In *Mycoplasma capricolum* and *P. aeruginosa,* the CAA anticodon tRNA abundance is found to be a little below average (West and Iglewski, 1988; Yamao et al., 1991). No count of the relative abundance of different tRNAs in *N. gonorrhoeae* has been made, however, in *E. coli* the abundance of tRNAs is clearly correlated with codon usage in mRNA (Dong et al., 1996). This would imply that if the TTG codon is common in *Neisseria gonorrhoeae*, the CAA tRNA will be common also. In *N. gonorrhoeae*, the TTG Leu alternative start codon is more common in the genome than the normal ATG Met codon (Table 23) (NCBI GenBank Flat File Release 160.0, 2007; West and Clark, 1989).

Table 23: *Neisseria gonorrhoeae* Codon Usage Database (https://www.kazusa.or.jp/codon/)

Neisseria gonorrhoeae **[gbbct]: 397 CDS's (130618 codons)**

fields: [triplet] [frequency: **per thousand**] ([number])

UUU 20.4(2661)	UCU 10.6(1382)	UAU 17.1(2237)	UGU 2.7(352)
UUC 22.3(2910)	UCC 15.0(1964)	UAC 22.7(2959)	UGC 5.1(662)
UUA 7.7(1010)	UCA 6.6(867)	UAA 1.7(221)	UGA 0.8(110)
UUG 31.2(4073)	UCG 9.5(1243)	UAG 0.5(66)	UGG 11.9(1548)
ALT		STOP	
CUU 9.5(1247)	CCU 7.8(1018)	CAU 9.1(1193)	CGU 10.3(1349)
CUC 8.4(1095)	CCC 12.0(1570)	CAC 11.7(1533)	CGC 21.7(2830)
CUA 1.9(245)	CCA 2.5(324)	CAA 26.4(3447)	CGA 3.1(409)
CUG 27.4(3574)	CCG 13.9(1817)	CAG 15.1(1973)	CGG 7.6(994)
AUU 21.0(2747)	ACU 11.6(1520)	AAU 21.5(2807)	AGU 6.8(889)
AUC 23.0(3005)	ACC 20.9(2725)	AAC 26.9(3514)	AGC 17.0(2216)
AUA 5.6(732)	ACA 7.3(957)	AAA 49.5(6466)	AGA 4.6(597)
AUG 22.5(2937)	ACG 12.4(1619)	AAG 12.9(1685)	AGG 3.9(506)
START			
GUU 21.3(2784)	GCU 12.1(1587)	GAU 25.4(3323)	GGU 20.1(2627)
GUC 19.0(2480)	GCC 41.2(5376)	GAC 26.3(3440)	GGC 50.6(6612)
GUA 13.2(1725)	GCA 21.0(2746)	GAA 44.3(5790)	GGA 8.8(1150)
GUG 13.7(1783)	GCG 21.7(2829)	GAG 11.5(1504)	GGG 8.1(1057)
ALT			

Coding GC 50.78% 1st letter GC 54.69% 2nd letter GC 40.92% 3rd letter GC 56.73%

12.12.1.2 Neighbouring nucleotide context

One of the factors influencing stop codon readthrough is the surrounding nucleotide context (Cassan and Rousset, 2001). A recent review of *cis*-acting factors reveals a wide range of identified motifs promoting readthrough. However, the review concludes that the influence of *cis* elements is likely to differ between genes, and it remains difficult to predict the rate of readthrough of any stop codon without additional experimental data (Palma and Lejeune, 2021).

Studies largely agree that the most established mRNA element influencing stop codon readthrough is the identity of the nucleotide immediately downstream of the stop codon (+4) (Chemla et al., 2018).

12.12.1.2.1 Neighbouring nucleotide context in eukaryotes

Much has been written on the neighbouring nucleotide motifs influencing stop codon readthrough in eukaryotes (Beryozkin et al., 2023). The mammalian TGA stop codon is the most permissive to

readthrough and the TAA stop codon is the least permissive (Wangen and Green, 2020). A +4 purine (A/G) favours translation termination, whereas a pyrimidine (C/T) facilitates readthrough (Tate and Mannering, 1996), a cytosine in particular (Wangen and Green, 2020). Another study shows a +4 cytosine is the most favourable nucleotide for promoting TGA and TAA readthrough, but readthrough of TAG is most efficient when thymidine is +4 (Manuvakhova et al., 2000). Other studies suggest the downstream viral hexamers CARYYA (where R is a purine and Y is a pyrimidine) and yeast CARNBA (where N is any of the four nucleotides and B can be T, C or G) favour stop codon readthrough (Harrell et al., 2002; Namy et al., 2001). Yet further studies in mammals suggest that the sequence T STOP C is a consensus sequence for efficient readthrough (Floquet et al., 2012), and the triplet AGC immediately upstream and downstream is required for readthrough of a nonsense mutation (Yesmin et al., 2020).

12.12.1.2.2 Neighbouring nucleotide context in bacteria

For the purposes of this study, we decided to focus on studies describing the effects of neighbouring nucleotide motifs influencing stop codon readthrough in bacteria.

A study in *E. coli* found the gonococcal *nosZ* premature stop codon TAG to be the most frequently suppressed stop codon (Chemla et al., 2018). Other studies find *E. coli* TGA codons are particularly susceptible to readthrough (Baggett et al., 2017; Fan et al., 2017). In *E. coli* and *S. typhimurium*, TGA can be reassigned to tryptophan (Hatfield and Diamond, 1993; Parker, 1989). It has been shown in *E. coli* that stop codons are readthrough with varying efficiency, depending on the +4 nucleotide in the following order of efficiency: G > A > C > T (Chemla et al., 2018).

Readthrough in *Salmonella enterica* primarily involve TAG; and cytosine is the most frequent +4 base (Feng et al., 2022). Reassignment of the TAG to an amino acid has been documented in *E. coli* (Johnson et al., 2012).

Based on these studies, and the finding that the gonococcal *nosZ* premature stop codon is a TAG with a +4 cytosine (Figure 24), we tentatively suggest it may be likely to be readthrough. The

gonococcal *nosD* premature stop codon is a TAG with a +4 guanine, suggesting it may be somewhat permissive to readthrough. The gonococcal *nosR* premature stop codon is a TAA with a +4 thymidine, suggesting it is unlikely to be readthrough.

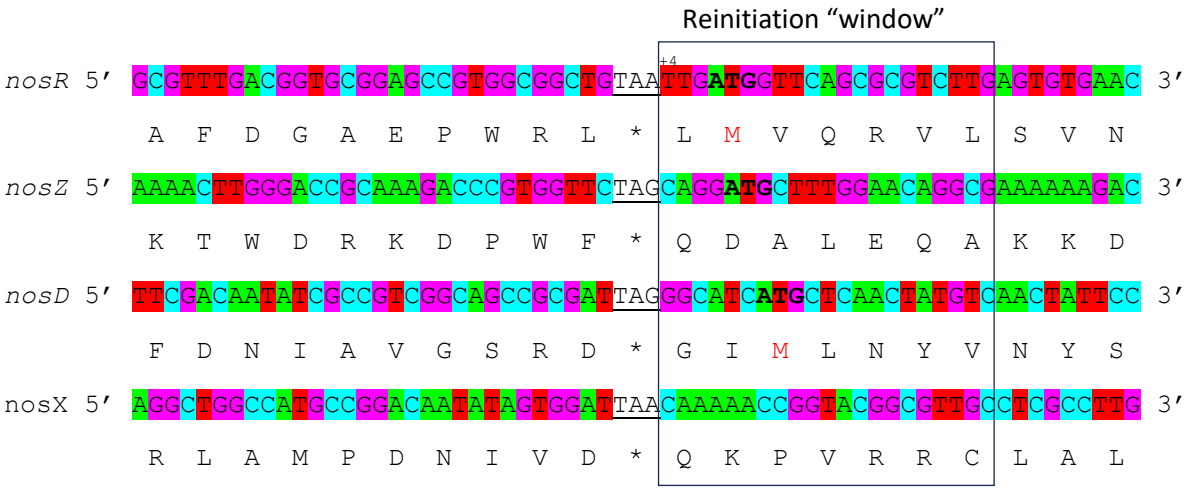


Figure 24: Gonococcal *nos* premature stop codons and surrounding regions.
Downstream ATG codons within reinitiation window in bold.

Reinitiation of translation at ATG occurs in a 7-codon window downstream of the *E. coli* stop codon (Karamyshev et al., 2004). In-frame downstream ATG start codons occur in the reinitiation window of *nosR* and *nosD*, suggesting reinitiation of translation may occur. An out-of-frame ATG start codon occurs in *nosZ*, which translates to the sequence MLWNRRKKTVSSWKKPPKPYAKATKYACI*, which has no homology to any known protein.

12.13 Visualising reinitiation of NosZ protein translation

Possible reinitiation sites for gonococcal nosZ translation were mapped onto the crystal structure of the homologous *Achromobacter cycloclastes* NosZ protein (PDB: 2IWF). The gonococcal *nosZ* premature stop codon is unique to *N. gonorrhoeae* (Figure 17 and Figure 23), and occurs at the 534th codon, between the Cu_A and Cu_Z sites. However, the 538th codon is an alternative start codon TTG, meaning potentially only four amino acids are deleted should reinitiation occur (Figure 25A). The next alternative start codon is not until the 563rd codon GTG (a 29 amino acid deletion, Figure 25B) and the next ATG start codon is not until the 565th codon (a 31 amino acid deletion, Figure 25C).

These deletions occur in a region of the homodimer distant to the catalytic Cu_Z sites and the interface between the two monomers (Figure 25A). A nine-nucleotide deletion between the gonococcal *nosZ* premature stop codon and the next reinitiation codon likely corresponds to a three amino acid deletion in an area of the enzyme distal to the active site (Figure 25A). We hypothesise such a deletion may not impact NosZ function, since the N- and C-terminal regions may still fold together to form an intact functional enzyme.

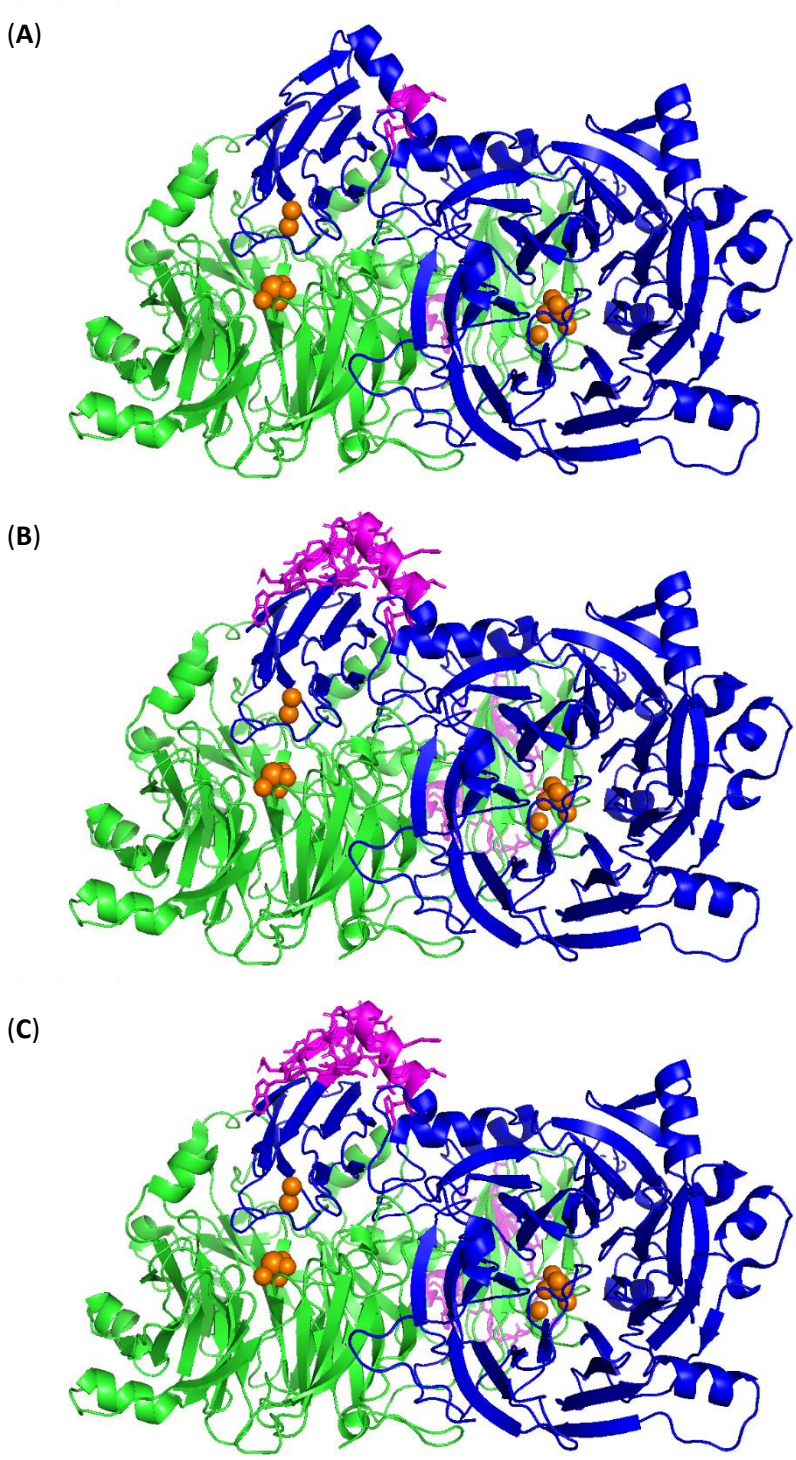


Figure 25: Predicted effects of the *N. gonorrhoeae* FA1090 nosZ premature stop codon.
Deletions plotted on the crystallized NosZ homolog from *Achromobacter cycloclastes* (PDB: 2IWF). (**A**) The four-residue deletion from premature stop codon 534 to TTG alternative start codon 538. (**B**) The twenty nine-residue deletion from premature stop codon 534 to GTG alternative start codon 563. (**C**) The thirty-one residue deletion from premature stop codon 534 to ATG standard start codon 565.

12.14 Discussion: Bioinformatics of the *nos* operon

The *N. gonorrhoeae* strain FA1090 *nos* operon is comprised of seven genes, *nosRZDFYL*, most of which have been partially characterized. Based on differences in operon structure, we suggest that *N. gonorrhoeae* and *N. meningitidis* both independently inactivated *nos* since these species diverged.

Based on current knowledge, we cannot make accurate predictions as to the probability that the gonococcal nonsense mutations will be readthrough based solely on the surrounding nucleotide context (*cis* elements). However, there remain six reasons to hypothesise that the particular premature stop codons in the gonococcal *nos* operon may not result in NosZ inactivation:

(1) Other than the premature stop codons, FA1090 has high nucleotide sequence and protein structural homology across the entire *nos* operon to the functional *nos* operons of other species (Figure 14, Figure 16 and Figure 20), suggesting most gonococcal Nos proteins may still be functional if the premature stop codons are readthrough.

(2) The gonococcal *nosR*, *nosZ* and *nosD* nonsense mutations are single-base substitutions (Figure 17 and Figure 21), the least deleterious mutation type. The *nosX* gene appears to have a highly deleterious 5'-end deletion (Figure 16G) but is not part of the *nos* operon. Furthermore, while the core *nosZDFYL* cluster is found in every N_2O-reducing prokaryote, *nosR* and *nosX* are not ubiquitous and are instead distributed by clade (Simon et al., 2004; Zumft and Kroneck, 2007).

(3) High conservation of *nos* premature stop codons and lack of other random mutations in the *nos* operon across homologous and disparate gonococcal isolates suggests a positive selection pressure on *nos* which may reflect a functionality (Golparian et al., 2020) (Table 22).

(4) Past research suggests that a truncated *P. denitrificans* NosZ lacking the C-terminal Cu_A domain is formed in low Cu conditions which still retains functionality (Felgate et al., 2012). The *P. denitrificans* NosZ has high structural homology to the gonococcal NosZ (Figure 20B and Figure 22C), suggesting a similarly truncated gonococcal NosZ may also possess functionality.

(5) The gonococcal *nosR*, *nosZ* and *nosD* genes contain nearby downstream ATG start codons which may allow re-initiation of translation (Karamyshev et al., 2004), and nearby downstream alternative TTG start codons (Belinky et al., 2017) (Figure 24).

(6) The NosZ premature stop codon occurs close to the C-terminus of the protein in a region of the crystal structure distal to the active sites and monomer-monomer interface (Figure 25).

13 Chapter 4: Expression of the gonococcal *nos* operon

13.1 Sequencing of *N. gonorrhoeae* F62 strain *nos* operon shows homology to FA1090

Analysis of the published genome of the gonococcal strain FA1090 (NCBI genome ASM684v1, https://www.ncbi. nlm.nih.gov/datasets/genome/GCF_000006845.1/) suggested that the *nos* operon contains three novel nonsense substitution mutations not present in other *Neisseria* species (Figure 17). These premature stop codons occur in the genes *nosR* (the putative transcription regulator and electron donor to NosZ), *nosZ* (the N_2O reductase enzyme), and *nosD* (a subunit of the sulphur transporter to NosZ).

However, a previous study claimed the gonococcal F62 strain *nosZ* gene is interrupted by a frameshift indel mutation causing a premature stop codon (Overton et al., 2006). A frameshift mutation would presumably cause the loss of the downstream reading frame and therefore total loss of the 3'-end of *nosZ*. The *nosR* gene is described by their study as degenerate, but no further description is given (Overton et al., 2006). Their study did not present sequencing data or describe whereabouts in the genes such frameshift mutations occur.

Based on this previous literature studying the gonococcal strain F62, we wanted to determine if the F62 strain *nos* operon was different to the nonsense-mutated *nos* operon present in FA1090. The anatomical location of F62 isolation was not disclosed. However, since F62 was isolated from the United States in the 1960s (Kellogg et al., 1963), we predicted it to be highly similar to FA1090 isolated in the following decade.

A BLASTn search found 100% identity of nucleotide sequence between FA1090 *nosZ* (NCBI genome ASM684v1, gene locus tag NGO_07410) and F62 *nosZ* (NCBI genome ASM16353v1, gene locus tag NGNG_RS0106100). There was also 100% homology in the FA1090 and F62 promoter regions upstream of *nosR*. Furthermore, Sanger sequencing of the genomic DNA extracts from FA1090 and F62 (DBS Genomics) revealed identical nonsense SNPs in the *nos* operon; no indel mutations were found in the F62 sequencing which would cause premature stop codons (Appendix 8). Both FA1090 and F62 strains used in this study were obtained from W. Shafer (Emory) and had previously been

used for Djoko et al., AAC (2015) (Djoko Group tube codes were KN0003 and KN0004, respectively). The F62 strain *nos* operon was found to be identical to the published NCBI FA1090 and F62 genomes. Compared to other *Neisseria*, both F62 and FA1090 genomes contain a C/A → T SNP at position 1138 (amino acid 380) in *nosR*, a G → A SNP at position 1601 (amino acid 534) in *nosZ*, and a C → T SNP at position 769 (amino acid 257) in *nosD*. These findings lead us to conclude that the sequencing in Overton et al (2006) experienced an error such as inadequate base calling which presented as a frameshift mutation. This study found that the F62 *nosRZD* stop codon regions were sometimes poorly amplified (Appendix 8). Poor amplification may lead to sub-optimal sequencing which might explain why previous authors believed there had been a single-base deletion.

It should be noted that the *nosZ* premature stop codon (TAG at residue 534) was conserved between FA1090 and the current *N. gonorrhoeae* reference genome (strain TUM19854, NCBI genome ASM1303007v1) (Appendix 9). There were only two SNPs in the FA1090 vs TUM19854 *nosZ* (**A**GC→**G**GC S141G and G**G**C→G**A**C G491D), and neither SNP causes a deleterious mutation such as a premature stop codon or frameshift.

13.2 Culture methods

This study used a microaerobic broth culture method modified from Knapp and Clark (1984). This involved static cultures with a 3:5 volume:vessel ratio, supplemented with and without 2 mM NO_2^- and left to grow for 8 hrs until OD_{600} ~ 0.5. No previous study used the exact microaerobic culture setup employed by this study (Table 24). Hence, direct comparison of our findings to previous literature is challenging. However, it is assumed in the literature that liquid cultures shaking at ≤ 100 rpm are microaerobic, allowing comparison to studies such as Whitehead et al., 2007. Many previous studies made use of anaerobic chambers, and have shown that gonococci struggle to grow anaerobically in broth and plate cultures, probably due to glucose depletion (Spence et al., 2008).

Table 24: Methods of culturing microaerobic *N. gonorrhoeae*

Study	Method of microaerobic growth	Incubation	End WT OD_{600}
Solid media			
Householder et al., 1999	5% CO_2 anaerobic chamber, NO_2^- disk agar plates	20 hr	N/A
Householder et al., 2000	5% CO_2 anaerobic chamber, NO_2^- disk agar plates	20 hr	N/A
Isabella and Clark, 2011b	5% CO_2 anaerobic chamber, 5 mM NO_2^- agar plates	20-24 hr	N/A
Atack et al., 2013	5% CO_2, agar plates	-	N/A
Liquid media			
Hoehn and Clark, 1992	Half-filled flasks, slow shaking, 2 mM NO_2^-	18-24 hr	-
Mellies et al., 1997	Full flasks, no shaking, 2 mM NO_2^-	7 hr	0.24
Lissenden et al., 2000	1-1.5:2.5 volume:vessel, 75 RPM, 5 mM NO_2^-	5 hr	0.5-0.9 (650 nm)
Whitehead et al., 2007	3:5 volume:vessel, 100 RPM, 5 mM NO_2^-	8 hr	0.7
Atack et al., 2014	0.9:1 volume:vessel, 25 RPM, ? NO_2^-	18 hr	0.2
Tzeng et al., 2023	1.2:1.5 volume:vessel, 100 RPM, 5 mM NO_2^-	Overnight	-
This study	3:5 volume:vessel, no shaking, +/- 2 mM NO_2^-	8 hr	0.5

For aerobic growth, this study used a method for shaking gonococcal cultures with a 1:5 volume:vessel ratio, 200 RPM shaking, supplemented with and without 2 mM NO_2^-, and left to grow until autolysis (Table 25). This methods is similar to previous studies (Atack et al., 2014).

Table 25: Methods of aerobically culturing *N. gonorrhoeae*

Study	Method of aerobic growth	Incubation	End WT OD_{600}
	Solid media		
Clark et al., 1987	Normal atmosphere agar plates, +/- NO_2^-	18-24 hr	N/A
Hoehn and Clark, 1992	5% CO_2 incubator agar plates	18-24 hr	N/A
Isabella and Clark, 2011b	Normal atmosphere agar plates, no NO_2^-	14-16 hr	N/A
Householder et al., 1999	5% CO_2 incubator agar plates	1-4 hr	N/A
	Liquid media		
Mellies et al., 1997	1:10 volume:vessel, 120 RPM shaking, no NO_2^-	-	-
Householder et al., 2000	240 RPM shaking baffled flask	-	-
Lissenden et al., 2000	1:2.5 volume:vessel, 125 RPM shaking, 5 mM NO_2^-	4 hr	1.5 (650 nm)
Atack et al., 2013	1:2.5 volume:vessel, 200 RPM	-	-
Atack et al., 2014	1:5 volume:vessel, 200 RPM	5 hr	1.0
Tzeng et al., 2023	1:50 volume:vessel, 200 RPM	Overnight	-
This study	1:5 volume:vessel, 200 RPM, +/- 2 mM NO_2^-	9 hr	1.2

13.3 *N. gonorrhoeae* requires NO_2^- for static growth but not shaking growth

Neisseria gonorrhoeae was thought to be an obligate aerobe until it was shown it can respire anaerobically when supplemented with NO_2^- as an alternative terminal electron acceptor (Knapp and Clark, 1984), suggesting it is instead a facultative anaerobe (Clark et al., 1987; Isabella and Clark, 2011b). NO_2^- is the substrate for the nitrite reductase AniA, and can be supplemented at 2 mM periodically during static growth without causing toxicity (Firth, 2023). NO_2^- was chosen as the supplemented initial denitrification substrate in this study since gonococci lack a NO_3^- reductase, and $^{\bullet}NO$ and N_2O gases are harder to supplement into multiple small cultures.

During infection, *N. gonorrhoeae* is thought to inhabit microaerobic niches within the host such as inside neutrophils (Cohen and Sparling, 1992). As the starting point for our research, we wanted to confirm the previously observed effect of O_2 depletion on growth of our *N. gonorrhoeae* cultures.

We hypothesised that as a facultative anaerobe, gonococcal cultures which were shaking (and therefore aerated) would be more oxygenated, therefore would grow faster than static (microaerobic) cultures.

As hypothesised, shaking gonococcal cultures grew to a higher OD_{600} faster than static cultures (Figure 26). Shaking gonococcal cultures did not require NO_2^- for growth, unlike static cultures. This suggests that our shaking cultures were truly aerobic while static cultures were truly microaerobic (since shaking cultures did not need to respire anaerobically via NO_2^- denitrification but static cultures did). The dependence of static cultures on NO_2^- for growth highlights the gonococcal need for denitrification in the absence of O_2; cytochrome *cbb*$_3$ oxidase does not work under these conditions.

Previous studies suggest both NO_2^- and $^{\bullet}NO$ are bacteriostatic agents due to their ability to inhibit protein activity (Guo and Gao, 2021). Hence, we hypothesised NO_2^- has an inhibitory effect in shaking gonococcal cultures where denitrification was not required. However, NO_2^- did not seem to have any inhibitory effect on shaking cultures, suggesting it was either metabolised (possibly via denitrification) or was non-toxic at 2 mM, as has been shown previously (Firth, 2023).

Denitrifying respiration is not as efficient as aerobic respiration, so microbial growth is slower in low O_2 cultures (Knowles, 1996). This was found in our study; shaking cultures reached stationary phase much quicker than static cultures. Growth of shaking culture was not affected by NO_2^-. Shaking cultures had a short lag phase (< 1hr), an exponential doubling time of ~ 1 hr, and reached a stationary phase at 5 hr (~ 1.1 OD_{600}). This corroborates previous findings regarding aerobic gonococcal growth (Firth, 2023; Morse and Bartenstein, 1974). Additionally, we found that shaking gonococci autolysed within 4 hr of reaching stationary phase, as evidenced by a drop in OD_{600} at 9 hr. Previous literature indicates that *N. gonorrhoeae* does not survive long at stationary phase before autolysis, usually thought to be due to the depletion of glucose (Elmros et al., 1976; Spence et al., 2008).

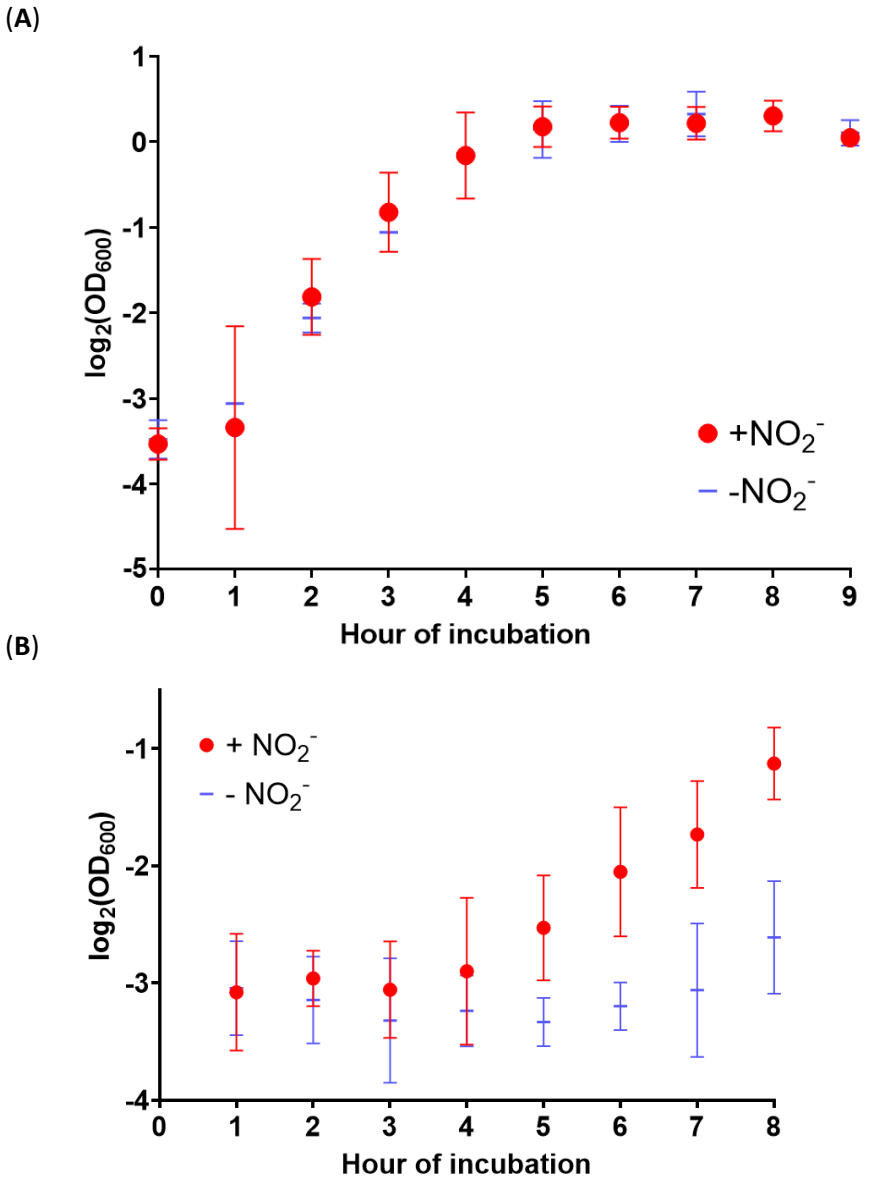


Figure 26: Effect of NO_2^- on shaking and static FA1090 gonococcal culture growth.
No cells control used as blank. (**A**) Shaking cultures grown over 9 hr (200 RPM shaking 50 mL cultures in 250 mL conical flasks). Data points represent the mean of n = 2. $NaNO_2$ (2 mM, red closed circles) was supplemented at 0, 5 and 7 hr. Error bars represent standard deviation. (**B**) Static cultures grown over 8 hr (30 mL cultures in 50 mL Falcon tubes). Data points represent the mean of n = 6. $NaNO_2$ (2 mM, red closed circles) was supplemented at 0, 5 and 7 hr. Error bars represent standard deviation.

Static cultures supplemented with NO_2^- had a 4-hr lag phase, followed by exponential growth, reaching OD_{600} ~ 0.5 at 8 hr. Static cultures without NO_2^- maintained a consistent OD_{600} ~ 0.1 throughout growth. These findings corroborate that NO_2^- is required for full microaerobic growth of gonococci (Firth, 2023; Whitehead et al., 2007). It is interesting to note that Whitehead et al (2007) showed partial growth in poorly aerated cultures without NO_2^-, whereas results from our lab show no growth without NO_2^-.

The 8 hr cultures without NO_2^- used in this study were thought to be viable, as previous studies suggest (Householder et al., 1999), but this was not experimentally tested (e.g. by restreaking). It is unknown if these cultures were non-dividing or were simultaneously dividing and undergoing autolysis at equal rates so as to maintain a constant OD_{600}.

13.4 Static gonococcal cultures are inhibited at 5 µM Cu

Two denitrification enzymes require Cu, AniA and NosZ. AniA requires 6 Cu atoms per active trimer (Barreiro et al., 2022) and is known to function in gonococci, being the major induced outer membrane protein in anaerobic cultures (Cardinale and Clark, 2000). NosZ requires 12 Cu atoms per active homodimer.

If each denitrification enzyme contributes equally to anaerobic respiration in terms of number of electrons transferred to drive ATP synthesis (Chen and Strous, 2013; Nicholls and Ferguson, 2003), NosZ is a less efficient use of Cu per active site compared to AniA. As such, we hypothesised that expression of gonococcal NosZ may be Cu-dependent. However, before starting gene and protein expression studies with variable Cu concentration, we were interested in how changing Cu concentration affected gonococcal growth. We hypothesised that excess Cu would inhibit gonococcal growth due to mismetallation of proteins and resulting toxicity.

Adding 1 µM Cu to gonococcal cultures did not appreciably increase growth (Figure 27), suggesting the GCBL media used in this study was already Cu-sufficient for gonococcal growth and in the micromolar range. GCBL is estimated to contain ~1 µM Cu (K. Djoko, personal communication). Hence, this study did not assess limitation of Cu. Concentrations of supplemented Cu greater than 5 µM increasingly inhibited gonococcal growth, corroborating previous findings that *N. gonorrhoeae* is susceptible to Cu at this concentration (Djoko et al., 2012; Firth, 2023). Once again, we found static cultures without NO_2^- could survive but not reproduce.

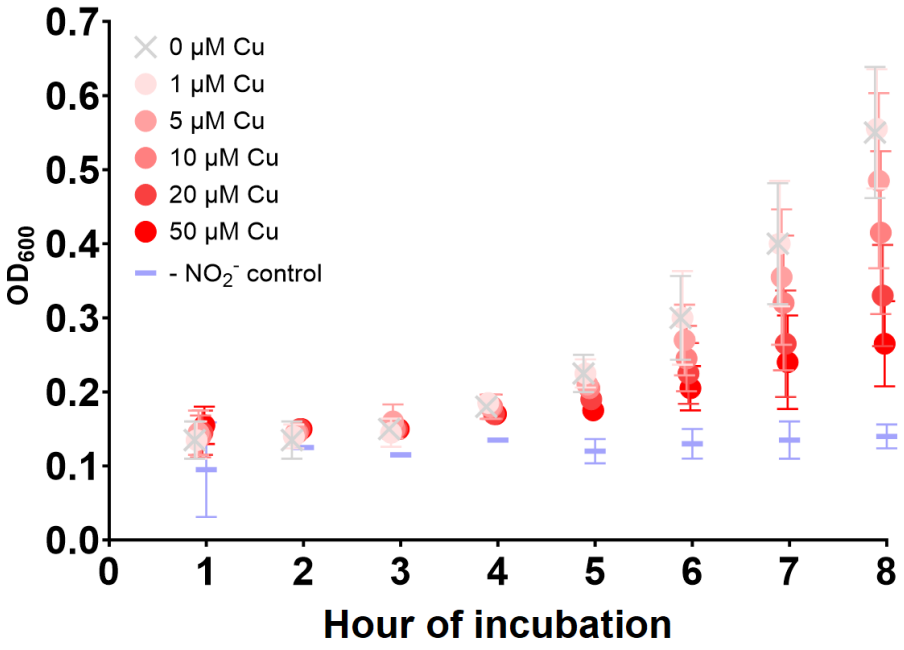


Figure 27: Effect of Cu on static gonococcal culture growth.
Growth of *N. gonorrhoeae* FA1090 with increasing Cu: 0 (light pink) – 50 (dark red) µM over 8 hr in microaerobic conditions (static 30 mL cultures in 50 mL Falcon tubes). $CuSO_4$ was supplemented at the start of growth. $NaNO_2$ (2 mM) was supplemented at 0, 5 and 7 hr. Data points represent the mean of n = 4. Error bars represent standard deviation. No cells control used as blank.

13.5 The gonococcal *nos* operon is fully transcribed into mRNA

Before studying NosZ protein expression, we wanted to determine if the gonococcal *nos* operon was first transcribed into mRNA. It is known that *Pseudomonas aeruginosa nosZ* is transcribed alongside the upstream *nosR* gene (Arai et al., 2003). However, while previous studies have confirmed expression of gonococcal *nosR* (Whitehead et al., 2007), no studies have previously studied the expression of gonococcal *nosZ*. Since gonococcal *nosR* (the upstream gene in the *nos* operon) is known to be transcribed, due to the polycistronic expression of prokaryotic operons (Osbourn and Field, 2009) we hypothesised that the entire gonococcal *nos* operon would be transcribed into mRNA.

Static 8 hr gonococcal cultures with NO_2^- were pelleted, RNA extracted, purified and cDNA synthesised. The cDNA from individual *nos* genes identified as containing premature stop codons (*nosR*, *nosZ* and *nosD*) were successfully used as a template in PCR (Figure 28). This suggests that these three individual *nos* genes are normally transcribed from the FA1090 genome.

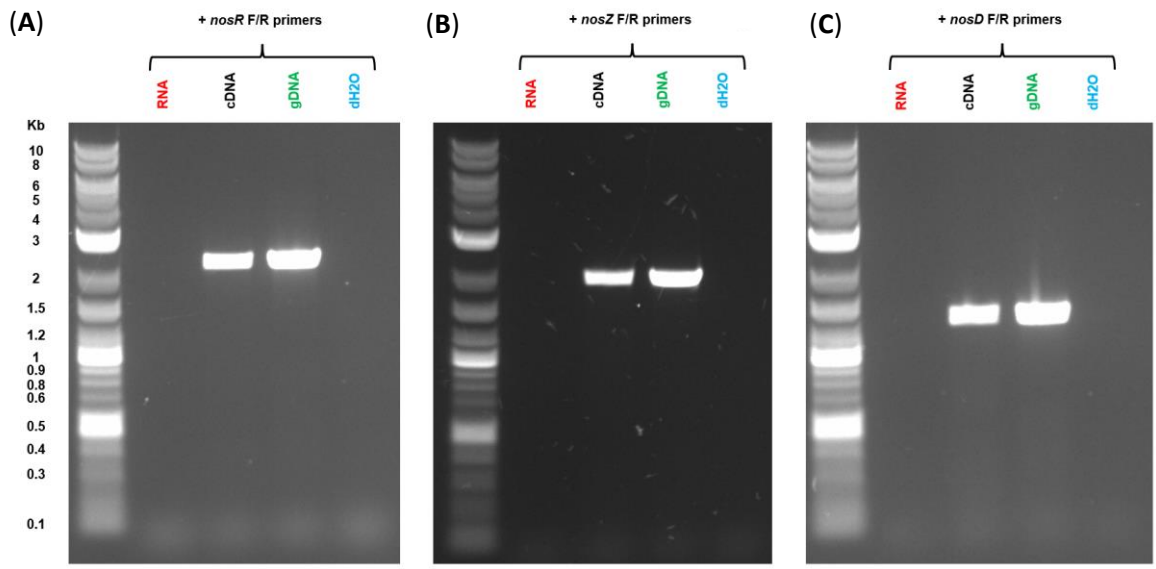


Figure 28: PCR with cDNA template generated from static 8 hr *N. gonorrhoeae* culture RNA extracts.
RNA extracts used as a negative control for persistent gDNA contamination. (**A**) PCR product amplified from nosR-full-Fv8 and nosR-full-Rv7 primers generating band size 2271 bp. (**B**) PCR product amplified from nosZ-full-F and nosZ-full-R primers primers generating band size 1968 bp. (**C**) PCR product amplified from nosD-full-F and nosD-full-R primers primers generating band size 1340 bp.

Transcription of the entire gonococcal *nos* operon into mRNA was then demonstrated by amplifying a series of PCR overlapping products spanning the entire operon from culture cDNA (Figure 29).

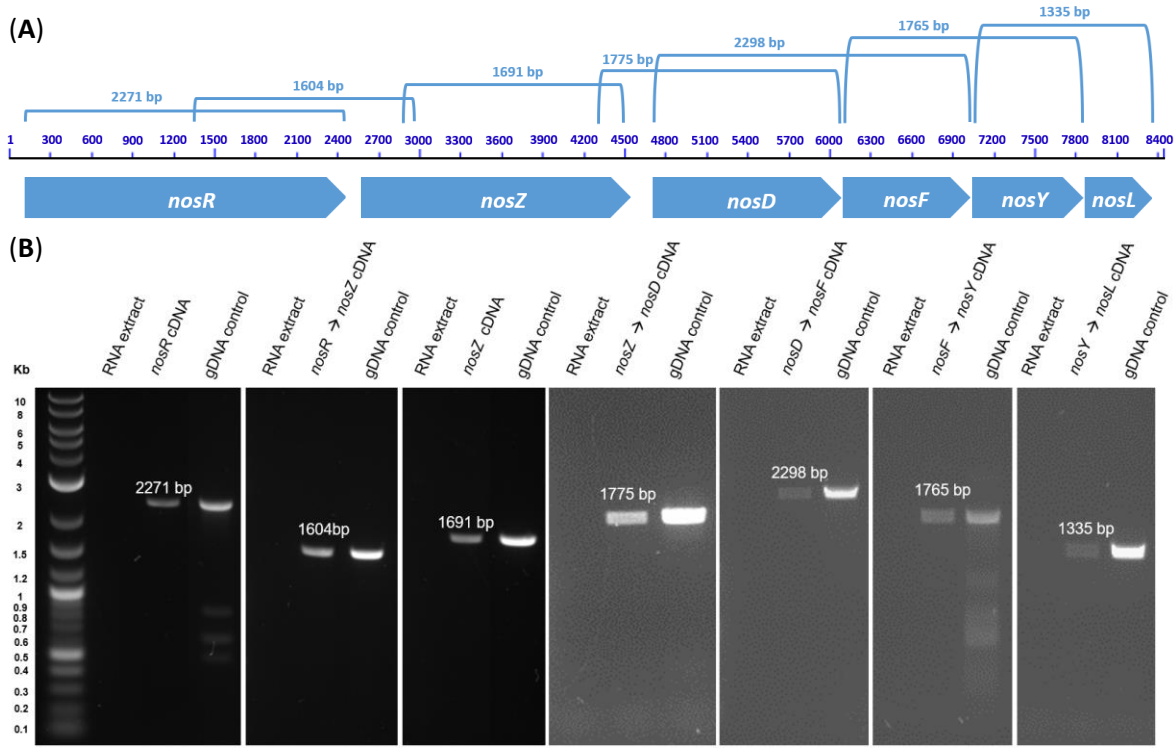


Figure 29: Transcription of the gonococcal *nos* operon in static 8 hr cultures.
(**A**) Diagram representing the seven overlapping PCR products generated from gonococcal cDNA. (**B**) Composite image consisting of seven overlapping PCR 1% agarose gel images. RNA extract as a negative control, gDNA as a positive control.

There are different reasons why a premature stop codon in the putative *nos* operon transcriptional regulator NosR may not affect *nos* operon transcription: 1) the necessity of NosR in gonococcal *nos* operon transcription has not been confirmed, 2) it is unknown if gonococcal NosR is important for basal transcription or upregulation of transcription, and 3) should NosR be required for gonococcal *nos* expression, a prematurely stopped NosR may still have function. Furthermore, the necessity of NosR in other species is debated. Some studies have found NosR is essential for *Pseudomonas stutzeri nosZ* expression (Cuypers et al., 1992; Wunsch and Zumft, 2005), and a *Bradyrhizobium japonicum nosR* mutant accumulates N_2O when cultured microaerobically in the presence of NO_3^- (Velasco et al., 2004). However, other studies suggest that *Pseudomonas aeruginosa* NosR is not required for activity of the *nosR* promoter (Arai et al., 2003). Furthermore, while the cluster *nosZDFYL* is always found in N_2O-reducing prokaryotes, the *nosR* and *nosX* genes are missing from certain clades such as Gram-positives and deltaproteobacteria (Simon et al., 2004; Zumft and Körner, 2007), suggesting they are not required for N_2O reduction.

Since the *nos* operon is expected to form one continuous mRNA transcript, we expected all *nos* gene amplicons to have similar band intensity (Figure 28 and Figure 29). However, certain amplicons occurred at a greater intensity than others, with the *nosY* → *nosL* amplicon being particularly faint. This likely reflected aborted transcription, or degradation of the mRNA by exonucleases.

13.6 Gene expression of static and shaking gonococci with and without NO_2^-

Before analysing gonococcal cell culture extracts for NosZ protein, we determined the expression of the various genes in gonococcal cultures, including genes involved in denitrification, anaerobiosis, and Cu homeostasis. See Table 26 for a list of genes studied in qPCR gene expression studies.

13.6.1 Description of target genes for qPCR analysis

13.6.1.1 *Housekeeping gene gyrA*

The housekeeping gene *gyrA* topoisomerase was used as a positive control in qPCR for gene expression. In bacteria, the DNA gyrase type II topoisomerase is a heterotrimer comprised of two A subunits encoded by *gyrA* and two B subunits encoded by *gyrB*. DNA gyrase is the only enzyme

capable of unwinding the DNA double helix. Due to this essentiality, DNA gyrase is useful as a qPCR housekeeping gene (Wen et al., 2016).

13.6.1.2 *Denitrification genes*

The genes encoding the denitrification enzymes *aniA*, *norB* and *nosZ*, as well as the denitrification Cu chaperones *accA* and *nosL* were assessed in parallel. The NO_2^- reductase, AniA, is the first enzyme in the gonococcal denitrification pathway given the absence of a NO_3^- reductase. AniA is the major anaerobically induced outer membrane protein in *N. gonorrhoeae* and is the only known outer membrane protein that is present during anaerobic growth but absent during aerobic growth (Cardinale and Clark, 2000; Clark et al., 1987; Hoehn and Clark, 1992). The periplasmic Cu chaperone to AniA, AccA, is essential for AniA function in pathogenic *Neisseria* (Jen et al., 2015) and microaerobic survival in *N. gonorrhoeae* (Firth, 2023).

Gonococcal $^{\bullet}NO$ reductase is encoded by a single gene, *norB* (Householder et al., 2000). While there are homologs in the FA1090 genome to the *P. denitrificans norQ* (gene ID 75501945) and *norD* (gene ID 75501944) accessory protein genes, gonococcal NorB is not thought to require the accessory proteins seen in other species (Kahle et al., 2018). The gonococcal $^{\bullet}NO$ reductase NorB is required for anaerobic growth (due to $^{\bullet}NO$ toxicity) and is induced by $^{\bullet}NO$ (Householder et al., 2000).

Pseudomonas stutzeri NosZ is thought to be supplied with Cu via the chaperone NosL (Müller et al., 2022). However, NosL does not appear to be essential for formation of the Cu sites in *Pseudomonas putida* NosZ (Wunsch et al., 2003). Early studies supplemented growth media with Cu, leading some to suggest a dedicated chaperone may still be essential under conditions of metal limitation (Prasser et al., 2021). NosL was recently found to be essential for the assembly of the NosZ Cu_Z site in *Paracoccus denitrificans* under low Cu conditions (Bennett et al., 2019).

13.6.1.3 *Cuproprotein genes*

The gene expression of two Cu-dependent proteins once implicated in denitrification, *laz* and *sco*, was also determined in this study. The lipid-modified cupredoxin azurin (Laz) is a Type 1 site cuproprotein suggested to donate electrons to gonococcal cytochrome *c* peroxidase (Ccp), which

subsequently protects against hydrogen peroxide (Nóbrega et al., 2016). Neisserial Laz is no longer thought to interact with AniA (Aas et al., 2015; Baarda et al., 2018) and is dispensable for gonococcal growth under anaerobic conditions in the presence of NO_2^- (Cannon, 1989). The expression of meningococcal Laz under microaerobic conditions is slightly higher than that under aerobic conditions, yet it is thought that the meningococcal FNR does not regulate the expression of Laz (Deeudom et al., 2015). Gonococcal *laz* contains a putative FNR binding site, but is not known to be regulated by FNR (Whitehead et al., 2007).

Sco (synthesis of cytochrome oxidase) is normally a Cu chaperone to bacterial Cox (Siluvai et al., 2011), but gonococcal Sco is not required for assembly of Cox (Seib et al., 2003), and is instead thought to play a catalytic role as an antioxidant in the gonococcal periplasm to protect proteins from oxidative damage (Seib et al., 2006, 2003). ScoP was once thought to be a putative membrane-anchored chaperone for NosZ Cu_A metalation in *Pseudomonas*, but was subsequently found to be a dispensable element for NosZ biosynthesis (Wunsch et al., 2003).

We also determined expression of *copA* and *copA2*, since these Cu exporters could influence availability of Cu cofactor to AniA and NosZ. The inner membrane P_{1B}-type ATPase transporter CopA in *Streptococcus pyogenes* exports Cu of the cytosol and into the periplasm, providing Cu tolerance (Dao et al., 2023; Stewart et al., 2019). CopA is one of the few Cu homeostasis proteins present in gonococci (Djoko et al., 2012). In *Pseudomonas aeruginosa*, CopA and CopA2 are two structurally similar Cu-ATPases which are both essential for virulence. CopA2 also catalyses cytoplasmic Cu efflux into the periplasm, but at a lower rate than CopA. It is suggested that this could be due to differences in transport kinetics or requirement of periplasmic partner Cu-chaperone proteins specific for each Cu-ATPase (González-Guerrero et al., 2010). CopA2 has been suggested to be important, but not essential, for Cu delivery to Cox. In gonococci, CopA (NGO_0579) and CopA2 (NGO_0685) share 33% amino acid identity and 17% similarity.

13.6.1.4 *FNR-regulated genes*

The expression of various genes previously identified as being regulated by FNR in gonococci (Whitehead et al., 2007) was used to indicate the aerobicity of cultures in this study. Whitehead et al (2007) found the genes *ompU*, *aniA*, *accA*, *res*, *nosR and ccp* to be upregulated by FNR, and *aph* and *norB* to be downregulated by FNR in 8 hr cultures.

OmpU (Outer Membrane Protein U) is an iron uptake outer membrane protein (El-Rami et al., 2019; Grabowicz and Silhavy, 2017; Lv et al., 2020) which is also implicated in binding to host cells (Liu et al., 2015). OmpU was recently identified as a novel gonococcal antigen (El-Rami et al., 2019; Zielke et al., 2016). However, disagreement surrounds gonococcal *ompU* expression; Isabella and Clark (2011b) found *ompU* to be downregulated in response to anaerobic conditions while Whitehead et al (2007) found *ompU* to be FNR-induced. Iron may be required for enzymes expressed in low O_2 conditions, such as NorB.

Res is a subunit of a type III restriction-modification enzyme EcoP1 containing the endonuclease domain for cleavage of invading phage DNA (Murray et al., 2021). Aph is an aminoglycoside phosphotransferase, which provides resistance to aminoglycosides (such as kanamycin) via O-phosphorylation modification of the antibiotic (Wright and Thompson, 1999). The gonococcal gene most down-regulated by FNR was *aph* (Whitehead et al., 2007). It is not immediately clear why the *res* and *aph* genes are regulated by O_2 concentration.

The final gene included in our qPCR analysis was *ccp*. The *ccp* gene encodes a cytochrome *c* peroxidase which reduces H_2O_2 to water in the periplasm (Nóbrega et al., 2017). The *ccp* gene was not identified as being FNR-regulated by microarray analysis due to low expression levels, however it has been observed to be FNR-induced in other experiments (Lissenden et al., 2000; Whitehead et al., 2007). A summary of the genes measured for expression during this study is given in Table 26.

Table 26: List of genes studied in qPCR gene expression studies

Gene/ Locus tag	Protein	Description	Interaction with Cu
Housekeeping gene			
gyrA NGO_0629	DNA gyrase subunit A	Type II topoisomerase. Positive expression control. Expected to be consistently highly expressed throughout growth.	None.
NO_2^- reduction			
*aniA** NGO_1276	Nitrite reductase	First enzyme in gonococcal denitrification. Major anaerobically induced outer membrane protein but absent aerobically. Upregulated by FNR (Whitehead et al., 2007).	Requires 6 Cu per active enzyme
*accA** NGO_1215	AniA Cu Chaperone A	Periplasmic Cu chaperone to AniA. Upregulated by FNR (Whitehead et al., 2007).	Cu chaperone
NO reduction			
*norB** NGO_1275	Nitric oxide reductase	Second enzyme in gonococcal denitrification. Down-regulated by FNR (Whitehead et al., 2007).	None
N_2O reduction			
nosZ NGO_07405	Nitrous oxide reductase	Third and final enzyme in gonococcal denitrification, putative pseudogene (nonsense-mutated).	Requires 12 Cu per active enzyme
nosL NGO_1397	NosZ Cu chaperone	Periplasmic Cu chaperone to NosZ.	Cu chaperone
Cu homeostasis			
copA NGO_0579	Cu exporter to periplasm	Inner membrane cytoplasmic Cu exporter.	Cu exporter
copA2 NGO_0685	Cu exporter to periplasm	Cu chaperone to cytochrome *c* oxidase (Cox).	Cu exporter
laz† NGO_0994	Lipid-modified azurin	Cupredoxin which donates electrons to cytochrome *c* peroxidase (Ccp). Once thought to donate electrons to AniA.	Cu protein
sco† NGO_1237	Synthesis of cytochrome oxidase	Antioxidant cuproprotein. Once thought to be a membrane-anchored Cu chaperone for NosZ.	Cu protein
Regulated by FNR in low O_2 (*)			
*aph** NGO_1716	Aminoglycoside phospho-transferase	Confers resistance to aminoglycosides such as kanamycin. Downregulated by FNR (Whitehead et al., 2007).	None
*res** NGO_0546	Res subunit of EcoPI restriction enzyme	Part of restriction-modification system (bacteriophage resistance). Upregulated by FNR (Whitehead et al., 2007).	None
*ompU** NGO_1688	Outer membrane Fe porin	Upregulated by FNR (Whitehead et al., 2007).	None
*ccp** NGO_1769	Cytochrome *c* peroxidase	Catalyses $H_2O_2 \rightarrow H_2O$ using heme cofactor. Upregulated by FNR (Whitehead et al., 2007).	None

* - reported in the literature to be regulated by FNR in low O_2, † - once proposed to be involved in denitrification but now not through to be involved. Laz and Sco are no longer thought to be involved in denitrification, so we have classed them as Cu homeostasis-related proteins. The *nosZ* gene was amplified in qPCR using two primer pairs: one 5'-end pair against the region downstream of the signal peptide, and one 3'-end pair against the region downstream of the premature stop codon.

13.6.2 Denitrification genes are not upregulated by NO_2^- until 8 hr in static cultures

Simultaneous static cultures were grown with and without 2 mM NO_2^- and harvested every 2 hr to determine the effect of this denitrification substrate on gene expression via qPCR (Figure 30**,** Figure 31**,** Figure 32**,** Figure 33 and Figure 34). Expression at 0 hr was always included as an indicator of expression in initial aerobic conditions. No gene showed a significant difference in expression with addition of NO_2^- at 0 hr.

Various previous studies have established the effect of low O_2 as an activator of *aniA* and *norB* gene expression in *N. gonorrhoeae* (Atack et al., 2013; Householder et al., 2000, 1999; Isabella and Clark, 2011a; Whitehead et al., 2007). While NO_2^- is not thought to directly regulate the transcription of any gonococcal denitrification enzymes, •NO is known to induce expression of *aniA*, *norB* and *nosZ* (Figure 11). Hence, we expected NO_2^- to upregulate *aniA*, *norB* and *nosZ* indirectly via AniA reduction of NO_2^- to •NO. Expression of *nosZ* in microaerobic conditions is expected, given the FNR box upstream of gonococcal *nosR* and upregulation of *nosR* in microaerobic gonococcal cultures reported previously (Whitehead et al., 2007). Based on this understanding of the regulation of denitrification, we expected all denitrification genes tested (*aniA*, *accA*, *norB*, *nosZ*, and *nosL*) to be increasingly upregulated in static 2, 4, 6 and 8 hr cultures with NO_2^- as O_2 concentration decreased.

However, we found little change in expression of the denitrification genes *aniA*, *accA* and *nosZ* in static cultures before 8 hr. Only at 8 hr growth, cultures with NO_2^- experienced upregulation of the denitrification genes *aniA*, *accA* and *nosZ* (Figure 30) compared to cultures without NO_2^-. At 8 hr, *aniA* was upregulated by NO_2^- 8.1-fold, *accA* 2.7-fold, and *nosZ(N)* 5.8-fold. Our finding of *nosZ* expression corroborates a previous study which found induction of gonococcal *nosZ* transcription in response to NO_2^- (Overton et al., 2006) and findings in other species that *nosZ* is upregulated in microaerobic conditions (Fernández et al., 2019). Gonococcal *aniA*, *norB* and *ccp* are upregulated in biofilms thought to be low O_2 environments (Falsetta et al., 2008; Phillips et al., 2012). The expression of *nosZ* seen in 8 hr cultures with NO_2^- is as high as that of *aniA*, suggesting there may be many copies of *nosZ* mRNA to be translated. It is known that mRNA transcripts containing a

premature stop codon are generally degraded faster (Belasco, 2010). Hence, it is interesting that the *nos* operon mRNA appears to be as abundant as *aniA*. Our finding that *aniA* and *nosZ* are upregulated in 8 hr static cultures supports our understanding that these cultures are microaerobic.

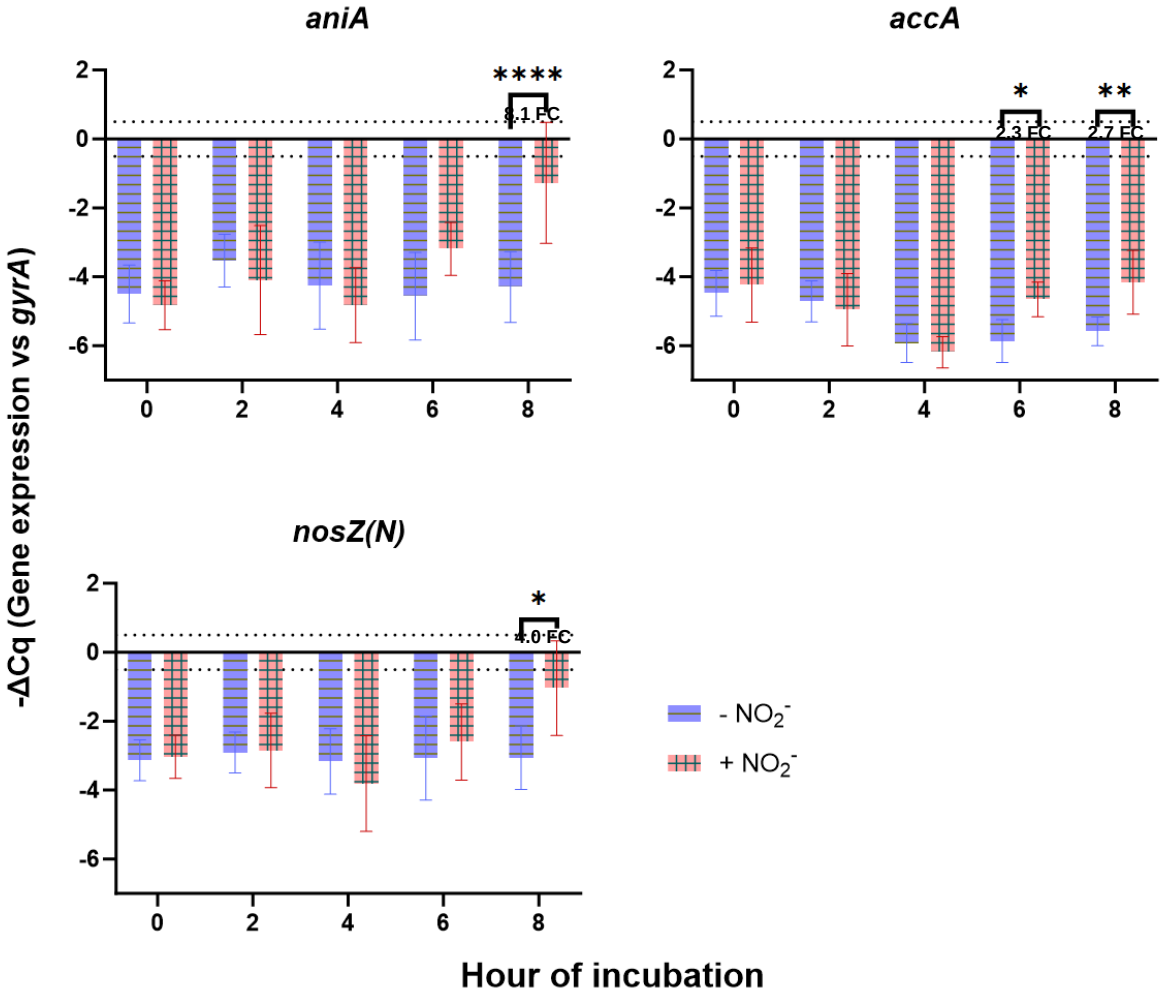


Figure 30: Expression of denitrification genes in static gonococcal cultures are upregulated at 8 hr. Cultures with NO_2^- (red hatched) or without NO_2^- (purple bars) were grown over 8 hr in microaerobic conditions (static 30 mL cultures in 50 mL Falcon tubes). $NaNO_2$ (2 mM) was supplemented at 0, 5 and 7 hr. Mean of n = 4 independent replicates ±SD. The *nosZ*(5'-end) gene was measured every hr for 8 hr, *nosZ*(3'-end) was measured at only 0 and 8 hr. Dashed horizontal lines represent the sensitivity limit of the assay ($log_2FC = \pm 0.5$). Only significantly different pairwise comparisons plotted. Data analysed with 2-way ANOVA. FC; fold change.

Reasons for the observed 8-hr lag in upregulation of denitrification genes are not immediately obvious. While previous studies often slowly shook microaerobic cultures to prevent cell clumping, this study used static cultures which were briefly vortexed before reading OD_{600}, which may have had the effect of oxygenating cultures. Hence, in the first 7 hr of static growth, gonococci may have been respiring aerobically using O_2 from the Falcon tube headspace, and only activated

denitrification after 7 hr when O_2 had been depleted. However, this does not fit with our growth data, which shows static 5 and 6 hr cultures supplemented with NO_2^- were clearly using that NO_2^- to grow compared to -NO_2^- cultures, which did not grow (Figure 26B). It is puzzling why there was no difference in expression of denitrification genes between 0 hr (OD_{600} ~ 0.12) and 6 hr (OD_{600} ~ 0.26) static cultures. One hypothesis is that basal expression of *aniA*, *norB* and/or *nosZ* and basal AniA, NorB and/or NosZ activity may be sufficient for anaerobic growth until the end of 8 hr cultures.

This study did not measure expression of cytochrome *c*bb_3 oxidase or culture O_2 level directly. Expression of denitrification and FNR-induced genes were our best estimation of O_2 level. Our static culture setup using capped tubes was thought to generate low O_2 (microaerobic), but not anaerobic conditions (Spence et al., 2008). Eukaryotic Cox will still function in low O_2 conditions (Chandel et al., 1997; Wikström et al., 2018) and bacterial *cbb*$_3$-type cytochrome *c* oxidases retains high activity in low O_2 (Ekici et al., 2012; Pitcher and Watmough, 2004; Preisig et al., 1993). However, if it both denitrification and aerobic respiration were occurring simultaneously in our static cultures, we would expect constant growth in cultures without NO_2^-. Since our static cultures without NO_2^- could only survive, not reproduce, we assumed aerobic respiration was inhibited in our static cultures.

The microaerobic gene expression results determined during this study are summarised in Table 27. A comparison of static 8 hr and shaking 3 hr gene expression fold change data is given in Appendix 10.

Table 27: Summary of gene expression in static gonococcal cultures over 8 hr

	Fold change ($2^{-\Delta\Delta Cq}$) between...			
Gene	**0 hr +NO_2^- cultures and 8 hr +NO_2^- cultures**	**8 hr -NO_2^- cultures and 8 hr +NO_2^- cultures**	**Expected microaerobic effect from literature?‡**	**Corroboration**
norB	10.6 (P = 0.0002; ***)	24.5 (P < 0.0001; ****)	↑	Yes
aniA	11.7 (P = 0.0001; ***)	8.1 (P < 0.0001; ****)	↑	Yes
nosZ (N)	5.7 (P = 0.0001; ***)	5.8 (P < 0.0001; ****)	↑	Yes
nosZ (C)	4.2 (P = 0.0024, **)	2.7 (P = 0.0194; *)	↑	Yes
copA2	1.7 (P = 0.1575; ns)	2.2 (P = 0.0361; *)	-	-
ompU	1.6 (P = 0.3619; ns)	1.8 (P = 0.2332; ns)	?	-
nosL	1.2 (P = 0.3819; ns)	2.4 (P = 0.0033; **)	↑	Yes
ccp	1.2 (P = 0.8178; ns)	1.2 (P = 0.8216; ns)	↑†	-
laz	1.1 (P = 0.7330; ns)	3.6 (P = 0.001; ***)	↑	Yes
aph	1.1 (P = 0.7415; ns)	2.3 (P = 0.0097, **)	↓	No
accA	1.1 (P = 0.8733; ns)	2.7 (P = 0.0063; **)	↑	Yes
copA	1.0 (P = 0.9715; ns)	1.4 (P = 0.0813; ns)	-	-
sco	0.7 (P = 0.4097; ns)	3.5 (P = 0.0084; **)	-	-
res	0.6 (P = 0.0561; ns)	0.5 (P = 0.0119, *)	↑	No

Genes ranked on effect of incubation time. ↑ indicates upregulation, ↓ indicates downregulation. Reductases underlined. † for a description of detecting gonococcal *ccp* transcripts, see Whitehead et al., 2007. ‡ for a description of regulation of denitrification gene expression, see section 10.8.2. Reductases underlined. Fold change effect size written as absolute multiples (x).

The strong upregulation of *aniA* and *norB* may reflect the various transcription factors regulating them compared to the simpler regulation of other genes (Figure 11). The gonococcal *aniA* gene is known to be induced by NarP, FNR, NtrY, NsrR (derepression) and possibly Fur, Nnr, Cu, and PrrA/ActR. The gonococcal *norB* gene is known to be induced by NtrY, NsrR (derepression), and possibly Fur, FNR (derepression), NorR and Cu. By comparison, however, the gonococcal *nosR* is only possibly regulated by FNR, NsrR (derepression) and Cu.

13.6.2.1 Denitrification enzymes (but not Cu chaperones) are upregulated over 8 hr static growth with NO_2^-

Since little change in gene expression was seen prior to 8 hr, the expression of additional denitrification genes (*nosZ(C)*, *nosL* and *norB*) was determined at 0 and 8 hr.

All denitrification genes tested (*aniA*, *accA*, *norB*, *nosZ(N)*, *nosZ(C),* and *nosL*) were upregulated in cultures with NO_2^- compared to cultures without NO_2^- at 8 hr (Figure 31). When the expression of all static genes at 8 hr was combined into a 2-way ANOVA, NO_2^- was a statistically significant source of variation ($P < 0.0001$; ****). This suggests that the *nos* operon is upregulated normally in response to NO_2^- in 8 hr static cultures.

There is a difference in the gene expression profiles of the denitrification enzymes compared to the other genes tested. When we compared expression of static cultures with NO_2^- at 0 and 8 hr, the only genes with statistically significant upregulation were the three denitrification enzymes: *aniA* 11.7-fold*, norB* 10.6-fold, *nosZ(N)* 5.7-fold and *nosZ(C)* 4.2-fold. In static cultures without NO_2^-, expression of *aniA, norB* and *nosZ* remained unchanged over the course of 8 hr growth, while the expression of *nosL* and *accA* decreased slightly. This suggests that expression of the Cu chaperones was unchanged with NO_2^- but was downregulated slightly without NO_2^- in microaerobic conditions (*accA* 2.2-fold and *nosL* 1.7-fold). While *aniA* and *accA* genes are separated in the genome by 63 kb, *nosZ* and *nosL* are part of the same transcriptional operon. Hence, finding differential expression of *nosZ* and *nosL* suggests a post-transcriptional regulation differentially affecting these genes.

NarQ is the NO_2^- sensor which activates the transcriptional regulator NarP to induce *aniA* expression in most species. However, it is thought that gonococcal NarP activates *aniA* constitutively rather than responding to NO_2^- via NarP (Overton et al., 2006). Our data suggest that gonococci do sense NO_2^- to upregulate *aniA*. However, this upregulation of *aniA* by NO_2^- may occur indirectly via NO_2^- reduction to •NO and induction of *aniA* by NO-dependent FNR and NsrR.

While Whitehead et al (2007) found *accA* to be upregulated by FNR in cultures both with and without NO_2^-, this study determined *accA* to be downregulated 2.2-fold in cultures without NO_2^- over

8 hr and upregulated 2.7-fold in cultures with NO_2^- at 8 hr. This disparity could be due to differences in growth in cultures without NO_2^- between our two studies; Whitehead et al report that cultures without NO_2^- grew at a constant rate rather than an exponential, whereas in this study, cultures without NO_2^- remained at constant OD_{600} over 8 hr.

Aside from *aniA*, *norB* and *nosZ*, the remaining genes studied were not significantly changed in cultures with NO_2^- between 0 and 8 hr. However, when we compared gene expression of 8 hr cultures with and without NO_2^-, in addition to upregulation with NO_2^- of *aniA* 8.1-fold, *accA* 2.7-fold, *norB* 24.5-fold, *nosZ(N)* 5.8-fold, *nosZ(C)* 2.7-fold, and *nosL* 2.4-fold, the genes *accA*, *laz*, *sco*, *aph*, *copA2* and *nosL* were all upregulated with NO_2^- between 2- and 4-fold.

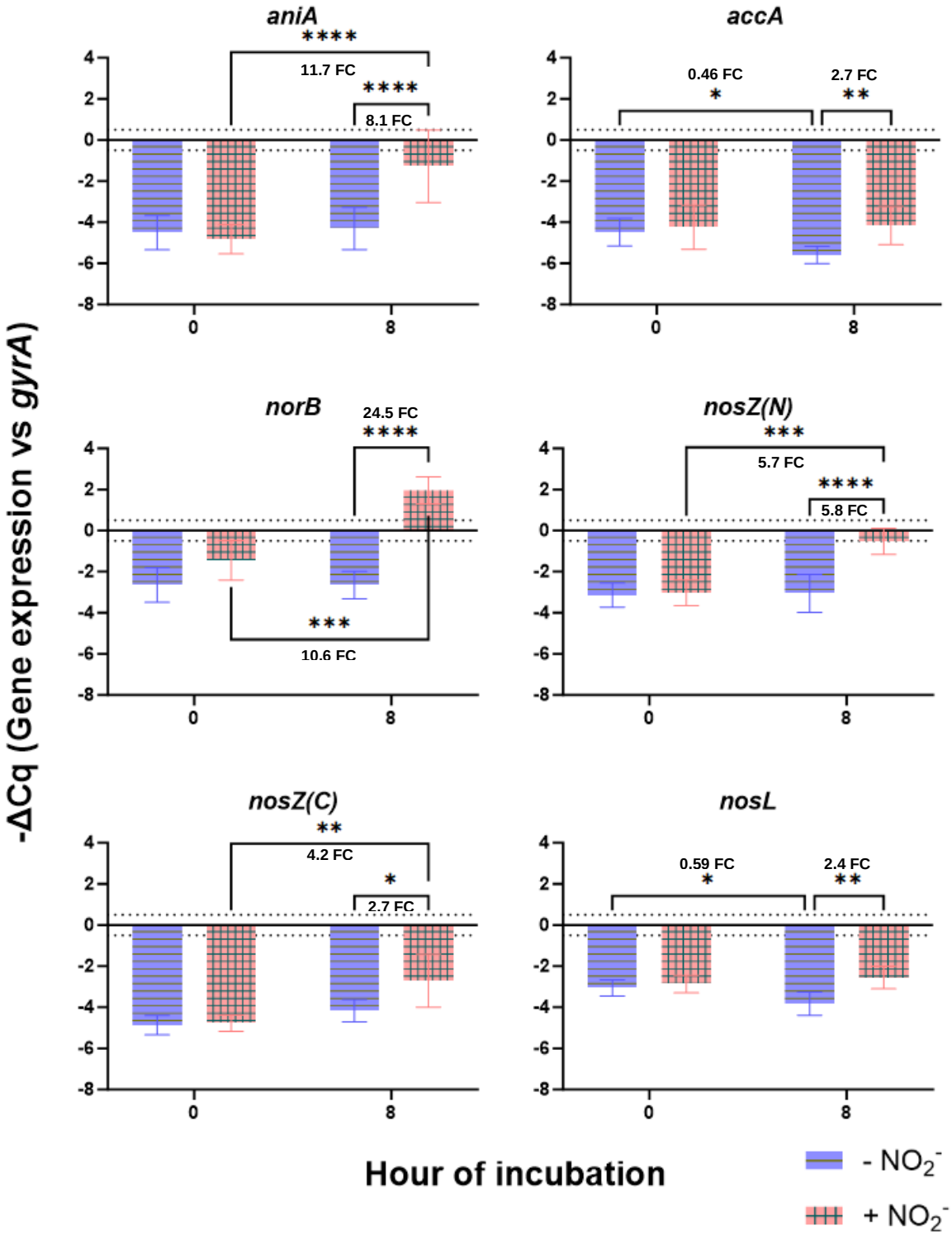


Figure 31: Expression of denitrification genes in response to NO_2^- in static gonococcal cultures at 0 and 8 hr. Cultures with NO_2^- (red hatched) or without NO_2^- (purple bars) were grown over 8 hr in microaerobic conditions (static 30 mL cultures in 50 mL Falcon tubes). $NaNO_2$ (2 mM) was supplemented at 0, 5 and 7 hr. Mean of n = 3 independent replicates ±SD. Dashed horizontal lines represent the sensitivity limit of the assay ($log_2FC = \pm 0.5$). Only significantly different pairwise comparisons plotted. Data analysed with 2-way ANOVA. FC: fold change.

13.6.2.1.1 Differences in nosZ N- and C- terminus transcript abundance

Bacterial mRNAs are polycistronic, meaning one mRNA strand is transcribed for an entire operon, with different genes in the operon being individually translated by separate ribosomes. Assuming polycistronic transcription of the *nos* operon, we may expect all *nos* genes to produce the same mRNA levels in cells. Therefore, we hypothesised that qPCR would reveal identical expression levels of the *nosZ(N)*, *nosZ(C)* and *nosL* genes. However, there was a statistically significant difference between expression of *nosZ(N)* and *nosL* with *nosZ(C)* at 0 hr with NO_2^- in static cultures (Figure 32A). There were also statistically significant differences between expression of *nosZ(N)* with *nosZ(C)* and *nosL* at 8 hr with NO_2^- (Figure 32B).

Differences in gene expression for the two primer pairs against the *nosZ* suggest that the 5'-end of the *nosZ* region of the *nos* operon mRNA is twice as abundant than the 3'-end. We hypothesised that this difference in abundance of the 5'- and 3'-end regions could relate to decay of the *nos* operon mRNA.

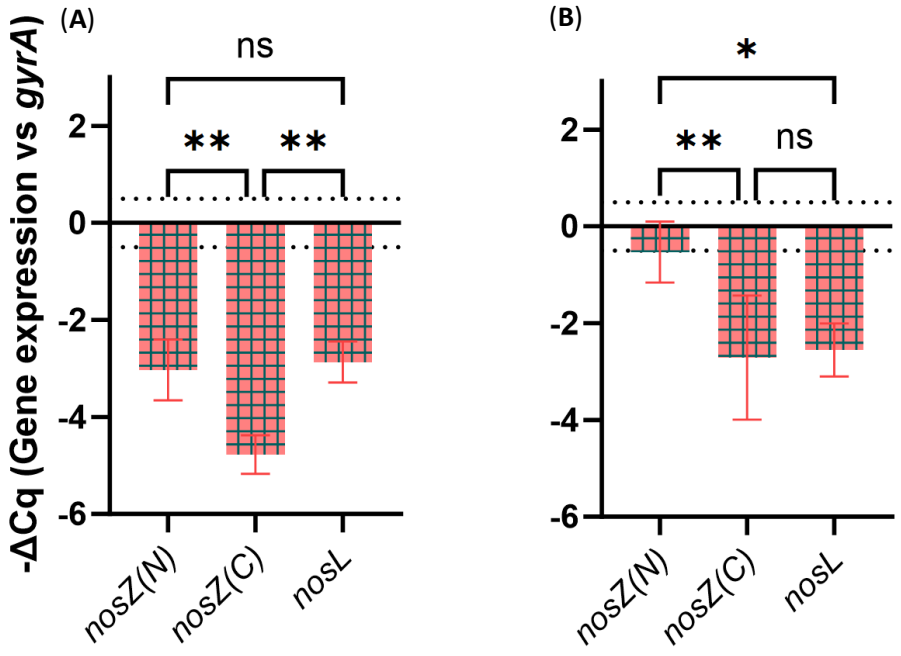


Figure 32: Differences in *nos* gene expression in static 0 hr (A) and 8 hr (B) NO_2^--supplemented cultures. Cultures with NO_2^- were grown over 8 hr in microaerobic conditions (static 30 mL cultures in 50 mL Falcon tubes). $NaNO_2$ (2 mM) was supplemented at 0, 5 and 7 hr. Mean of n = 4 independent replicates ±SD. Dashed horizontal lines represent the sensitivity limit of the assay ($log_2FC = \pm 0.5$). Data analysed with 2-way ANOVA.

13.6.2.1.1.1 mRNA decay

It is known that mRNA decay plays a key role in regulating gene expression. Bacterial mRNAs are not post-transcriptionally spliced, and are thought to have a short half-life (40 sec - 1 hr), with both 5' and 3'-end mRNA degradation pathways described (Richards et al., 2008). The association of several genes in one mRNA (as in a polycistronic prokaryote operon) does not necessarily mean that equimolar amounts of each protein are translated. Due to a variety of stabilising and destabilising structures (such as hairpins and single-stranded regions) within the primary mRNA transcript, nucleases are known to split polycistronic RNA into a series of mRNAs of different lengths with different half-lives which in turn lead to different quantities of translated proteins for different genes (Rauhut and Klug, 1999).

While much is known about nonsense-mediated mRNA decay in eukaryotes, no analog of the eukaryotic nonsense-mediated decay system is known in prokaryotes. However, a destabilizing effect of premature stop codons on bacterial mRNA stability has been observed (Morse and Yanofsky, 1969; Nilsson et al., 1987), leading others to suggest that a uncharacterized nonsense-mediated decay-like quality control mechanism exists in prokaryotes (Li and Lynch, 2020). Nonsense mutations within 26 codons of the 5'-end of the *E. coli bla* gene lead to destabilisation of the entire mRNA. However, nonsense mutations further downstream do not have this destabilising effect (Nilsson et al., 1987). Based on these findings, we suggest the premature stop codons in gonococcal *nosR*, *nosZ* and *nosD* genes are unlikely to increase mRNA decay since these codons occur at least halfway through each gene (*nosR* 49%, *nosZ* 81%, *nosD* 57%).

It may be the case that the *nos* operon mRNA is degraded by 3'-terminus and 3'-to-5' exoribonucleases (Richards et al., 2008). We would then expect the *nosL* mRNA to be the least abundant of all the *nos* genes since it is at the downstream end of the operon. There is a drop in expression of *nosZ(C)* and *nosL* compared to *nosZ(N)* at 8 hr which fits this hypothesis (Figure 32B). This also fits the finding of fainter PCR amplification bands for the downstream *nos* operon genes *nosDFYL* (Figure 29B).

However, static culture 0 hr *nosL* expression was very similar to *nosZ(N)* (Figure 32), suggesting that the *nosL* region in the *nos* mRNA was as abundant as *nosZ* region at this time. Hence, we suggest the *nosZ* 3'-end region of the *nos* operon mRNA may experience endoribonuclease-mediated degradation during initial aerobic growth, whereby the nuclease cleaves internal mRNA phosphodiester bonds (Belasco, 2010; Laalami et al., 2014; Richards et al., 2008). In bacteria, the recognition and degradation of nonsense mRNA is executed by the ordinary cellular apparatus for RNA degradation (Belasco, 2010). Premature release of *E. coli* ribosomes at premature stop codons expose internal mRNA sites for cleavage by the 5'-end-independent endoribonuclease E (Baker and Mackie, 2003).

13.6.2.1.1.2 Alternative transcription units

Another explanation which may explain the differences in expression of *nos* operon regions is the alternative initiation and/or termination of transcription by RNA polymerase at different regions of the operon creating alternative transcript isoforms. Premature termination of transcription causes a greater abundance of mRNA containing the 5'-end than the entire gene. Some studies have found that the majority of bacterial transcription termination is premature and associated with translated regions, i.e. within open reading frames (D'Halluin et al., 2023). In eukaryotes and archaea, such use of alternative transcription termination sites results in transcriptome 3' end length diversity (Dar et al., 2016; LaForce et al., 2022; Reyes and Huber, 2018). The generation of multiple transcript isoforms may result in unequal numbers of *nos* gene mRNA transcripts in qPCR and may explain our finding that the *nosZ(N)* region transcript is more abundant than *nosZ(C)* or *nosL* (Figure 32).

13.6.2.2 Copper homeostasis genes are not upregulated during 8 hr static cultures

We hypothesised that *laz* would be upregulated in static 8 hr cultures regardless of supplementation with NO_2^-. While *laz* is not thought to be FNR-dependent (Whitehead et al., 2007), and it is known that meningococcal Laz is not essential for growth under anaerobic conditions (Baarda et al., 2018), meningococcal Laz expression increases under O_2-limiting conditions (Deeudom et al., 2015). Gonococcal Laz expression has been shown to increase ~3-fold under O_2-limiting conditions (Zielke

et al., 2016, 2014), which is not corroborated by this study (Figure 33). Since little is known about regulation of *sco* (Seib et al., 2006), we hypothesised expression of *sco* would remain unchanged during microaerobic growth.

The expression of the *copA*, *copA2*, *laz* and *sco* genes did not significantly change in cultures with NO_2^- between 0 and 8 hr in static cultures (Figure 33). This suggests that the shift to microaerobic conditions during 8 hr static gonococcal culture growth had a minimal effect on Cu homeostasis. However, 8 hr cultures with NO_2^- upregulated *copA2* 2.2-fold, *laz* 3.6-fold and *sco* 3.5-fold. Upregulation of *copA2* is consistent with our hypothesis of increased periplasmic Cu demand to metalate denitrification enzymes. However, since change in *copA2* expression with NO_2^- at 8 hr was relatively small (2.2-fold) and due to the lack of upregulation of *copA* (1.4-fold) also, we would hesitate to draw any conclusions from this without further study. Avenues of further enquiry should be to determine the periplasmic vs cytoplasmic Cu content of microaerobic gonococci via ICP-MS, and to determine the regulation of *copA* and *copA2* in gonococci.

Upregulation of *laz* with NO_2^- at 8 hr (3.6-fold) was as predicted, supporting our hypothesis that these cultures were truly microaerobic. Upregulation of *sco* with NO_2^- at 8 hr (3.5-fold) was a novel finding which contributes to our understanding of this gene in bacteria.

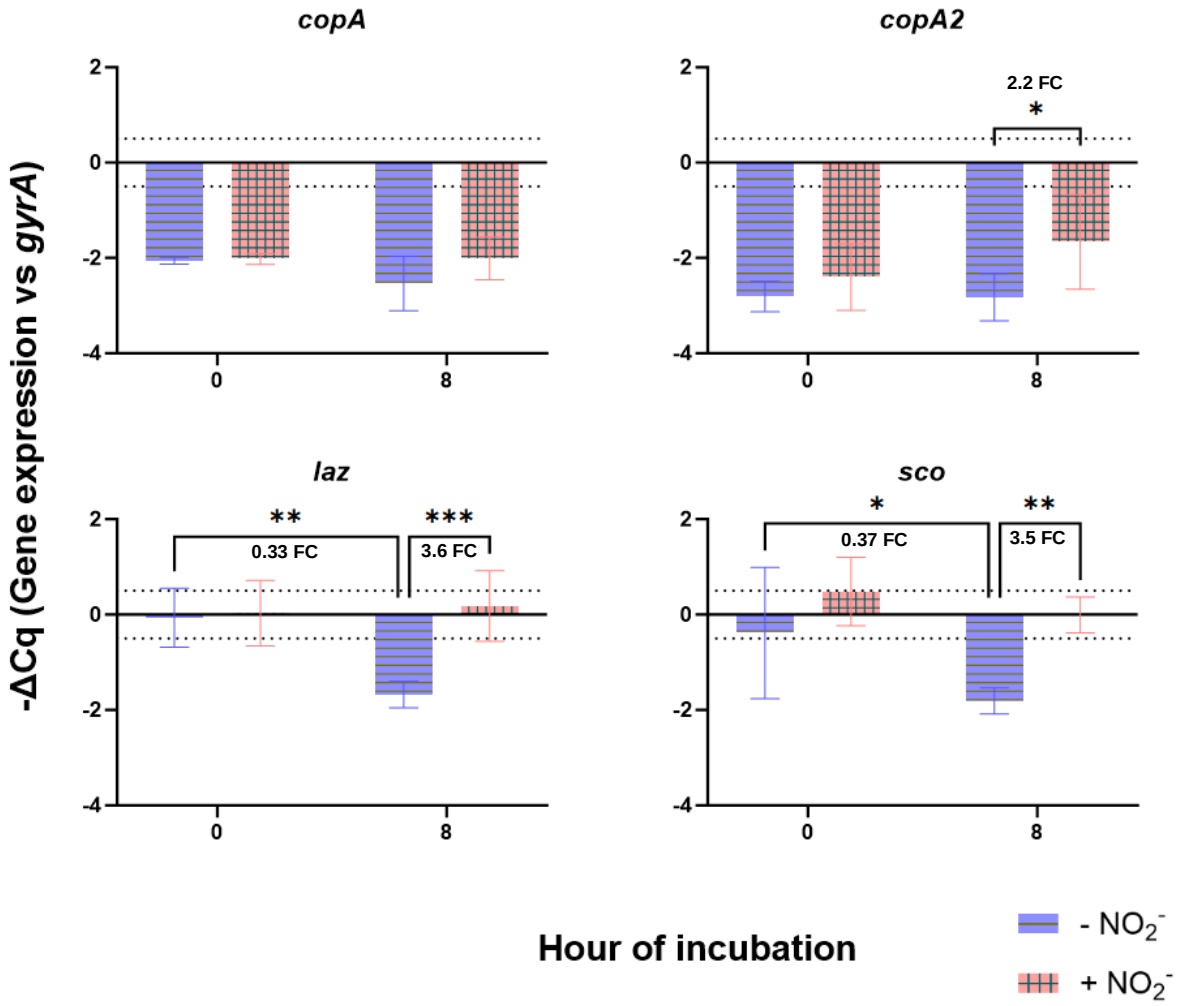


Figure 33: Expression of Cu homeostasis genes in static 8 hr gonococcal cultures supplemented with NO_2^-. $NaNO_2$ (2 mM) was supplemented at 0, 5 and 7 hr. Mean of n = 4 independent replicates ±SD. Only significantly different pairwise comparisons plotted. Data analysed with 2-way ANOVA.

13.6.2.3 FNR-dependent genes are expressed at 8 hr in static cultures

To assess the O_2 concentration of static and shaking cultures, we studied the expression of various genes thought to be upregulated by FNR, a transcription factor which senses low O_2 (Edwards et al., 2010). We hypothesised that static cultures would only upregulate FNR-dependent genes at 8 hr, and shaking cultures would not upregulate FNR-regulated genes since these cultures were expected to be more aerated.

In a previous study, the following genes were found to be upregulated by FNR: *ompU*, *aniA*, *accA, res* and *ccp*, while the genes *aph* and *norB* were downregulated by FNR (listed in order of change in transcript abundance) (Whitehead et al., 2007). Cytochrome *c* peroxidase (Ccp) is also suggested to be upregulated in low O_2 in other studies (Atack and Kelly, 2006). There is no FNR site upstream of

FA1090 *ompU*, but there are FNR sites upstream of *aniA*, *accA*, *res*, *ccp*, *aph* and *norB* (Table 29). These genes were tested for expression in static 0 and 8 hr cultures in this study (Figure 34**,** results summarised in Table 28**)**.

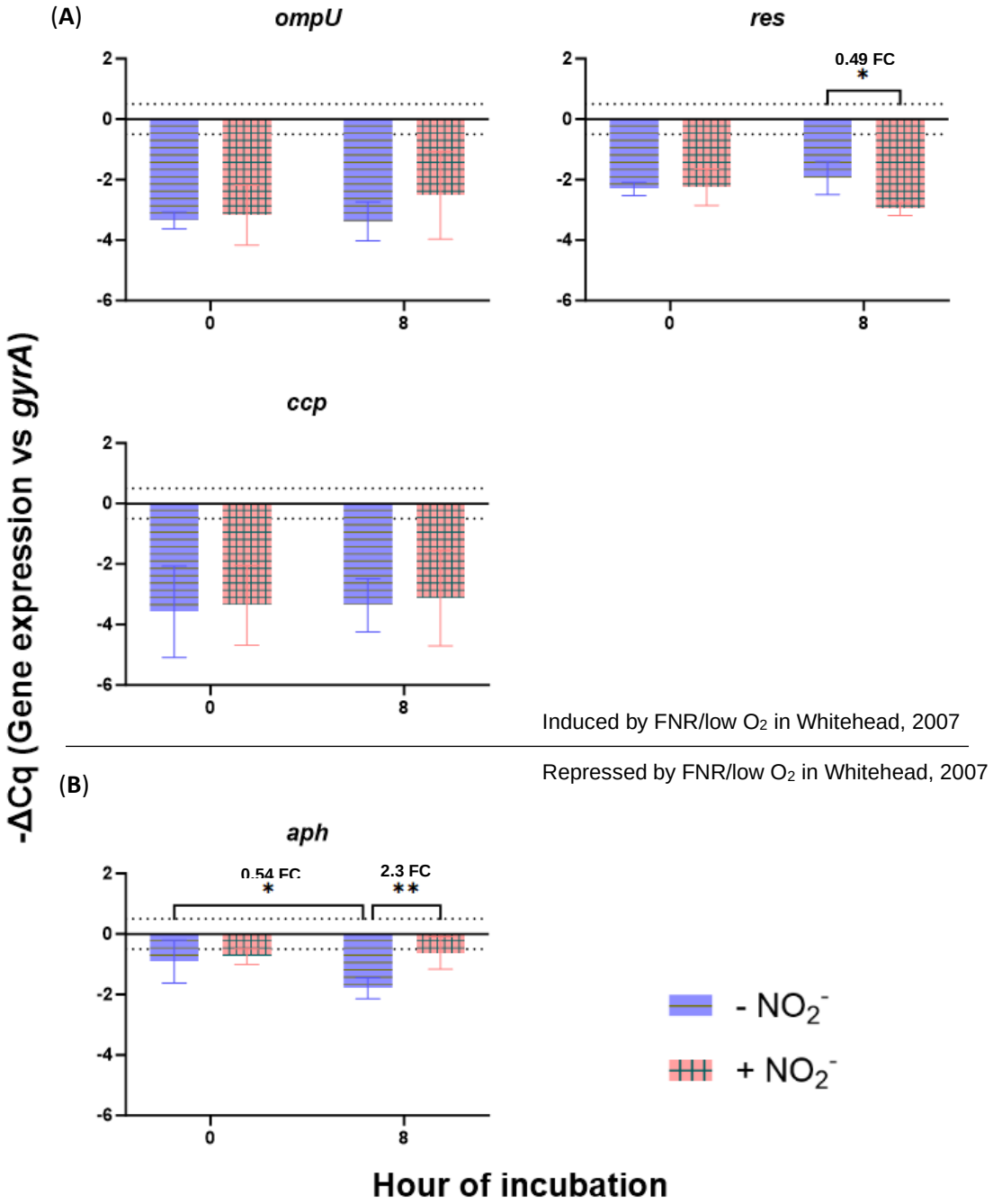


Figure 34: Expression of FNR-dependent genes in static 0 hr and 8hr gonococcal cultures.
$NaNO_2$ (2 mM) was supplemented at 0, 5 and 7 hr. (**A**) Genes upregulated by FNR in Whitehead et al (2007). (**B**) Genes downregulated by FNR in Whitehead et al (2007). Mean of n = 3 independent replicates ±SD. Only significantly different pairwise comparisons plotted. Data analysed with 2-way ANOVA.

Of the seven FNR-regulated genes tested, only *aniA* exhibited similar gene expression under microaerobic conditions in this study compared to Whitehead et al (2007). The *aniA* gene was upregulated in cultures with NO_2^- over 8 hr, and upregulated with NO_2^- at 8 hr.

Unlike Whitehead et al (2007), this study found appreciable quantities of *ccp* transcript; *ccp* mRNA was as abundant as *ompU* or *accA*. Whitehead et al. (2007) detected gonococcal Ccp protein by staining proteins separated by SDS-PAGE for heme-dependent peroxidase activity. Their study found that while gonococcal *ccp* expression was induced by FNR, expression was very low, to the extent that the quantity of the *ccp* transcript was insufficient to generate a statistically significant signal above background noise. We hypothesise difference in primer design between this study and the *ccp* probes from the Pan Neisseria Array v2 used in Whitehead et al (2007) may account for this disparity in *ccp* amplification.

While Whitehead et al (2007) found *ompU* and *res* to be upregulated by FNR in cultures both with NO_2^-, this study determined expression of both genes to unchanged and downregulated 2-fold at 8 hr with NO_2^-, respectively. While accurate comparisons with other studies regarding the speed of gene upregulation are limited by the different culture methods used in this study, it should be noted that Whitehead et al (2007) saw upregulation of *ompU* and *res* within 8 hr. *Vibrio cholerae* OmpU is thought to be regulated by the Fe sensor Fur, and by quorum sensing via ToxR (Ganie et al., 2022). Hence, either higher Fe level or lower 8 hr culture OD_{600} in this study compared to Whitehead et al (2007) may explain our finding of reduced *ompU* expression.

Little research has been undertaken on the environmental factors regulating gene expression of the *res* Type III EcoP1 restriction-modification system. Generally, restriction-modification systems are induced upon detection of unmethylated DNA, such as those possessed by bacteriophages (Rodic et al., 2017). The EcoP1 *res* gene is transcribed from an FNR site present within the EcoP1 *mod* gene (Rao et al., 2014). Transcription of *res* may also be regulated through the activity of an antisense promoter within the *res* gene (Sharrocks and Hornby, 1991). It is not clear what environmental

factor in our setup would cause repression of the *res* gene in static cultures in the presence of NO_2^-; this is an avenue for further inquiry.

A major difference between the findings of this study and Whitehead et al (2007) was between gene expression of *aph* and *norB*. Whitehead et al (2007) found *aph* and *norB* to be repressed by FNR in cultures both with and without NO_2^-. However, this study determined *aph* to be unchanged over 8 hr in cultures without NO_2^- and upregulated 2.3-fold in 8 hr cultures with NO_2^-. Furthermore, this study determined *norB* to be upregulated 10.6-fold over 8 hr in cultures with NO_2^- and upregulated 24.5-fold at 8 hr in cultures with NO_2^-. The difference between the findings of these two studies may be because Whitehead et al (2007) described gene expression as a ratio of transcript abundance between RUG7001 (an F62 strain expressing an *aniA::lacZ* fusion at the site of the *proAB* genes (Silver and Clark, 1995) and RUG7001 *Δfnr* strains, while our study analysed the expression of only the WT FA1090 strain. As such, it may be the case that FNR does regulate gonococcal *ompU*, *res*, *aph* and *norB* as described by Whitehead et al (2007), but that the overall expression of these genes is overridden by other transcription factors in a WT strain as described by this study.

Little research has been done on the regulation or expression of the *aph* gene. Presumably, *aph* is induced upon exposure to aminoglycoside antibiotics. There is evidence to suggest that aminoglycosides bind the leader RNA of genes for aminoglycoside acetyl transferase (AAC) and aminoglycoside adenyl transferase (AAD) resistance enzyme (Jia et al., 2013). However, it is not yet clear if the aminoglycoside phosphotransferase (*aph*) gene is under the control of a similar riboswitch (Woegerbauer et al., 2014).

The lack of upregulation of putative FNR-regulated genes prior to 8 hr in this study, and the lack of upregulation of some FNR-regulated genes at 8 hr such as *ompU* and *res* highlights the challenge in making direct comparisons between studies since culture conditions may greatly influence experimental outcome. The expression of FNR-regulated genes in static cultures determined during this study are summarised in Table 28.

Table 28: Comparison of FNR-regulated gene expression between this study and Whitehead et al (2007)

	Ratio of *fnr+/Δfnr* transcript intensity (Whitehead 2007)		Ratio of *narP+/narP* transcript intensity (Overton 2006)	8 hr -ΔCq (gene expression vs *gyrA*) (this study)		Change between...		Corroboration
Gene	**-NO_2^-**	**+NO_2^-**		**8 hr -NO_2^-**	**8 hr +NO_2^-**	**0 hr +NO_2^- and 8 hr +NO_2^-**	**8 hr -NO_2^- and 8 hr +NO_2^-**	
ompU	↑ 6.6	↑ 12.27		-3.38	-2.53	-	-	No
aniA	↑ 3.56	↑ 6.02	↑ 3.22	-4.3	-1.27	↑	↑	Yes
accA	↑ 3.52	↑ 4.57	↓ 0.25	-5.59	-4.16	-	↑	Partial
res	↑ 5.2	↑ 2.31		-1.95	-2.97	-	↓	No
ccp	↑ ???	↑ ???		-3.36	-3.13	-	-	Partial
aph	↓ 0.18	↓ 0.14		-1.79	-0.62	-	↑	No
norB	↓ 0.28	↓ 0.60	↑ 3.98	-2.66	1.96	↑	↑	No

Genes ranked based on transcript abundance in Whitehead et al., 2007 expression results. Key: ↑, up-regulation of gene; ↓, down-regulation of gene; -, no change in expression of gene.

13.6.2.4 Adherence to FNR site consensus sequence does not influence gene expression

Putative FNR sites are found upstream of the FA1090 genes *aniA*, *accA*, *nosR*, *laz*, *res*, *aph*, *norB*, *ccp* and *dnrN* (Table 29).

Table 29: Putative regulatory boxes in the *N. gonorrhoeae* FA1090 genome

Box	FA1090 genome locus	Gene	Sequence (5' → 3')	Notes
Denitrification genes				
NarPQ	NGO_1276	*aniA*	TAGTTATAAAGTATTA	
Fur			TATTAGAAGCGTCATTTTA	
NsrR			AGTTCATATTTTATGAATT	
FNR			TTGACTTAAATTAA	After NsrR & NarPQ boxes; -95 bp.
FNR	NGO_1215	*accA*	TTAATCTACATCAA	After NarPQ box; -80 bp.
NarPQ			TAATCCTTTAGGATTA	
FNR	NGO_07420	*nosR*	TTGATTTGCATCAA	Before the NsrR box; -78 bp.
NsrR			GATTCATATAATATCAATA	
-	NGO_07405	*nosZ*	–	FNR before *nosR*
-	NGO_1397	*nosL*	–	FNR before *nosR*
FNR	NGO_0994	*laz*	TTGATTTCCGTAAA	-33 bp.
-	NGO_1237	*sco*	–	
-	NGO_0579	*copA*	–	
-	NGO_0685	*copA2*	–	
-	NGO_1688	*ompU*	–	
FNR	NGO_0546	*res*	TTGAATGTGATTAT	-113 bp, inside another gene
FNR	NGO_1716	*aph*	TTGAGTGAAAATAA	-92 bp, inside *mod* gene
NsrR	NGO_1275	*norB*	CATTCATATTTTGTGAATT	
FNR			TGAATTTTAAACAA	After Fur & NsrR boxes; -40 bp.
FNR	NGO_1769	*ccp*	TTGATTTTCCGCAA	
Regulator genes				
NsrR	NGO_0653	*dnrN*	GATTCTTAAAGTATGAATG	
FNR			ATGTTCAAAACCAA	
Nnr	NGO_1579	*fnr*	TTGATATGTGTCAA	
Miscellaneous genes with identified regulatory boxes				
FNR	NGO_2047	lipoprotein	TGGAAATGAAACAA	
FNR	NGO_1131	hypothetical protein	TTCAGCTGCTTCAA	
FNR	NGO_0167	hypothetical protein	TTGATTGAAATCAA	
NsrR	NGO_1073	hypothetical protein	CATTGAAAATACAAGAACC	
NsrR	NGO_0753	ATPase	AATTCATGTTATTTGAATT	
NarPQ	NGO_1370	membrane protein	TAGTCATAAAGAATTA	

The lack of an FNR site upstream of FA1090 *ompU* prompted Whitehead et al (2007) to suggest that potential FNR half-sites present in the *ompU* promoter region may facilitate FNR binding, implying FNR binding is highly promiscuous. It should be noted that although this study identified an FNR-like binding site upstream of FA1090 *laz*, since *laz* was not been previously identified as an FNR-

regulated gene (Whitehead et al., 2007), for the purposes of this study *laz* was not classed as an FNR-regulated gene. We suggest the guanine at the 10th base in the putative *laz* FNR site may inhibit FNR binding (Table 30). Whitehead 2007 showed genes upregulated by FNR were *ompU*, *aniA*, *accA, nosR*, *res and ccp*, while genes downregulated by FNR were *aph* and *norB*. They found these genes to possess different FNR binding sites but did not hypothesise as to how these different binding sites may influence gene expression in low O_2.

This study adopted the approach of Whitehead et al. (2007) to identify putative FNR binding sites with similarity to the *E. coli* consensus sequence, `TTGATNNNNATCAA`, within 200 base pairs upstream of the translation start codon of FA1090 genes. The exact *E. coli* consensus sequence sequence revealed 6 hits in the entire FA1090 genome, with only one promoting a gene of interest, *nosR*. Hence, we then expanded this search to include the consensus sequence `TKRANNNNNMNYAW`. This revealed FNR sites with varying homology to the *E. coli* consensus sequence upstream of *aniA*, *accA*, *nosR*, *laz*, *res*, *aph*, *norB*, *ccp* and *dnrN*. None of these eight FNR sites in question are identical; all seem to have at least two bases changed in pairwise comparisons and all but the site preceding *nosR* break the *E. coli* consensus sequence (Table 30). This suggests each of these genes may experience different levels of regulation by FNR.

Table 30: Alignment of *N. gonorrhoeae* FA1090 gene FNR sites to *E. coli* consensus sequence

			Expression results from this study	
	Consensus SNPs	SNPs unique to gene	With NO_2^- (at 8 hr)	0 to 8 hr (with NO_2^-)
E. coli consensus TTGATNNNNATCAA				
nosR TTGATTTGCATCAA	0	-	↑	↑
aniA TTGACTTAAATTAA	2	5C	↑	↑
accA TTAATCTACATCAA		6C	↑	-
laz TTGATTTCCGTAAA		10G	↑	-
aph TTGAGTGAAAATAA	3	-	↑	-
norB TGAATTTTAAACAA		2G	↑	↑
ccp TTGATTTTCCGCAA		10C, 11G	-	-
res TTGAATGTGATTAT		5A, 14T	↓	

Genes ranked based on adherence to consensus sequence.

Of the FA1090 genes with upstream FNR sites, only *aniA*, *norB* and *nosZ* (*nosR*) were upregulated in static cultures with NO_2^- over 8 hr and upregulated with NO_2^- at 8 hr. The genes *laz*, *nosL, accA* and *aph* were only upregulated with NO_2^- at 8 hr. Expression of *ccp* was unchanged and *res* was downregulated with NO_2^- at 8 hr (Figure 34). Hence, we hypothesised there was a stepwise regulation of FNR activation occurring, whereby FNR initially bound to those sites with strongest adherence to a consensus sequence, and subsequently bound to sites with weaker adherence. The adherence to a FNR site consensus sequence may therefore influence expression of FNR-regulated genes. However, this study found no significant correlation between adherence to an FNR consensus sequence and upregulation in low O_2 cultures (Table 30). This finding that various FNR-regulated genes exhibited different expression profiles in static cultures is not surprising, given their variable FNR sites and the potential action of other transcription factors.

13.6.3 Static culture *aniA* and *nosZ* expression unaffected by Cu

Previous research in other species suggests that Cu is required for activation of denitrification genes. A *Paracoccus denitrificans ΔnosL* strain has no denitrification phenotype under high Cu but fails to reduce N_2O under low Cu conditions (Bennett et al., 2019), suggesting a role for NosL only in low Cu. Furthermore, while addition of 5 µM Cu does not change expression of *Rhodobacter capsulatus nos* genes, addition of BCS represses *nosZ* and induces *nosL*. This suggests that *R. capsulatus* either compensates for Cu shortage by inducing NosL, or that NosL is involved in the biogenesis of a different unknown cuproprotein (Selamoglu et al., 2020). Similarly, a gonococcal *ΔaccA* strain fails to reduce NO_2^- unless supplemented with Cu, suggesting that in the absence of AccA, AniA is expressed as an *apo*-enzyme, but is only metalated if Cu increases. In the presence of BCS Cu chelator, gonococcal strains lacking the AccA C-terminal tail have reduced growth and NO_2^- consumption, suggesting a role for AccA in Cu starvation (Firth, 2023).

Expression of *B. diazoefficiens nir*, *nor* and *nos* operons are all repressed in low Cu (Pacheco et al., 2022). Low Cu represses *P. denitrificans nosZ* gene expression (Sullivan et al., 2013) and represses NosZ protein expression (Felgate et al., 2012; Sullivan et al., 2013; Woolfenden et al., 2013). The Cu-

mediated regulation of *P. denitrificans nos* is thought to require NosR, which contains two putative CXXXCP motifs Cu binding sites, and NosC, a *Paracoccus*-specific hypothetical protein of unknown function, although the mechanisms by which these proteins mediate this response to Cu warrant further investigation (Sullivan et al., 2013). Low Cu also represses *B. diazoefficiens nir* and *nor* in addition to *nos* (Pacheco et al., 2023). Supplementation of Cu increases N_2O reduction in wetland soils and stream sediments (Sharma et al., 2022; Yang et al., 2023). Furthermore, it is known that under low Cu conditions, photosynthetic algae switch from using an Cu-containing plastocyanin to a heme-containing cytochrome (Eriksson et al., 2004). However, there is no known Cu-independent mechanism for N_2O reduction, and in *P. denitrificans* N_2O reduction is inhibited in low Cu (Sullivan et al., 2013).

These findings that Cu influences expression of *nos* genes in other species gave us a rationale for investigating *nos* expression in *N. gonorrhoeae* in response to Cu. Having determined that the genes for various periplasmic cuproproteins (*aniA*, *accA*, *nosZ*, *nosL*) were upregulated in static 8 hr cultures with NO_2^-, we hypothesised that the gonococcal periplasmic Cu demand was increased in these cultures, which would then increase expression of Cu transporters *copA* and *copA2* that export cytosolic Cu into the periplasm. In static 8 hr cultures without NO_2^-, we hypothesised that the lack of denitrification would mean Cu demand would be unchanged.

We determined that 1 µM supplemented Cu was nutritional for static gonococcal growth, whereas 10 µM Cu was excess and 20 µM Cu highly inhibitory (Figure 27). There is no known Cu sensor in *N. gonorrhoeae*. In gonococci, the transcriptional activator of the cytoplasmic Cu exporter *copA* is not the Cu sensor CueR, but is instead thought to be NmlR, which is thought to respond to oxidative and/or nitrosative stress, not Cu (Kidd et al., 2005). It is unknown if concentrations of Cu as high as 10-20 µM are physiologically possible even inside the phagosome or Cu coil contraceptive environment. However, studies suggest the *E. coli* copper tolerance Cue system responds to extracellular 1-400 µM Cu with maximum expression at 50 µM (Stoyanov et al., 2001), while the Cus

and Pco copper tolerance systems respond to 5-500 µM Cu (Munson et al., 2000). Furthermore, the *Acinetobacter baumannii oprC* copper importer is downregulated by Cu concentrations as low as 4.8 µM (Thummeepak et al., 2020). These findings suggests that bacteria copper importers are inactivated in very low Cu, while copper tolerance systems sense a far wider range of Cu concentrations. However, these studies only describe responses to exogenous copper, and did not measure intracellular available Cu concentrations, which are expected to be very low due to the Irving-Williams series. Hence, we did not make predictions *a priori* regarding the effect of 0, 1 and 10 µM Cu exogenous Cu used in this study on expression of gonococcal *copA* and *copA2*.

13.6.3.1 Effect of Cu on expression of gonococcal denitrification genes

Static 8 hr cultures were grown as previously described with supplemented 1, 10 and 20 µM Cu, then harvested, RNA extracted and gene expression analysed using qPCR as previously described (Figure 35). To the best of our knowledge, no studies have investigated the effect of Cu supplementation on induction of gonococcal denitrification genes. We hypothesised that in a low Cu environment, gonococcal *nosZ* expression may be repressed since NosZ requires a large quantity of Cu to function (twelve per functional enzyme). Likewise, we also hypothesised that *nosZ* expression may be induced only in high Cu.

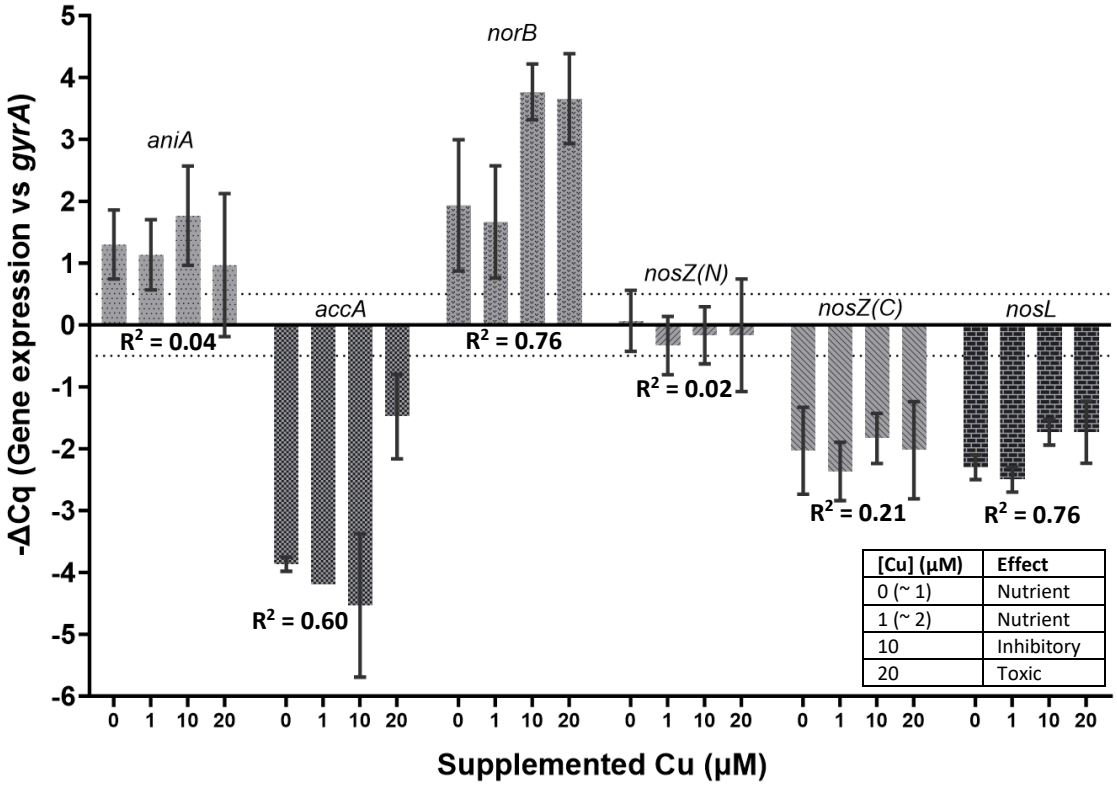


Figure 35: Effect of Cu concentration on expression of denitrification genes.
Static cultures grown over 8 hr in microaerobic conditions (30 mL cultures in 50 mL Falcon tubes). $NaNO_2$ (2 mM) supplemented at 0, 5 and 7 hr. Data points represent three independent replicates. Data analysed using linear regression.

Cu concentrations of 1-20 µM did not induce expression of gonococcal *aniA* or *nosZ*. This is contrary to findings in other species, but corroborates previous understanding of regulation of gonococcal denitrification, which is not known to be Cu-dependent. Instead, regulation of gonococcal denitrification likely depends on other transcription factors. However, 10-20 µM Cu did induce expression of the two copper chaperones, *accA* and *nosL*, as well as the Fe-dependent *norB*. The induction of *accA* by 20 µM Cu was 8-fold, *norB* by 10 µM Cu 4-fold, while *nosL* by 10 µM Cu less than 2-fold.

The gonococcal *norB* gene is known to be induced by •NO and low O_2 but finding induction of *norB* by Cu is novel. Unlike AniA and NosZ, NorB has no requirement for Cu, being instead dependent on heme for catalysis. Hence, the mechanism of this Cu-dependent induction of *norB* is not obvious, since an increased expression of *accA* will not necessarily increase concentration of •NO (the *norB* substrate) without cognate increased expression of *aniA*. We hypothesise that Cu may generate

increased •NO via an AniA-independent pathway (e.g. redox cycling with NO_2^-). It is known that under very low O_2 conditions, Cu-containing Cox can convert NO_2^- to •NO (Igamberdiev et al., 2010). Increased •NO would then be expected to induce expression of *norB*.

Once again, we see differential gene expression of the Cu-dependent denitrification enzymes *aniA* and *nosZ* compared to their cognate chaperones, *accA* and *nosL*. It appears that the concentration of the Cu chaperone mRNA is kept low, and upregulated in response to Cu while the expression of the cognate enzymes is unchanged regardless of Cu concentration. It is unknown if AccA and NosL have other roles outside of metalating AniA and NosZ; these findings suggest gonococcal AccA and NosL may act as non-specific scavengers of excess and inhibitory Cu.

This study found that Cu induces expression of the Cu chaperones *accA*, *nosL* and Fe-dependent *norB*, but not the genes encoding the Cu-dependent enzymes *aniA* or *nosZ*. This suggests *nosZ* is not repressed to conserve scarce Cu, nor is *nosZ* upregulated when Cu is plentiful. Instead, regulation of the denitrification pathway likely depends on other transcription factors.

13.6.3.2 *Effect of Cu on expression of gonococcal laz, sco and copA*

After determining the effect of Cu concentration on the expression of gonococcal denitrification genes, we studied the effect of Cu on the expression of *laz*, *sco* and *copA* (Figure 36). Laz and Sco are Cu-binding proteins. Laz has no established regulatory link to Cu so was not expected to be affected by changing Cu concentration. Sco, however, is involved with protection from oxidative stress (Seib et al., 2003), which is known to be increased in high Cu (Cervantes-Cervantes et al., 2005; Liu et al., 2023). Hence, we hypothesised that *sco* may be be upregulated in toxic Cu to defend against subsequent oxidative stress.

The *Shewanella oneidensis copA* copper exporter is highly induced under anaerobic conditions with 100 µM Cu (Toes et al., 2008) and *Erwinia amylovora copA* is strongly induced by 500 µM Cu (Águila-Clares et al., 2017). The *E. coli copA* is induced by Cu via CueR (Stoyanov and Brown, 2003). We hesitate to use gonococcal *copA* expression as an indicator of cytoplasmic Cu concentration since,

rather than being regulated by CueR as in other species, gonococcal *copA* is instead regulated by NmlR, which responds to oxidative and/or nitrosative stress (Djoko et al., 2012; Kidd et al., 2005). However, Cu is known to induce oxidative stress, so may possibly indirectly activate NmlR and subsequently *copA*.

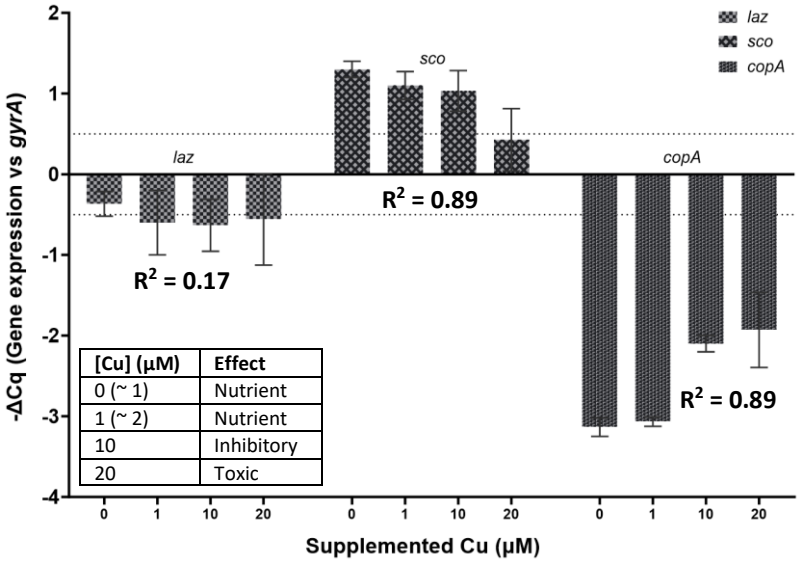


Figure 36: Effect of Cu concentration on expression of *laz*, *sco* and *copA*.
Static cultures were grown over 8 hr in microaerobic conditions (30 mL cultures in 50 mL Falcon tubes). $NaNO_2$ (2 mM) was supplemented at 0, 5 and 7 hr. Data points represent three independent replicates. Data analysed using linear regression.

The expression of *laz* was unaffected by all Cu concentrations tested in this study, as predicted. Contrary to our hypothesis, *sco* expression was not upregulated by 1-10 µM Cu and was instead downregulated. This may be because *sco* does not respond to oxidative stress directly, or it may be because 10 µM Cu was insufficient to generate oxidative stress. Following our hypothesis, the expression of *copA* was upregulated 2-fold in 10 µM Cu. A summary of the effect of Cu on microaerobic gonococcal gene expression is given in Table 31.

Table 31: Gonococcal static culture gene expression with added Cu

	No supplemented Cu		Supplemented Cu
Gene	**Fold change between 0 hr $+NO_2^-$ cultures and 8 hr $+NO_2^-$ cultures**	**Fold change between 8 hr $-NO_2^-$ cultures and 8 hr $+NO_2^-$ cultures**	**Linear regression**
aniA	11.7 (P < 0.0001; ****)	8.1 (P < 0.0001; ****)	- (R^2 = 0.0392, P = 0.801; ns)
norB	10.6 (P < 0.0002; ***)	24.5 (P < 0.0001; ****)	↑ (R^2 = 0.7584, P = 0.009; **)
nosZ(N)	5.7 (P = 0.0001; ***)	5.8 (P < 0.0001; ****)	- (R^2 = 0.0221, P = 0.9002; ns)
nosZ(C)	4.2 (P = 0.0024, **)	2.7 (P = 0.0194; *)	- (R^2 = 0.2073, P = 0.6119; ns)
nosL	1.2 (P = 0.3819; ns)	2.4 (P = 0.0033; **)	↑ (R^2 = 0.7557, P = 0.011; *)
laz	1.1 (P = 0.7330; ns)	3.6 (P = 0.001; ***)	- (R^2 = 0.1741, P = 0.6893; ns)
accA	1.1 (P = 0.8733; ns)	2.7 (P = 0.0063; **)	↑ (R^2 = 0.5976, P = 0.108; *)
copA	1.0 (P = 0.1575; ns)	1.4 (P = 0.0361; *)	↑ (R^2 = 0.8869, P = 0.0001; ***)
sco	0.7 (P = 0.4097; ns)	3.5 (P = 0.0084; **)	↓ (R^2 = 0.8932, P = 0.0018; **)

Genes ranked on effect of incubation time. Reductases underlined. P-values for supplemented Cu cultures were calculated based on a linear regression of -ΔCq gene expression vs Cu concentration.

13.6.4 Shaking cultures upregulate denitrification genes within 1 hr

We wished to determine gene expression of shaking cultures to establish a control for aerobic conditions. Shaking cultures were set up as described for static growth with the following modifications: 200 rpm shaking 50 mL cultures in 250 mL conical flasks were used instead of static 30 mL cultures in 50 mL Falcon tubes. *N. gonorrhoeae* is a facultative anaerobe, meaning it can grow in a low O_2 environment via denitrification but grows optimally in an aerobic environment via a sole cbb_3-type cytochrome *c* oxidase (Turner et al., 2005; Zhong et al., 2024). This corroborates our finding that shaking gonococcal cultures grew quicker than static cultures (Figure 26). At 4 hr, shaking cultures were still growing exponentially but after 5 hr, cultures were thought to be entering stationary phase (Figure 26A). Hence, we harvested shaking gonococcal cultures at 0, 1, 2, 3 and 4 hr and determined gene expression via qPCR.

Expression of *aniA* is induced strongly during anaerobic growth with NO_2^- relative to aerobic growth without NO_2^- (Clark et al., 1988). However, it is thought NO_2^- slightly induces gonococcal *aniA* and significantly induces *norB* in aerobic conditions (Tzeng et al., 2023). Having established the upregulation of *aniA*, *nosZ* and *norB* in static 8 hr cultures with NO_2^-, we hypothesised that O_2 would repress expression of these genes in shaking cultures via inhibition of FNR. We expected 2 mM NO_2^- to have no effect on the expression of denitrification genes in shaking cultures, since O_2 would be inhibiting FNR and thus inhibiting denitrification. We predicted *laz* to be downregulated in aerobic conditions based on the finding of meningococcal Laz upregulation in microaerobic conditions (Deeudom et al., 2015) and predicted *sco* to not change based on the limited information regarding its regulation.

Initially, expression of the genes *aniA*, *accA*, *nosZ(N)*, *nosZ(C)* and *nosL* were tested every hr for 4 hr in shaking cultures. Contrary to our hypothesis, we found that expression of all these genes were rapidly upregulated in shaking cultures, peaking in expression at 3 hr (

Figure 37). Incidentally, the OD_{600} of 3 hr shaking cultures was roughly equivalent to 8 hr static cultures. Hence, expression of subsequent genes was only measured at 0 and 3 hr. Most denitrification genes (*aniA*, *accA*, *nosZ(N)*, *nosL* and *norB* but not *nosZ(C)*) were expressed faster and to a greater level in shaking cultures than in static cultures.

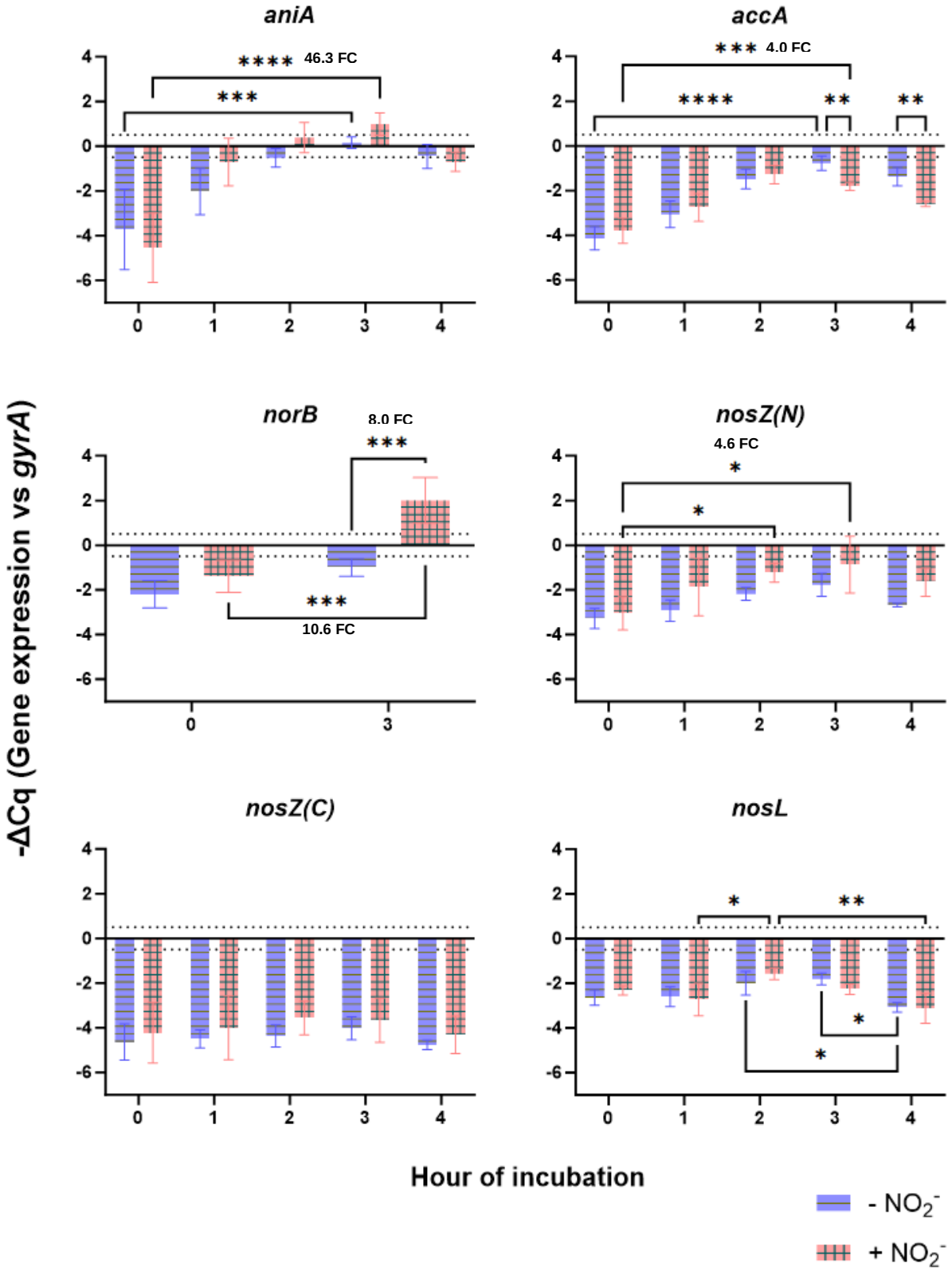


Figure 37: Expression of denitrification genes over shaking 4 hr gonococcal cultures.
Cultures with NO_2^- (red hatched) or without NO_2^- (purple bars) were grown over 4 hr in shaking conditions (200 RPM shaking 50 mL cultures in 250 mL conical flasks). $NaNO_2$ (2 mM) was supplemented at 0 hr. Mean of n = 3 independent replicates ±SD. Dashed horizontal lines represent the sensitivity limit of the assay ($log_2FC = \pm 0.5$). Not all significantly different pairwise comparisons plotted. Data analysed with 2-way ANOVA.

13.6.4.1 NO_2^- has minimal effect on shaking gonococcal culture gene expression

Survival of shaking gonococcal cultures was not NO_2^--dependent as was the case for static cultures, suggesting shaking cultures did not require denitrification to grow (Figure 26). Furthermore, our qPCR data shows that the induction of denitrification genes in shaking cultures was not as heavily NO_2^--dependent as was the case for static cultures. A major exception to this observation was *norB*, which is significantly upregulated (P = 0.0009 ;***) by NO_2^- in 4 hr shaking cultures such that it was the most expressed gene at 3 hr this project studied. This may reflect increased concentrations of O_2 and/or •NO in shaking cultures (Figure 11B). NO_2^- also caused a slight but significant decrease in expression of *accA* at 3-4 hr (P = 0.0097-0.0032; **).

However, when analysing all genes across 4 hr shaking growth, only *nosZ(N)* and *norB* had a statistically significant change in expression with added NO_2^- (2-way ANOVA *nosZ(N)* P = 0.0048; **, *norB* P = 0.0018; **). When the expression of all shaking genes at 3 hr was combined into a 2-way ANOVA, NO_2^- was a statistically insignificant source of variation (P = 0.9693).

This lack of denitrification gene upregulation by NO_2^- in our shaking cultures suggests denitrification was not required for shaking culture growth. However, the upregulation of the denitrification genes *aniA*, *accA*, *norB* and *nosZ* in these cultures (even without NO_2^-) suggest denitrification may be occurring in addition to aerobic respiration via Cox (which is known to function even in low O_2). Simultaneous denitrification and aerobic respiration (*aerobic denitrification*) has been shown in other species (Householder et al., 2000; Yang et al., 2020; Zumft, 1997), and it is known that both *N. meningitidis* and *N. gonorrhoeae* can simultaneously cometabolize O_2 and NO_2^- as electron acceptors during O_2-limited growth (Rock et al., 2005; Tzeng et al., 2023). The upregulation of the denitrification genes *aniA*, *accA*, *norB* and *nosZ* in shaking cultures without NO_2^- suggests that other factors affecting expression of denitrification (such as cell density) may be more important regulators than NO_x substrate concentration.

13.6.4.2 Copper homeostasis genes are not upregulated during shaking 3 hr cultures

Similar to static cultures, the expression of the Cu exporter genes *copA* and *copA2* were largely unchanged throughout shaking culture growth (Figure 38). Shaking cultures do not see the NO_2^--dependent upregulation of *copA2* which was seen in static cultures, however, the difference in *copA* and *copA2* -ΔCq gene expression between 8 hr static and 3 hr shaking cultures was not statistically significant (2-way ANOVA). As such, we hesitate to draw conclusions as to the effect of shaking on gonococcal culture *copA2* expression.

Identification of a trend in *laz* expression was challenging. In shaking cultures both with and without NO_2^-, expression slightly fluctuated over the course of 4 hr (within 2-fold change). Expression of *sco* in shaking cultures was upregulated over 2-fold within 2 hr regardless of NO_2^-. This was unlike 8hr static cultures which only upregulated *sco* with NO_2^-. This finding of *sco* upregulation over the course of shaking growth was novel, but potentially aligns with the role of Sco in synthesis of Cox.

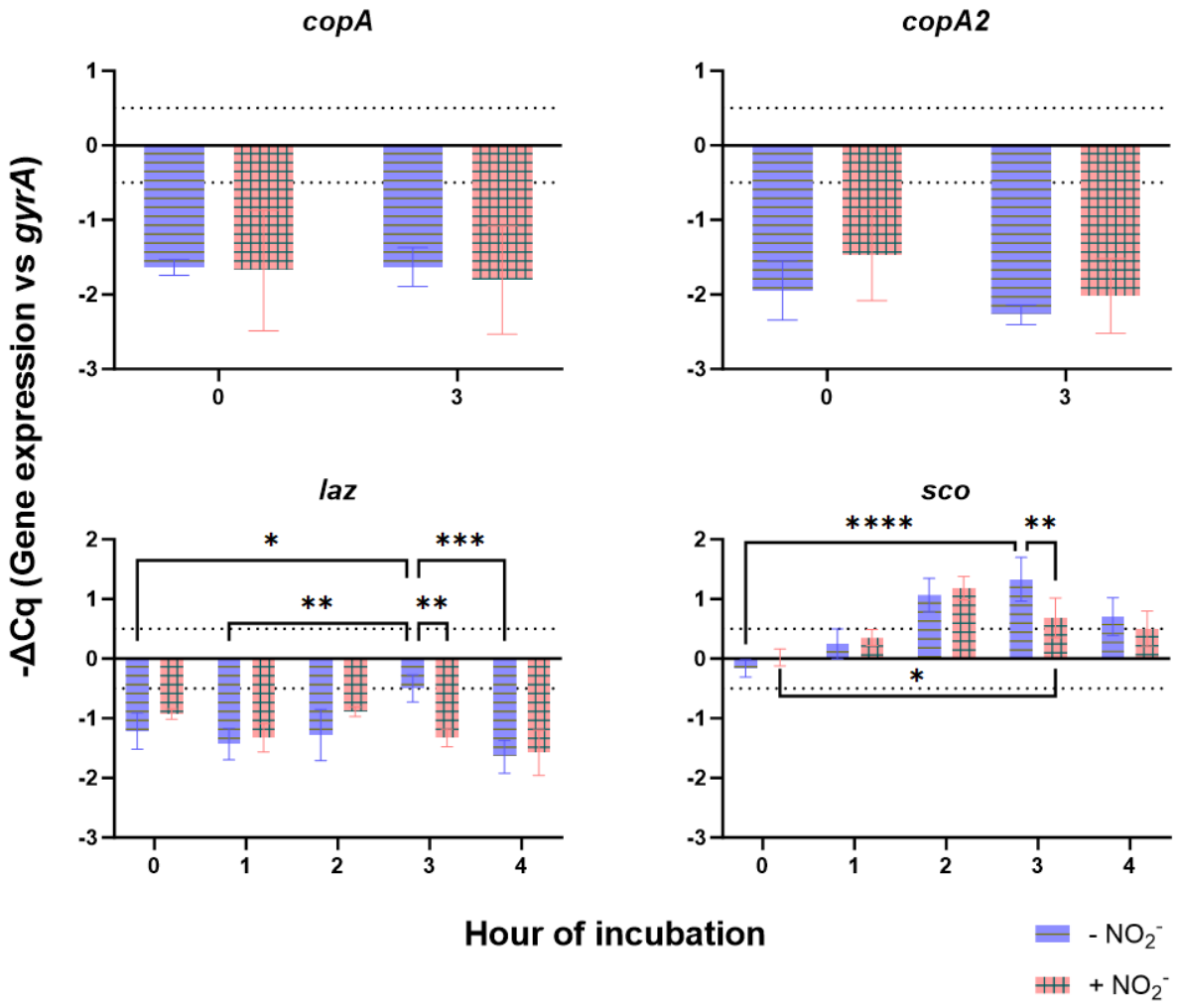


Figure 38: Expression of Cu homeostasis genes in shaking 4 hr gonococcal cultures supplemented with NO_2^-. $NaNO_2$ (2 mM) was supplemented at 0 hr. Mean of n = 3 independent replicates ±SD. Not all significantly different pairwise comparisons plotted. Data analysed with 2-way ANOVA.

13.6.4.3 FNR-dependent genes are upregulated within 3 hr in shaking cultures

Previous research suggests AniA is the only known gonococcal outer membrane protein present during anaerobic growth but absent during aerobic growth (Clark et al., 1987). Hence, finding the *aniA* gene highly expressed in our shaking cultures suggests these cultures were not aerobic. Upregulation of additional denitrification genes (*accA*, *norB*, *nosZ(N)*, *nosL*) within 3 hr further suggests that shaking cultures became increasingly microaerobic and increasingly required denitrification within 3 hr. We hypothesise that this was due to increased culture cell density in shaking cultures compared to static cultures (Figure 26) depleting available O_2 faster than could be replenished by shaking, and subsequently inducing transcription of denitrification genes via FNR.

FNR is thought to regulate gonococcal *aniA*, *accA*, *ompU*, *res*, *aph*, *norB*, *nosR*, and *ccp* (Whitehead et al., 2007), and this study showed upregulation of *aniA*, *accA*, *res*, *aph* and *norB* with NO_2^- in static cultures (Figure 31 and Figure 34). We thought to use these genes as an indicator of the aerobicity of shaking cultures. Given the unexpected upregulation of denitrification genes in shaking cultures, we hypothesised that these cultures were actually microaerobic due to increased cell density, and that FNR-induced genes would be upregulated similar to static growths.

As hypothesised, similar to static cultures, the FNR-induced *aniA* and *accA* genes were upregulated over the course of shaking culture growth. However, this upregulation was not NO_2^--dependent. The expression of *norB* in 3 hr shaking cultures was very similar to 8 hr static cultures (upregulation over 3 hr growth and at 3 hr with NO_2^-). This suggests an increase in microaerobic conditions in shaking growths. Expression of *ompU* and *res* was unchanged between 8 hr static and 3 hr shaking cultures (Figure 39).

The only genes which differed in their expression profiles between shaking and static cultures were *ccp* and *aph*. The *ccp* gene was upregulated over 3 hr shaking cultures (regardless of NO_2^-, Figure 39), whereas *ccp* expression was unchanged in static cultures over 8 hr (Figure 34). Conversely, expression of *aph* did not change in shaking cultures (Figure 39**)**, while it was upregulated by NO_2^- in 8 hr static cultures (Figure 34). The causes of these differences are not immediately apparent. Determining the mechanisms behind the differential expression profiles of these genes could be an avenue for further enquiry.

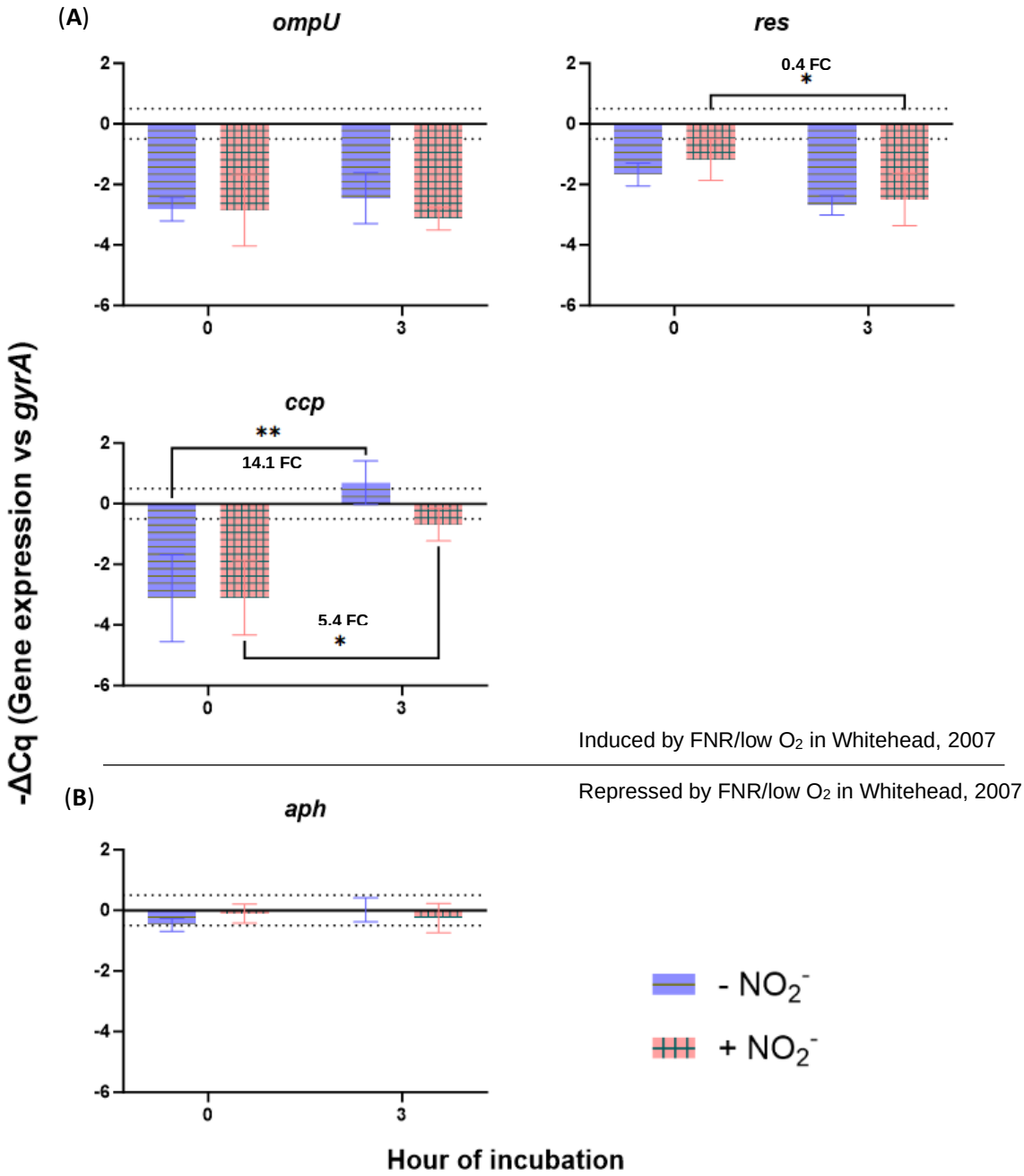


Figure 39: Expression of FNR-dependent genes in shaking 0 hr and 3 hr gonococcal cultures.
$NaNO_2$ (2 mM) was supplemented at 0 hr. (**A**) Genes upregulated by FNR in Whitehead et al (2007). (**B**) Genes downregulated by FNR in Whitehead et al (2007). Mean of n = 3 independent replicates ±SD. Only significantly different pairwise comparisons plotted. Data analysed with 2-way ANOVA.

13.7 Copper storage protein *csp* is not expressed in gonococci

The Cu storage protein *csp* gene (NGO_1701) was also initially included amongst the genes

measured in this study. Csp is thought to play a role in bacterial cytoplasmic Cu resistance after the

first layer of Cu resistance (the CsoR/CopZ/ATPase efflux system) becomes saturated (Straw et al., 2018; Vita et al., 2016).

A pair of primers against the *csp* gene: *csp*-qPCR-F (5' CTCAGGGCGACACGTCTATG 3') and *csp*-qPCR-R (5' CAGAGGGGAATTTTGTGCGG 3') successfully generated a product in Q5 PCR against a genomic DNA extract template (Figure 40

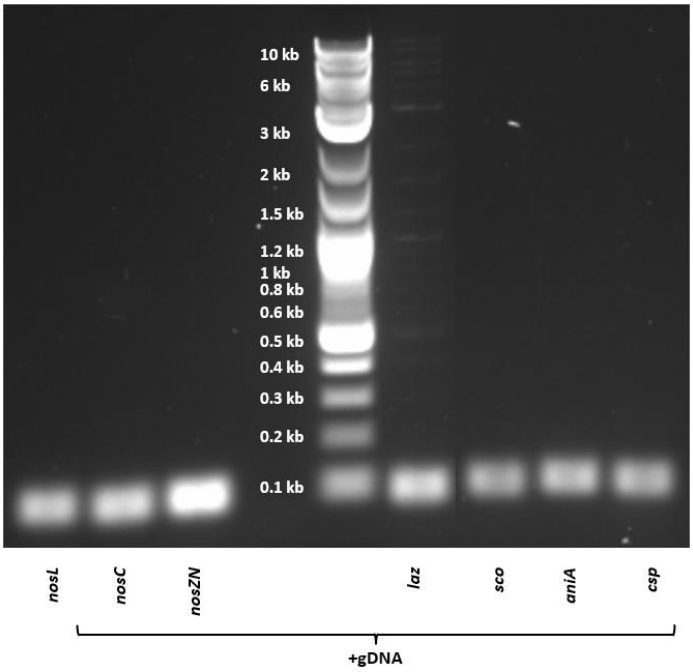


Figure 40: Gonococcal gDNA Q5 PCR amplification using qPCR primer pairs.
Various primers, including a pair against the *csp* gene, amplified gonococcal gDNA extracts. 1% agarose gel.

NGO_07415	787,278	L221	C			A	A				Y

).

However, unlike all other genes studied, these *csp* primers did not generate a product in LUNA qPCR against cDNA reverse transcribed from static culture 8 hr RNA extracts (Figure 41A). We hypothesised that one or both of the *csp* primers had a secondary structure which prevented qPCR amplification. However, while the *csp*-qPCR-R primer had two quadruplex GGGG and TTTT motifs, *in silico* checks suggested no self-dimers, primer dimers or secondary structures would be formed by this primer pair (see Methods Section 11.5).

To check for degradation of the *csp* qPCR primer master stocks, a second set of *csp* qPCR primers were ordered and tested (*csp*-qPCR-Fv2/Rv2) which had the same sequence as the first set of

primers. This second set of new primers were also not amplified in qPCR (Figure 41A). Only using 20 times the cDNA template concentration normally used in qPCR (200 ng rather than 10 ng) was the Cq for *csp* reduced below 30, the threshold for background noise (Figure 41B). This amplification of the *csp* gene only with excess template suggests *csp* was not expressed in *N. gonorrhoeae*. This corroborates previous studies which failed to detect a gonococcal Csp protein (L. Stewart, personal communication).

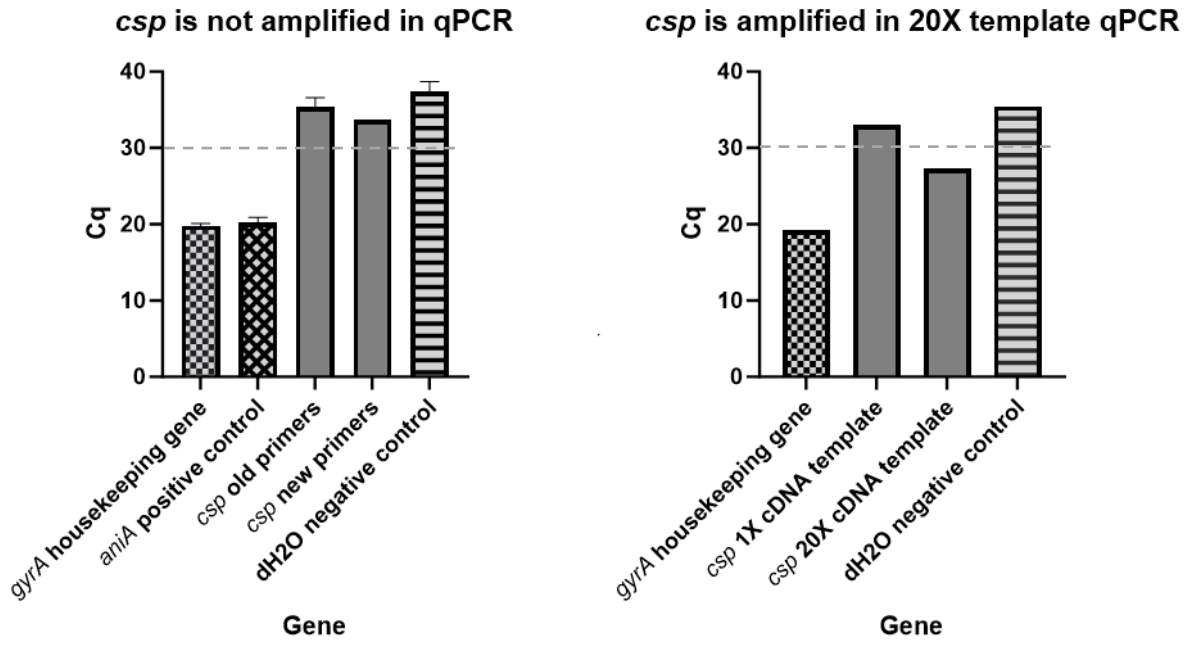


Figure 41: Transcription of gonococcal *csp* gene is highly downregulated.
(**A**) Threshold cycle (Cq) values for *csp* amplification during qPCR with 1x (10 ng) cDNA template per reaction. Mean of n = 6 independent replicates ±SD. (**B**) Threshold cycle (Cq) values for *csp* amplification during qPCR with 1x cDNA template *gyrA*, 1x template *csp* and 20x (excess) template *csp* reactions. N = 1 replicate. Background threshold for no amplification was set at 30 cycles (dashed lines).

Previous literature claims that *N. gonorrhoeae* possesses the periplasmic Csp1 (Dennison et al., 2018), however, the presence of Csp1 in *N. gonorrhoeae* has not been experimentally validated; the *csp* gene (UniProt accession: C1I025) in *N. gonorrhoeae* has only been identified by protein homology searches (Vita et al., 2015). A previous study tested the Cu binding properties of recombinantly overexpressed *N. gonorrhoeae* Csp1 (Alsharif, 2020). However, their study did not assess wild-type expression of the native gene (e.g. qPCR) or protein (e.g. using Western blot).

The finding of *csp* repression in *N. gonorrhoeae* contributes to the growing body of literature which suggests *N. gonorrhoeae* has a minimalist Cu homeostasis pathway (Antoine et al., 2019). This specialization of the Cu homeostasis pathway is hypothesised to be due to the obligate parasitic nature of *N. gonorrhoeae* (Rivera-Millot et al., 2023). An avenue for further enquiry should be to determine if gonococcal *csp* can be upregulated under certain conditions.

13.8 Discussion: Expression of the gonococcal *nos* operon

Since static gonococcal cultures required NO_2^- to grow and *N. gonorrhoeae* is not thought to possess an alternative respiratory pathway such as dissimilatory nitrate reduction to ammonium (DNRA), we propose static cultures were dependent on denitrification throughout 8 hr growth. However, denitrification genes (*aniA*, *accA*, *norB* and *nosZ*) were only upregulated at 8 hr. This suggests that the basal expression level of denitrification genes and/or Cox is sufficient for cultures up to OD_{600} ~ 0.4, after which point additional denitrification is required.

This study found evidence to suggest that the WT gonococcal *nos* operon is fully transcribed into mRNA. Hence, the *nos* operon may be functional on the mRNA level (e.g. to regulate transcription). Furthermore, the *nosZ* gene is upregulated in 8 hr static cultures with NO_2^-. The *nosZ* and *nosL* genes are expressed to a level comparable to that of the NO_2^- reductase, *aniA* (the major induced gonococcal outer membrane protein in anaerobic conditions), its Cu chaperone *accA*, and the $^{\bullet}NO$ reductase, *norB*. It should be noted that this finding of *nosZ* transcription does not imply readthrough of the *nosR* premature stop codon, since the NosR has not been confirmed to be required for *nos* transcription in *N. gonorrhoeae*.

While the denitrification enzymes *aniA*, *nosZ* and *norB* are upregulated over the course of static 8 hr, growth expression of the Cu chaperones *accA* and *nosL* are unchanged. This suggests different genes in the *nos* operon (*nosZ* vs *nosL*) experience different rates of mRNA decay. This also hints at additional roles for the AccA and NosL chaperones, possibly as Cu storage proteins or chaperones to other proteins.

Shaking gonococcal cultures did not require NO_2^- for survival, suggesting shaking cultures are primarily dependent on aerobic respiration. Denitrification genes (*aniA*, *accA*, *norB*, *nosZ* and *nosL)* were upregulated within 3 hr in shaking cultures regardless of NO_2^- supplementation, although we suggest denitrification is occurring within 3 hr cultures with NO_2^-. This finding of induction of denitrification genes in shaking cultures within 1 hr but with an 8 hr lag in induction in static cultures suggests there is another key factor influencing regulation of gonococcal denitrification genes which currently eludes us. An alternative explanation is that the rapid growth of gonococci in shaking cultures depletes most of the dissolved O_2, prompting upregulation of denitrification. The similar expression profiles of 3 hr shaking and 8 hr static FNR-regulated genes suggests a tend towards microaerobic conditions in these cultures.

There is a correlation in denitrification gene function and expression profile. The gonococcal denitrification enzymes *aniA*, *norB* and *nosZ* are upregulated over 8 hr static growth with NO_2^- and in response to NO_2^- at 8 hr and are also upregulated over 3 hr in shaking cultures with NO_2^-. The two chaperones *accA* and *nosL* are not upregulated over 8 hr static growth but are upregulated in response to NO_2^- at 8 hr. These genes are also upregulated less in static cultures than the denitrification enzymes.

14 Chapter 5: Expression of the gonococcal NosZ protein

Finding gene expression does not necessarily mean protein expression (or enzyme activity) is occurring. Hence, this study subsequently investigated expression of the gonococcal NosZ enzyme.

14.1 Sequencing of FA1090 *nosZ* mutant strain *nos* operons

To determine the expression and functionality of gonococcal NosZ, mutant gonococcal strains were generated during this study. A *nosZ* knockout strain was generated to determine if there was any growth defect in microaerobic conditions following loss of *nosZ*. Two strains of StrepII-tagged *nosZ* were also generated: one 5'-end tag (*strepII::nosZ*) and one 3'-end tag (*nosZ::strepII*). Samples from these strains were tested for NosZ protein expression on a Western blot.

To confirm that there were no off-target mutations in our strains, duplicate clones of the FA1090 WT, Δ*nosZ*, *strepII::nosZ* and *nosZ::strepII* strains were sent for whole-genome sequencing by MicrobesNG (University of Birmingham). Nucleotides in these strains which did not match the published FA1090 strain genome assembly ASM684v1 (which was the gonococcal reference genome at the start of this project) were labelled as genetic variants. In these variants, ≥90% of sequencing reads had the variant and the read depth was at least 10x. Across the eight clones, no variants were identified in the *nos* operon promoter region and only two variants were identified in the *nos* operon (Table 32).

The first *nos* variant occurs in *nosZ*; only one clone of each of the StrepII-tagged strains possessed the synonymous SNP this study used to domesticate the random BsaI recognition site occurring in the WT *nosZ*. However, the Golden Gate assembly appears to have succeeded in all *strepII*::*nosZ* and *nosZ*::*strepII* clones, suggesting the random BsaI recognition site occurring in the WT *nosZ* was not inadvertently cleaved during the Golden Gate reaction. Furthermore, the A and B clones of both *strepII*::*nosZ* and *nosZ*::*strepII* had identical phenotypes in static microaerobic cultures (Figure 42A), suggesting that these mutations are silent.

The second *nos* variant occurs in *nosR*; both *ΔnosZ* clones had a random synonymous CT**G** → CT**T** mutation. The potential source of random mutations such as this could be the frequent thawing and passaging of stocks in the laboratory. Our FA1090 WT *nos* operon sequence was found to be identical to the published FA0190 ASM16353v1 genome sequence.

Table 32: Mutations in mutant gonococcal strains *nos* operon

FA1090 nt	FA1090 Ref Allele Bottom Strand	WT A	WT B	*ΔnosZ* A	*ΔnosZ* B	*strepII::nosZ* A	*strepII::nosZ* B	*nosZ::strepII* A	*nosZ::strepII* B	Mutation type	Notes	Gene
789,110	A			.	.		G	G		Synonymous	NosZ BsaI domesticated site	*nosZ*
787,278	C			A	A					Synonymous	Random CTG → CTT synonymous mutation	*nosR*

A '.' in the sample column means that variant calling could not be performed at that location for that sample. This is usually due to the depth of reads being below the coverage threshold.

14.2 *ΔnosZ* and StrepII-tagged mutant growth rate indistinguishable from WT

Having determined the growth phenotype of static and shaking WT gonococcal cultures, we hypothesised that there would be an altered phenotype in the *ΔnosZ* strain. This is seen in *Paracoccus denitrificans ΔnosZ* strains, which do not reduce N_2O and have a reduced anoxic growth rate (Bergaust et al., 2012). If a functional NosZ enzyme is present in *N. gonorrhoeae* and contributes to anaerobic respiration, the *ΔnosZ* mutant would be expected to have inhibited growth in static cultures. To the best of our knowledge, this study represents the first time a *nosZ* knockout strain has been generated in *N. gonorrhoeae*.

Static 1 mL cultures of WT, *ΔnosZ*, *strepII*::*nosZ* and *nosZ*::*strepII* triplicate clones were grown in sealed cuvettes and OD_{600} values measured over 10 hr to compare growth (Figure 42A). While there was slight variation amongst the growth of different clones (Figure 42A), there was no significant differences between endpoint 10 hr OD_{600} of different strains (Figure 42B). This suggests that none

of the nucleotide variants between strains detected by whole-genome sequencing significantly altered static growth.

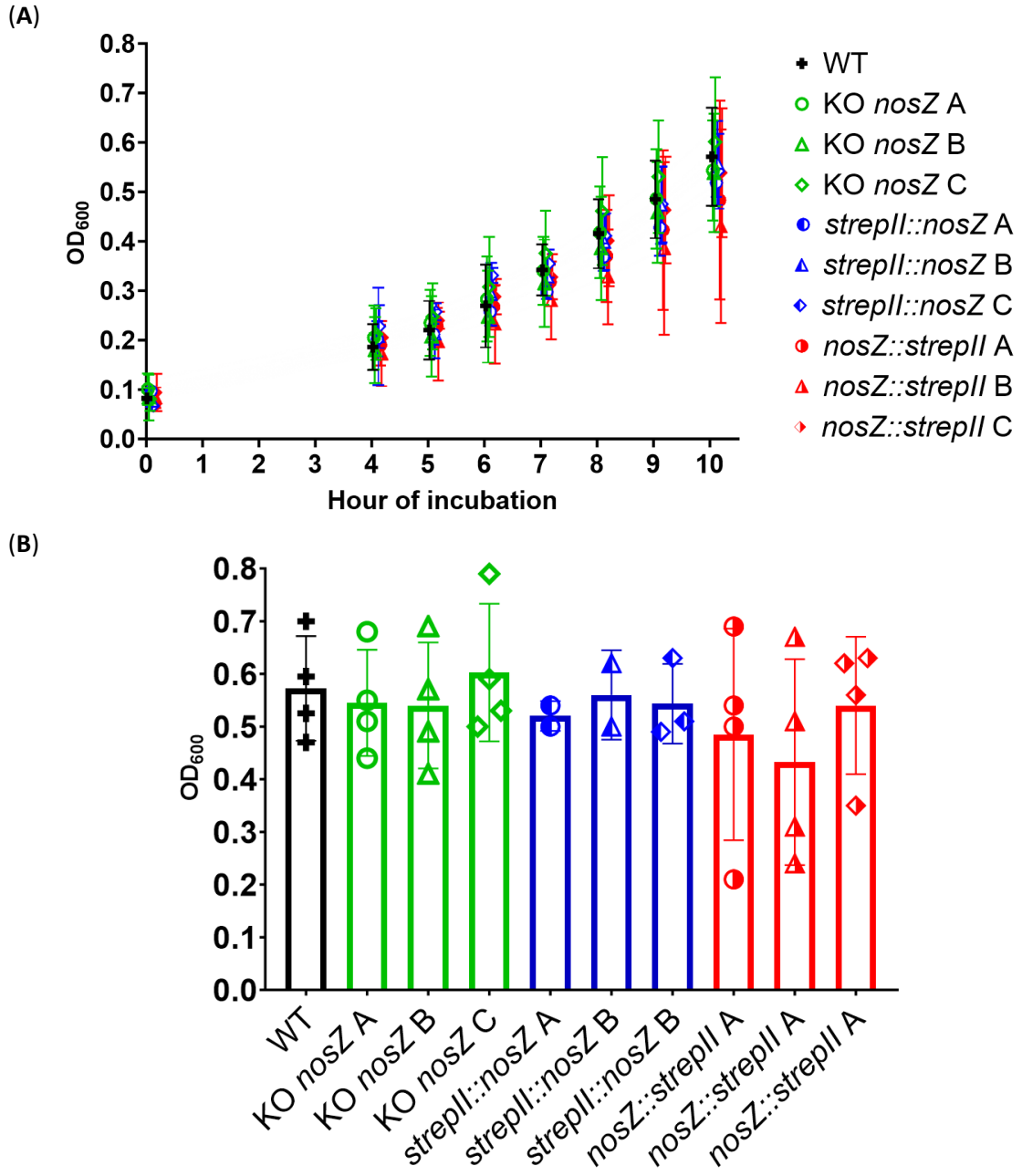


Figure 42: Static growth curves of WT, *ΔnosZ* and StrepII-tagged *N. gonorrhoeae* strains.
(**A**) Static cultures grown over 10 hr (1 mL cultures in sealed cuvettes). Data points represent the mean of n = 3. $NaNO_2^-$ (2 mM, red closed circles) was supplemented at 0, 5, 7, 8, 8.5, 9 and 9.5 hr. Error bars represent standard deviation. Data corrected for no cells control and nudged for clarity. (**B**) 10 hr endpoint OD_{600} data from triplicate clones. Data found to be statistically indistinguishable by one-way ANOVA.

14.3 Loss of *nosZ* does not affect expression of *nosL*, *aniA*, *norB* or *copA*

Having established the transcription of the WT gonococcal *nos* operon and the upregulation of *nosZ* in response to NO_2^-, we hypothesised that the loss of *nosZ* may alter gene expression in the *ΔnosZ::specR* strain. While the substrate of NosZ, N_2O, is an inert diffusible gas, and there are no known N_2O sensors in bacteria, we hypothesised that N_2O may still act indirectly to influence denitrification. It is known that a *Paracoccus denitrificans ΔnosZ* strain accumulates N_2O over time, which leads to a subsequent accumulation of •NO, likely via inhibition of NorB (Bergaust et al., 2012). •NO is known to induce expression of *aniA* and *norB* in gonococci via NsrR (Overton et al., 2006). Hence, we expected to see an upregulation of *aniA* and *norB* in a *ΔnosZ* strain.

To confirm that *nosZ* expression was abolished in the *ΔnosZ::specR* strain and study if loss of *nosZ* expression affected other gonococcal genes, we performed qPCR on cDNA synthesised from 8 hr static *ΔnosZ* and WT culture RNA extracts. Primers against *nosZ(N)*, *aniA*, *norB* and *copA* were included in the qPCR experiment.

These qPCR experiments revealed that expression of *nosZ* was successfully abolished in the *ΔnosZ* strain. The cDNA from *ΔnosZ* samples was either never detected (nd; Cq = 0.00) or only amplified above the background threshold after 30 cycles (Figure 43).

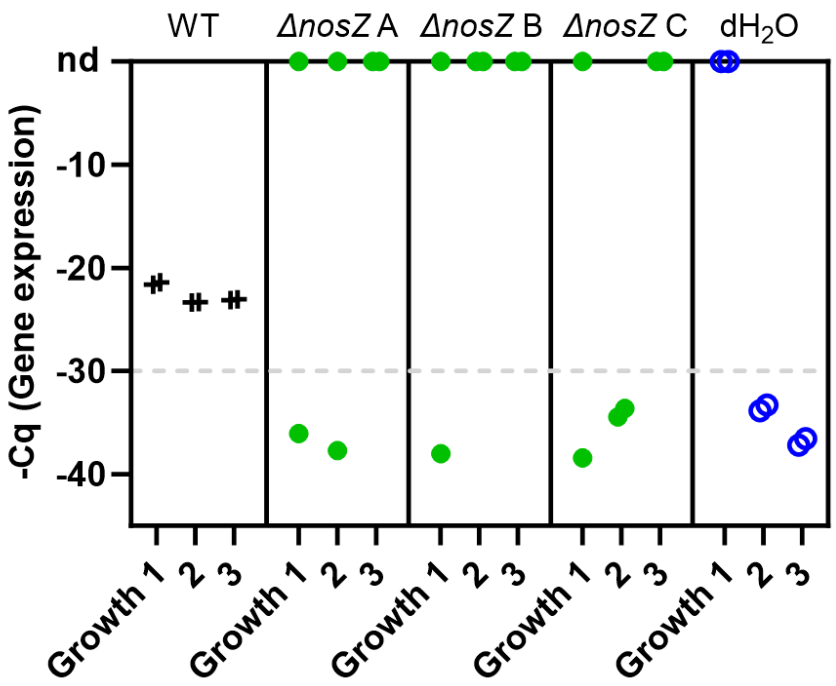


Figure 43: Expression of *nosZ* is abolished in *ΔnosZ::specR N. gonorrhoeae* strains.
N = 3 independent growths with two technical replicates. Threshold for a genuine amplification of sample template cDNA is within 30 cycles, denoted by dashed line. ND, PCR product not detected. Primers used were against *nosZ* 5'-end.

Contrary to our hypothesis, expression of *aniA* and *norB* was unchanged between the WT strain and three *ΔnosZ* clones (Figure 44). This adds support to our null hypothesis that WT gonococcal NosZ enzyme is non-functional. Expression of *nosL* and *copA* were also unchanged in the *ΔnosZ* strain, suggesting that the loss of *nosZ* and introduction of *specR* did not cause pleiotropic effects on downstream *nos* genes or other areas of the genome.

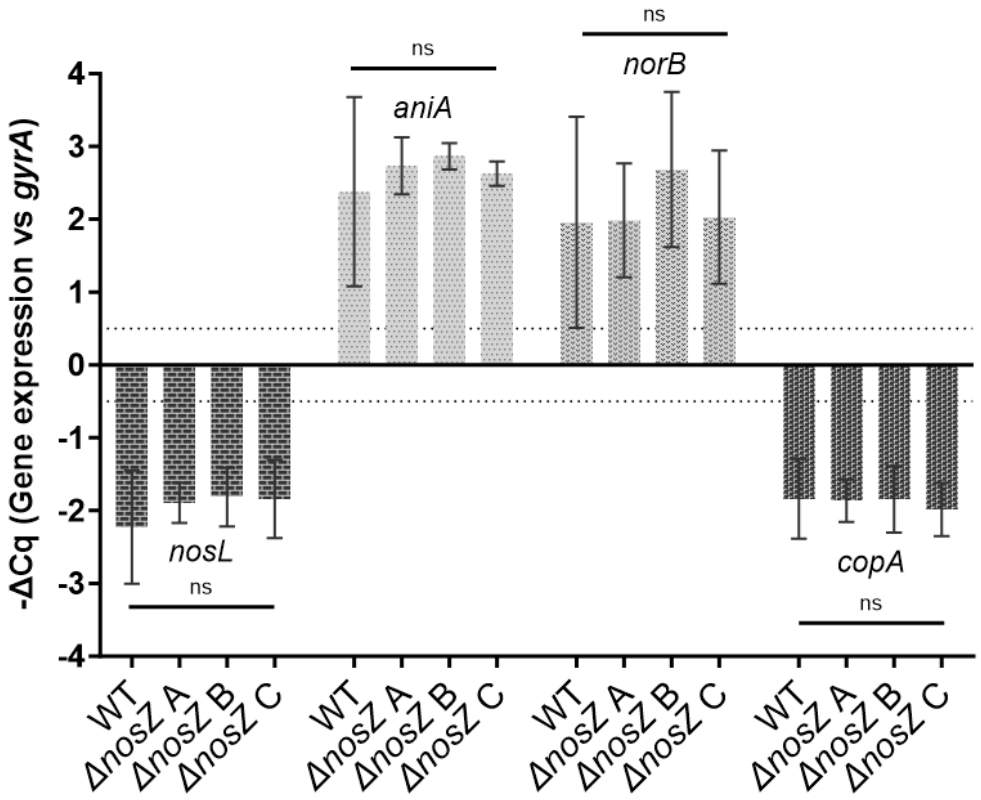


Figure 44: Effect of *nosZ* knockout on gene expression.
Static cultures grown over 8 hr in microaerobic conditions (30 mL cultures in 50 mL Falcon tubes). $NaNO_2$ (2 mM) supplemented at 0, 5 and 7 hr. Sample size of n = 3. Dashed horizontal lines represent the sensitivity limit of the assay ($log_2FC = \pm 0.5$). Data analysed using 1-way ANOVA.

14.4 A promoterless *specR* gene is translated from the *nosZ* start codon

The *ΔnosZ* strain generated in this study contained a spectinomycin resistance gene *specR* which was promoterless, instead beginning at the WT *nosZ* start codon. During generation of the *ΔnosZ* strain, to select for successful transformants, colonies were streaked onto 100 µg/mL Spc GC agar plates and left to incubate. The fact that the *ΔnosZ* strain grew on spectinomycin plates suggests that the SpecR protein was translated successfully from the WT *nosZ* start codon (Figure 45).

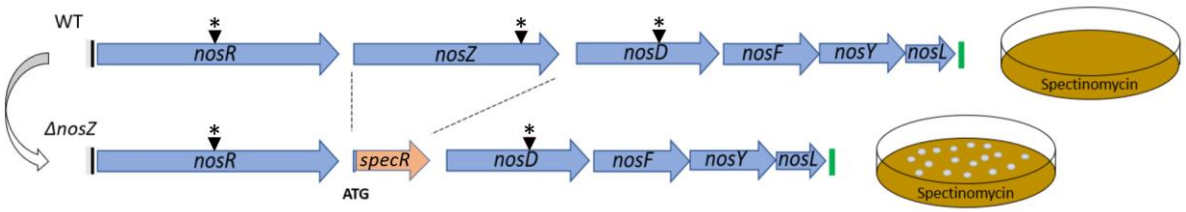


Figure 45: Selection of successful *ΔnosZ::specR* transformants on spectinomycin-containing GC agar plates.

14.5 Western Blots suggest the gonococcal NosZ protein is absent

A key aim of this study was to determine if a NosZ protein was present in microaerobic gonococcal cultures. It is currently unknown how oxygen concentration affects gonococcal N_2O reduction, but research has been previously carried out on this topic in other species. A previous study found the presence of a full-length 72 kDa *Bradyrhizobium diazoefficiens* NosZ protein in microaerobic cultures but an absence of protein in aerobic cultures via Western blot (Fernández et al., 2019). The authors did not elaborate on this total absence of NosZ from aerobic cultures, and other papers generally still detect limited N_2O reduction in aerobic cultures. Another study found different forms of *Paracoccus pantotrophus* NosZ upon exposure to different O_2 concentrations - aerobic NosZ preparations often resulting in an inactive form of the enzyme which has lost the Cu_Z site (Rasmussen et al., 2002). Another study tested the N_2O reduction of several bacterial species, finding differences in activity with changing O_2 concentration. N_2O reduction of *Stutzerimonas stutzeri* was not inhibited by O_2 (i.e., O_2 tolerant), *Paracoccus denitrificans* reduced N_2O in the presence of O_2 but slower than in the absence of O_2 (i.e., O_2 sensitive), and N_2O reduction of *Pseudomonas aeruginosa* did not occur when O_2 was present (i.e., O_2 intolerant) (Wang et al., 2023).

We predicted the possible lengths of different gonococcal NosZ protein isoforms on a Western blot. If readthrough of the gonococcal *nos* premature stop codons were to occur and the NosZ protein translated as one polypeptide, we would expect the protein to be exported to the periplasm via the N-terminal TAT signal peptide (Lee et al., 2006a) which would then be cleaved to leave a mature 68 kDa NosZ protein (Figure 46A). This full-length NosZ protein tagged with StrepII would produce a 69 kDa band on a Western blot (Figure 46A B).

Should stop codon suppression not occur, a prematurely truncated 54.5 kDa NosZ protein would be detected in only N-terminal tagged StrepII strains (Figure 46B). Subsequent reinitiation of translation at the next downstream TTG Leu would mean only 3 amino acids (0.3 kDa) are lost, while reinitiation at the next downstream ATG Met would mean 30 amino acids (3.3 kDa) are lost (Figure 46B).

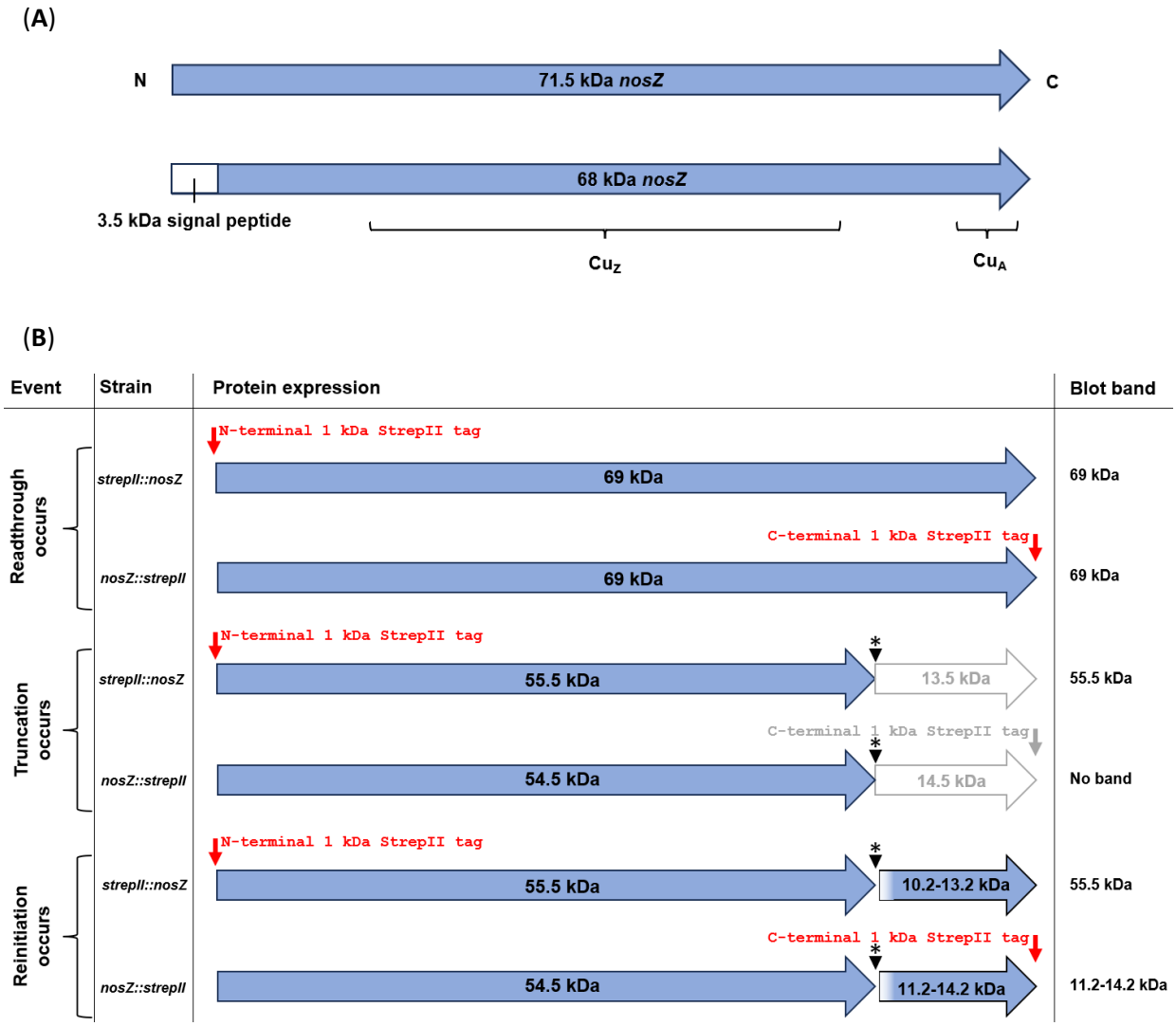
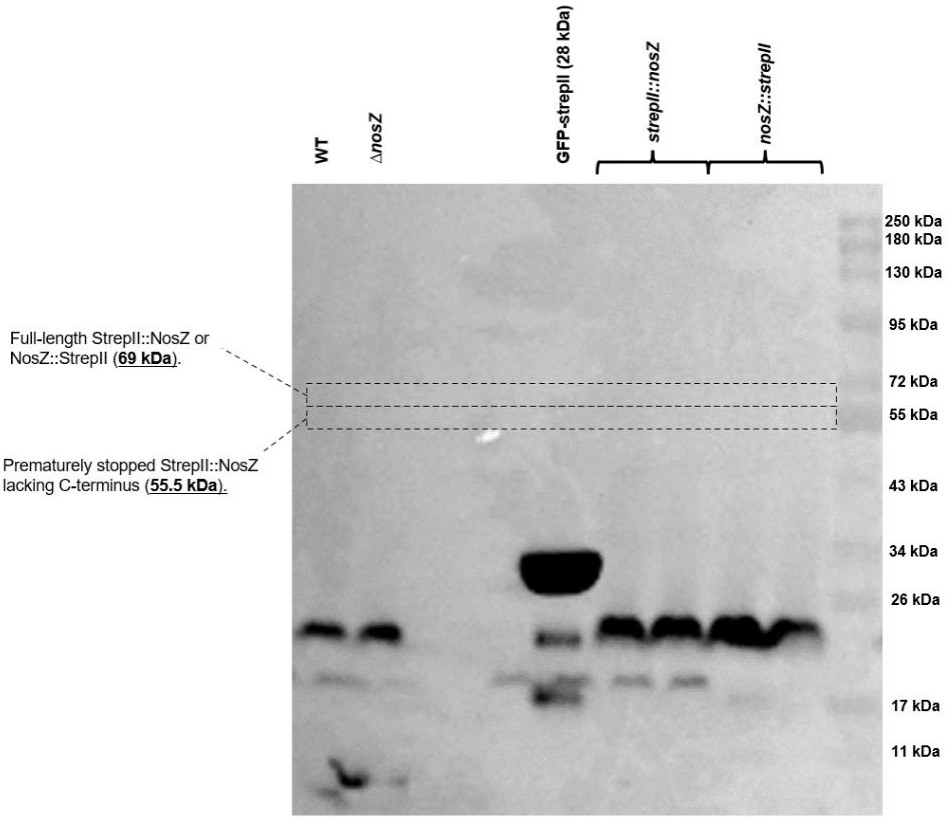


Figure 46: Expected band sizes of possible NosZ protein variants.
(**A**) Diagram of the WT NosZ protein. (**B**) Diagram of StrepII-tagged NosZ proteins. Protein sizes predicted using Peptide Synthetics peptide Mass Calculator (https://www.peptidesynthetics.co.uk/tools/).

The *N. gonorrhoeae* strains *strepII*::*nosZ* and *nosZ*::*strepII* (containing a StrepII tag at the *nosZ* N- or C-terminus) were grown in static and shaking cultures, then cell lysates probed by immunoblotting using a Streptavidin-HRP conjugate. Timepoints for harvesting cultures for Western blotting were based on qPCR experiments; static cultures were harvested at OD_{600} 0.4-0.5 and shaking cultures were harvested at OD_{600} 0.8-1.0. These cell densities were selected to match the cell densities of 8 hr static and 4 hr shaking cultures (Figure 26), which were timepoints when *nosZ* was upregulated in qPCR experiments (Figure 30 and Figure 37). However, this study detected no bands corresponding to any predicted NosZ protein of any length in both static and shaking cultures (Figure 47).

(**A**)



(**B**)

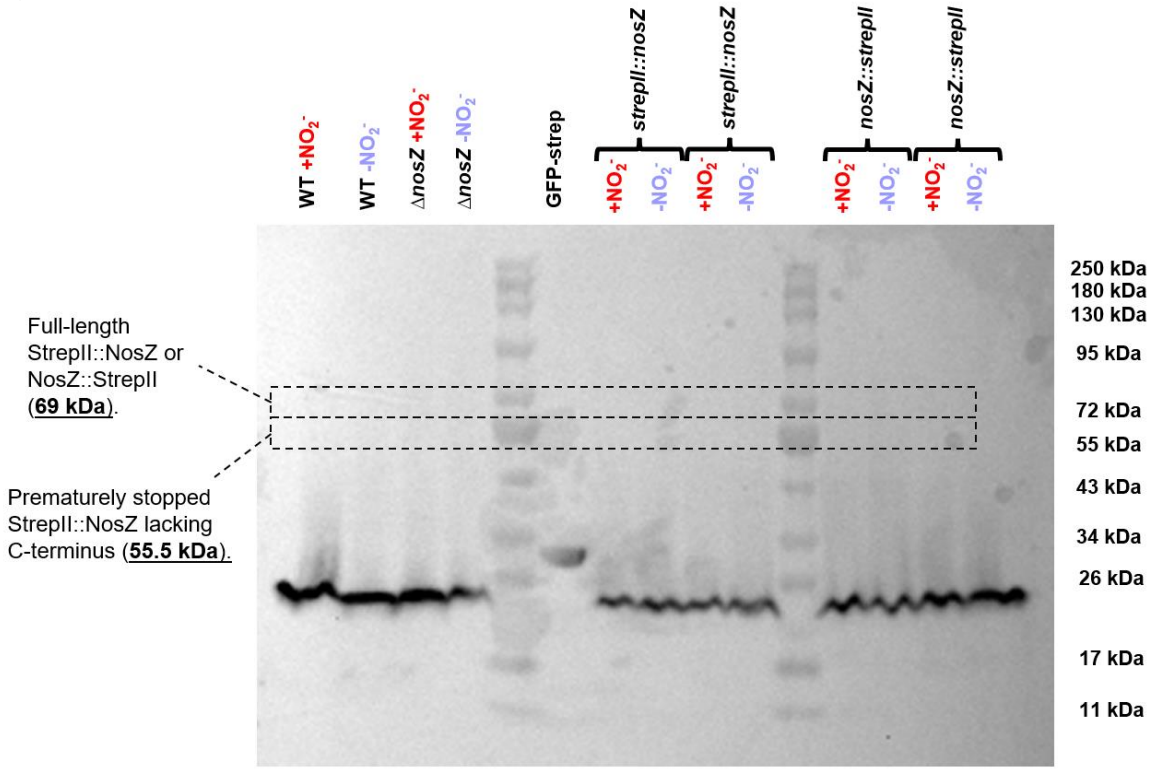


Figure 47: Representative Western blots of static and shaking *N. gonorrhoeae* culture lysates against StrepII. (**A**) Static 10 hr growth cultures supplemented with 2 mM NO_2^- (final OD_{600} ~0.44). (**B**) Shaking 4-6 hr growth cultures with and without NO_2^- (final OD_{600} ~1.0). Repeated lanes on a gel represent different clones. Region of expected NosZ protein bands shown in dashed boxes.

The only band seen on Western blot gels of *N. gonorrhoeae* was ~20 kDa and appeared in the WT and *ΔnosZ* strains which lack StrepII, as well as both N- and C-terminal StrepII-tag strains and in both static and shaking cultures. Since there is no exact StrepII nucleotide or protein sequence (TGGAGCCACCCGCAGTTCGAAAAA/WSHPQFEK) in gonococcal genome or proteome, we suggest that this ~20 kDa band represents an unidentified gonococcal protein containing a StrepII-like epitope which was non-specifically detected. The finding of this small StrepII-like gonococcal protein suggests there was sufficient gonococcal lysate added to the gel for protein detection.

The finding of non-specific binding by the Streptavidin-HRP conjugate was surprising, given the reported highly specific interaction between the StrepII tag and streptavidin (Johar and Talbert, 2017; Schmidt and Skerra, 2007; Terpe, 2003). To the best of our knowledge, no-one has used a StrepII tag before against raw whole-cell *N. gonorrhoeae* lysates. Previous studies have instead overexpressed the protein in *E. coli* (Jain et al., 2012), which would exclude the ~20 kDa strepII-like protein found in this study.

Based on our detection of *nosZ* mRNA in WT gonococci (Figure 28B), we propose it is more likely that the nascent NosZ protein was degraded before a stable polypeptide produced, rather than the *nosZ* mRNA being degraded. We suggest this rapid degradation of the NosZ polypeptide is the result of the premature stop codon in the *nosZ* gene. Based on the translation of SpecR from the WT *nosZ* start codon (Figure 45), we suggest that WT *nosZ* may be translated, at least as far as the premature stop codon. However, it should be noted that this study did not directly measure translation of the gonococcal NosZ protein. Hence, it is possible that WT gonococcal *nosZ* is never translated, which would explain the total absence of any length of N-terminal NosZ in Western blots.

We think it unlikely that the addition of a StrepII tag in both the N- and C-terminal locations of the mutants created in this study would cause degradation of the polypeptide. There is no evidence in the literature to suggest that the StrepII tag promotes protein instability or degradation. The StrepII tag is described as being short, biologically inert, proteolytically stable and does not interfere with

membrane translocation or protein folding (Schmidt and Skerra, 2007). Studies finding proteolysis of C-terminally fused StrepII tag see the same with other tags and attribute it to inherent susceptibility of a protein C-terminus to proteolysis (Kittur et al., 2015). There is evidence to suggest that the StrepII tag actually promotes protein stability; StrepII-tagged *Candida antarctica* lipase B has increased catalytic efficiency (Johar and Talbert, 2017) and addition of an N-terminal StrepII tag increases the stability of *Arabidopsis* SAUR proteins, with StrepII-tagged fusion proteins accumulating to a much higher level than untagged proteins (Spartz et al., 2012).

Analysis of the FA1090 NosZ protein sequence in the AlphaFold 3D sequence predictor (Jumper et al., 2021; Varadi et al., 2022) revealed the location of our N-terminal StrepII tag was in an unstructured region of the folded protein, therefore unlikely to inhibit protein folding (Figure 48A). It should be noted that the AlphaFold model of *N. gonorrhoeae* NosZ is truncated at the premature stop codon. When searching AlphaFold for the C-terminus of FA1090 NosZ, the primary result is the C terminus of the *Neisseria lactamica* 020-06 NosZ, which has high homology to the FA1090 amino acid sequence. This *N. lactamica* AlphaFold structure suggests our fused C-terminal StrepII-tag would also be on the exterior of the homodimer and therefore unlikely to inhibit protein folding (Figure 48B).

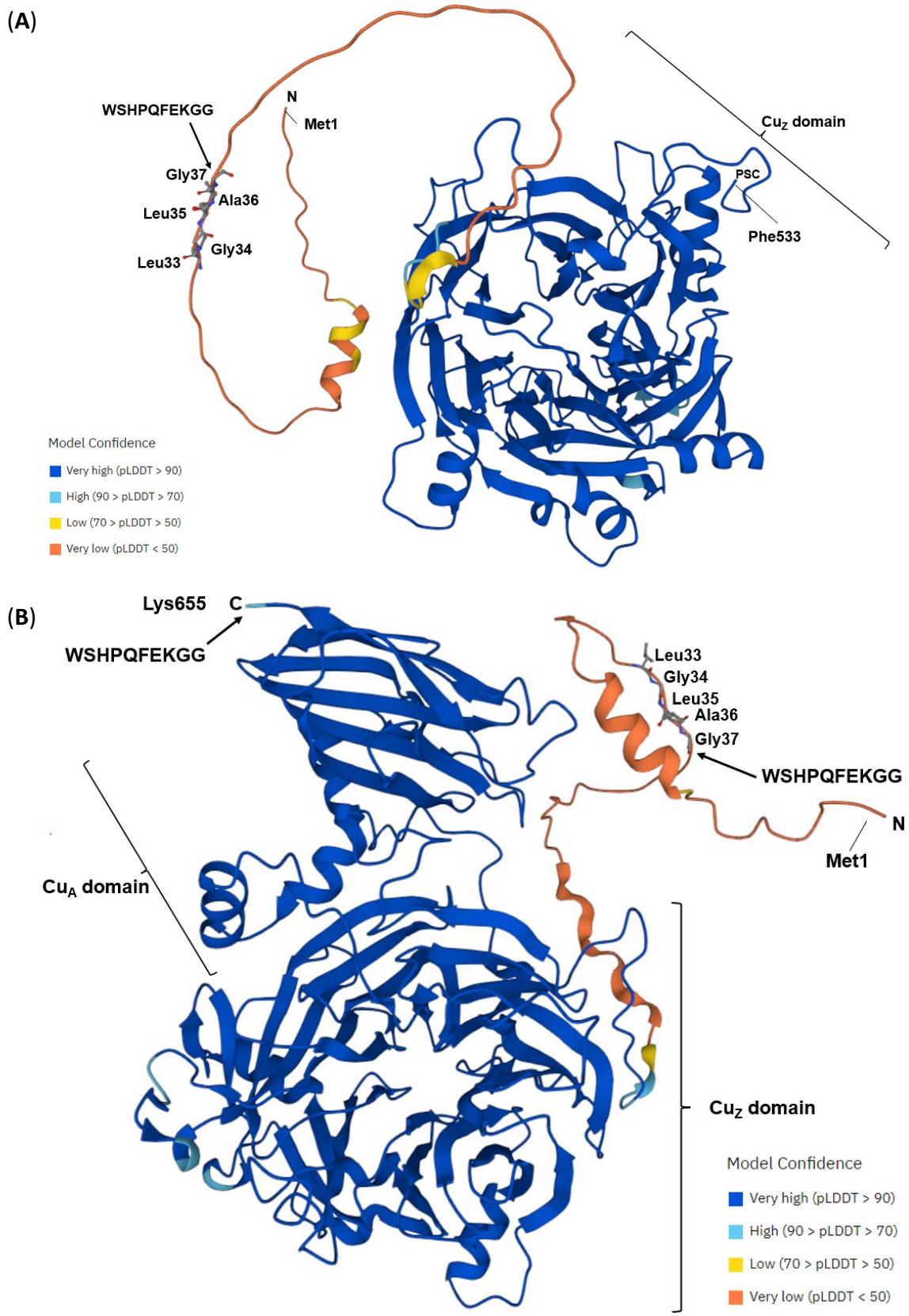


Figure 48: Predicted NosZ 3D structure reveals regions of StrepII tag insertion.
(**A**) *Neisseria gonorrhoeae* FA1090 NosZ AlphaFold (UniProt A0A0H4IT26, AF-A0A0H4IT26-F1) with N-terminal StrepII tag. (**B**) *Neisseria lactamica* 020-06 NosZ AlphaFold (UniProt E4ZES4, AF-E4ZES4-F1) with N- and C-terminal StrepII tags. Lys655 is the final residue before the final stop codon. Arrows indicate point of StrepII tag insertion.

14.6 Supplemented Cu does not stabilize gonococcal NosZ

Evidence from initial Western blots against static and shaking culture lysates suggest that a NosZ protein is not present in *N. gonorrhoeae*. We hypothesised that this may be due to a lack of translation of NosZ, or a lack of metalation of the NosZ leading to unfolding and degradation. *Pseudomonas stutzeri* NosZ requires Cu transfer from the membrane-anchored Cu chaperone NosL to NosD and then from NosD to the NosZ Cu_Z site (Müller et al., 2022). While some metalloproteins such as AniA are stable in their *apo* (metal-free) form (Jen et al., 2015), others will not correctly fold in the absence of metal, leading to increased instability and rapid degradation (Palm-Espling et al., 2012; Tottey et al., 2008).

In *Paracoccus denitrificans* cultures lacking Cu, N_2O reduction is inhibited (Felgate et al., 2012; Sullivan et al., 2013; Woolfenden et al., 2013). Furthermore, *Paracoccus denitrificans* cultures supplemented with low (0.5 µM) and high (13 µM) Cu produce different ratios of full-length and truncated NosZ protein (Felgate et al., 2012). In low Cu cultures, the full-length 65 kDa Cu_Z-Cu_A band is only very faintly detectable while the truncated 50 kDa Cu_Z band is still detected. In high Cu cultures, the 65 kDa Cu_Z-Cu_A NosZ is predominant while the 50 kDa Cu_Z band is also detected. This led the authors to conclude that even in high Cu, NosZ is vulnerable to normal proteolysis of the C-terminal Cu_A region. It is proposed that denitrifying bacteria thrive in a NO_3^--rich, Cu-deficient environment by partially shutting down this last step of denitrification (Felgate et al., 2012).

Hence, we thought to test the effect of Cu concentration on the level of gonococcal NosZ protein. We hypothesised that we did not detect NosZ in initial Western blots because the cultures which provided the pellets were starved of Cu, leading to degradation of the unfolded NosZ. We repeated Western blots against extracts from gonococcal culture supplemented with 0, 1 and 10 µM Cu.

It was thought that the background Cu content of our GCBL was ~1 µM (Djoko, personal communication). Hence, we supplemented cultures with 1 and 10 µM Cu (estimated two-fold and

ten-fold increases, respectively). At 10 µM, Cu was thought to be in excess since it causes a slight inhibition of growth (Figure 27).

No bands corresponding to any predicted NosZ protein of any length were detected on a Western blot even with added Cu (Figure 49). This suggests that either the NosZ protein was never translated or it was degraded due to truncation by the time of culture harvesting. Had a truncated N-terminal Cu_z-only NosZ polypeptide been translated in our cultures, it would likely have been metalated (unless the premature stop codon in gonococcal NosD prevents Cu insertion into NosZ).

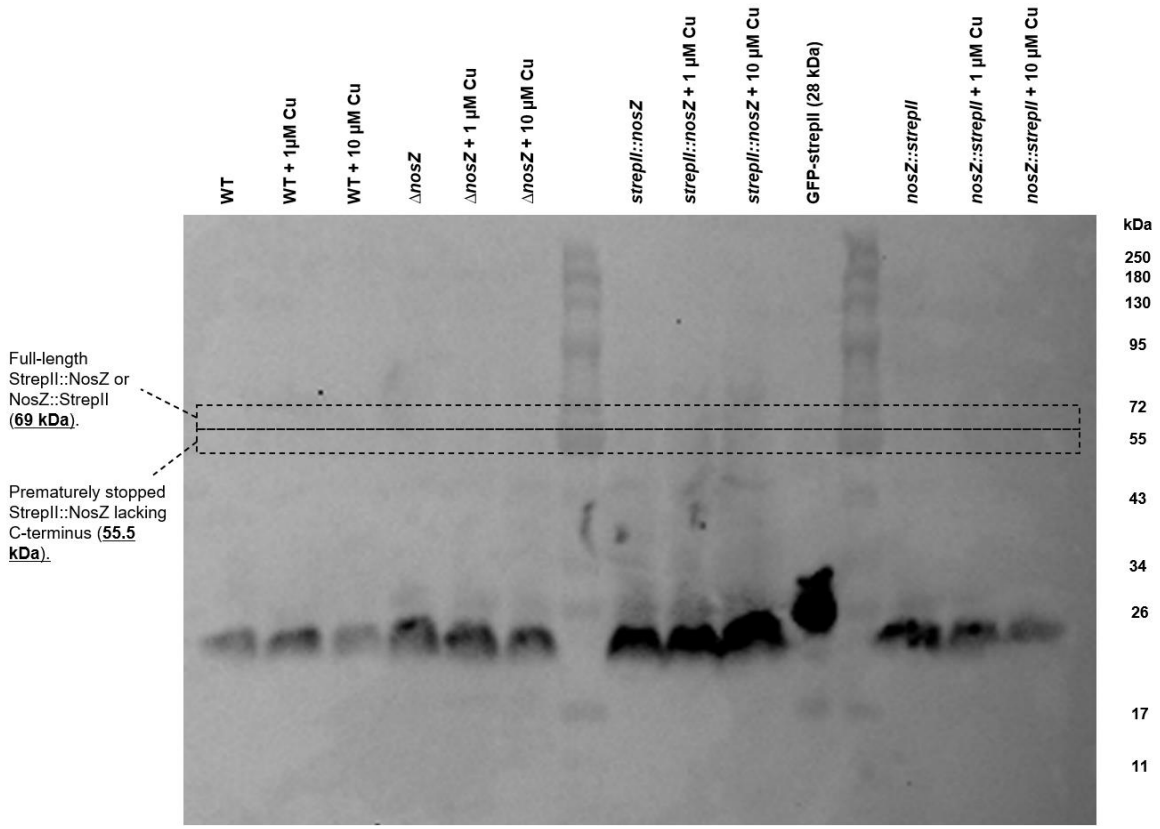


Figure 49: Representative Western blot of Cu-supplemented static *N. gonorrhoeae* culture lysates against StrepII.
Cultures were supplemented with 2 mM NO_2^- at 0, 5, 7, 8 & 9 hr and grown for 8-10 hr until OD_{600} ~0.44. Region of expected NosZ protein bands shown in dashed boxes.

14.6.1 Modelling the effect of NosZ truncation on protein unfolding

The C-terminal truncation of the NosZ protein at the FA1090 premature stop codon will remove the Cu_A site domain. The Cu_A site domain occurs at the NosZ monomer-monomer interface (Figure 50A). Hence, we hypothesise that loss of this domain may weaken binding of the two NosZ monomers and prevent formation of a functional homodimer, leading to protein degradation. It is known that the

Lon protease is present in *Neisseria gonorrhoeae*, and is thought to recognise exposed hydrophobic protein residues (Lee and Suzuki, 2008). The *N. lactamica* NosZ AlphaFold entry AF-E4ZES4-F1 was used to model the FA1090 full-length (Figure 50B) and nonsense-truncated NosZ (Figure 50C). However, this model did not reveal any obvious hydrophobic regions after truncation which may be attacked by proteases.

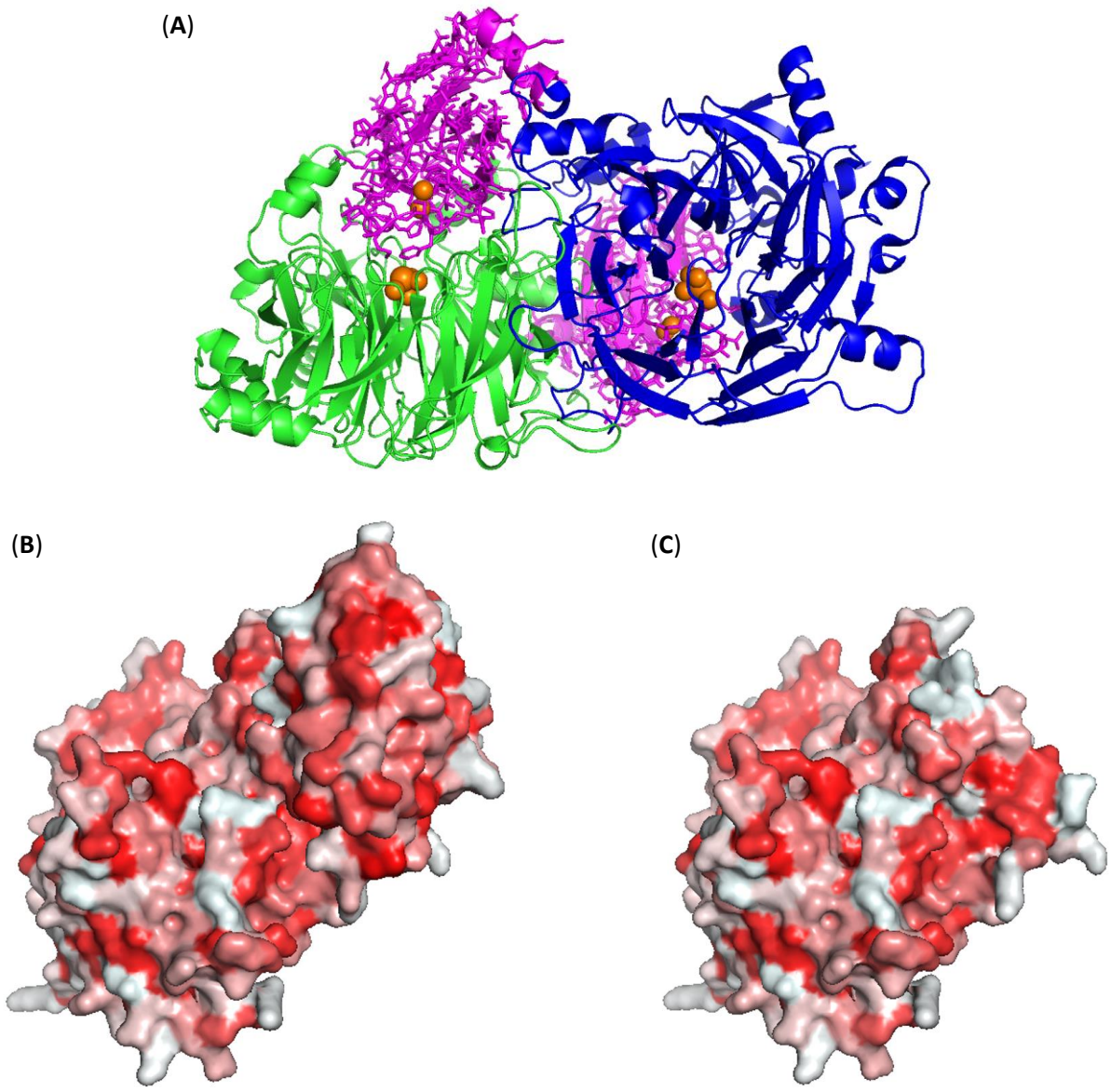


Figure 50: The NosZ C-terminal Cu_A site downstream from the FA1090 premature stop codon occurs at the monomer-monomer interface.
(**A**) *Achromobacter cycloclastes* NosZ homodimer (PDB 2IWF) shown as a ribbon structure (monomers in green and blue ribbons), Cu_A region in pink sticks. (**B**) *N. lactamica* (UniProt E4ZES4) NosZ monomer AlphaFold model. (**C**) *N. lactamica* (UniProt E4ZES4) NosZ monomer AlphaFold model with C-terminal truncation. Darker residues more hydrophobic, coloured according to hydrophobicity scale (Eisenberg et al., 1984).

The absence of a gonococcal NosZ contrasts with the findings of Felgate et al. (2012), which suggested *Paracoccus denitrificans* produces a stable truncated N-terminal NosZ that retains 40% catalytic activity of the full-length protein in low Cu. The *P. denitrificans* PD122 *nosZ* gene (NCBI assembly NC_008687.1, locus tag PDEN_RS21020) is not prematurely stopped, and Felgate et al (2012) do not propose a mechanism for how the *P. denitrificans* NosZ is truncated (e.g. premature translation termination or proteolysis of the C-terminus).

14.6.2 Modelling the secondary structure of *nosZ* mRNA

The lack of a detected gonococcal NosZ on Western blots suggests that either the protein is rapidly degraded (possibly due to nonsense truncation), or there is a lack of initial NosZ translation. If SpecR translation is not used to infer NosZ translation, there is the possibility that the WT *nosZ* mRNA is never translated. This may be due to the presence of mRNA structures such as hairpins in the *nosZ* mRNA; such structures have been suggested to repress translation in plants (Wang and Wessler, 2001) and *E. coli* (Espah Borujeni et al., 2017). Hairpins introduced early in the *E. coli mRFP1* mRNA effectively inhibit translation (Espah Borujeni et al., 2017). GC-rich mRNA sequences form more stable hairpins and reduce translation rate due to the higher energy barrier required to unfold them. For instance, the "13-GG" hairpin sequence **ATG**AAACAGAAC*GGCGCG*AUUAUC*CGCGCC*AAA inserted at position +13 in the *mRFP1* gene was found to significantly reduce translation (Espah Borujeni et al., 2017).

When an RNA molecule folds back on itself to form a three-dimensional structure, the rules of complementary base pairing determine the folding process, resulting in a pattern of Watson-Crick and GU pairings (helices) and intervening stretches of less regularly ordered nucleotides (loops) (Lorenz et al., 2011). As such, RNA secondary structure can be modelled as a graph wherein nodes represent nucleotides and edges between consecutive nodes represent the phosphate backbone.

RNAfold computes the minimum free energy (MFE) of an RNA sequence and predicts an optimal secondary structure (Zuker and Stiegler, 1981). RNAfold then generates a graph known as a

"mountain plot" which plots nucleotide position on the x-axis against the number of base pairs enclosing each nucleotide position on the y-axis (Vienna RNA Web Servers Help, accessed 2024). In a mountain plot, structured regions of RNA (i.e. helices) will form slopes while unstructured regions (loops) form flat areas. Hairpin loop structures will thus form flat-topped mountains while internal loops, bulge loops and multibranch loop (junctions) structures form low valley plains surrounding the mountains. A RNA molecule with more hairpin loops will have more peaks on a mountain plot, greater entropy (less structure), fewer helices and lower translation rate. For a more thorough description of mountain plots, see the diagram in Appendix 13. For a more thorough description of RNA secondary structures, see the diagram in Appendix 14. For an example of the effect of hairpin insertion on mountain plots, see Appendix 15.

To test our hypothesis that the gonococcal *nosZ* mRNA sequence hinders ribosome translation, RNA secondary structure was predicted using RNAfold (Lorenz et al., 2011). The RNAfold program could not compute the folding of the entire *nos* operon, so the region from the *nosR* stop codon to the *nosD* start codon was analysed instead. This region was compared to the mRNA sequences of *specR* (the spectinomycin resistance gene thought to be translated in KO mutants), *N. lactamica nosZ* (a close homolog to *N. gonorrhoeae*) and *P. denitrificans nosZ* (known to be translated *in vivo*).

RNAfold showed that the shorter *specR* mRNA has fewer hairpin loops than gonococcal *nosZ* (Figure 51A-B), while gonococcal *nosZ* mRNA is predicted to have multiple hairpin loops, the first occurring just over 100 nucleotides downstream of the start codon (Figure 51B).

The closest nucleotide homolog to the gonococcal *nosZ* is from *N. lactamica* 020-06. The *N. lactamica nosZ* sequence has 96% identity to *N. gonorrhoeae nosZ*. However, there are noticeable differences in the mountain plots (Figure 51B-C). While the *N. lactamica* sequence has an initial hairpin region at a similar position (~200-300 nt) to the first hairpin region in *N. gonorrhoeae*, there are fewer subsequent hairpin loops and more slope-stem regions (Figure 51C), suggesting more translation will occur before the hairpin-loop at ~1,500 bp is reached.

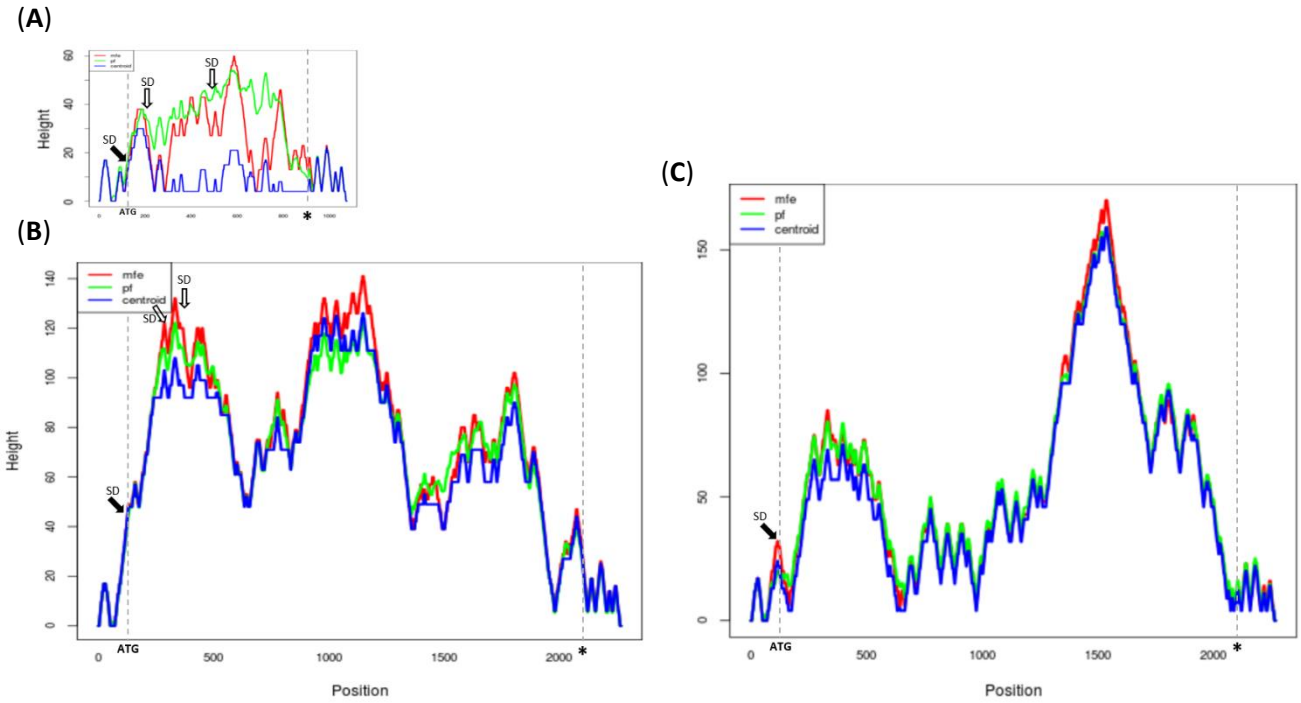


Figure 51: Predicting secondary structures in *nosZ* and *specR* mRNA.
(**A**) Mountain plot predicting secondary structure in *specR* mRNA. (**B**) Mountain plot predicting secondary structure in *N. gonorrhoeae* FA1090 *nosZ* mRNA. (**C**) Mountain plot predicting secondary structure in *N. latamica* 020-06 *nosZ* mRNA. Flanking regions from *nosR* stop codon to *nosZ* start codon (128 nt) and from *nosZ* stop codon to *nosD* start codon (172 bp) were included. Arrows indicate SD sequences. Graphs scaled to equalize height and position axes.

Paracoccus denitrificans PD1222 *nosZ* is known to be translated into a functional enzyme (Felgate et al., 2012) and thus provides a positive control for translation. Hence, we compared the *nosZ* mRNA secondary structures of the gonococcal FA1090 *nosZ* mRNA to the *Paracoccus denitrificans* PD1222 *nosZ* (NCBI gene ID 75503602) (Figure 52). While the gonococcal sequence has a large hairpin region soon after the start of the mRNA (less amenable to ribosome translation), the *Paracoccus denitrificans* sequence has a more pronounced slope-stem region at the start of the mRNA (suggesting a helix region which may be more amenable to initial ribosome translation).

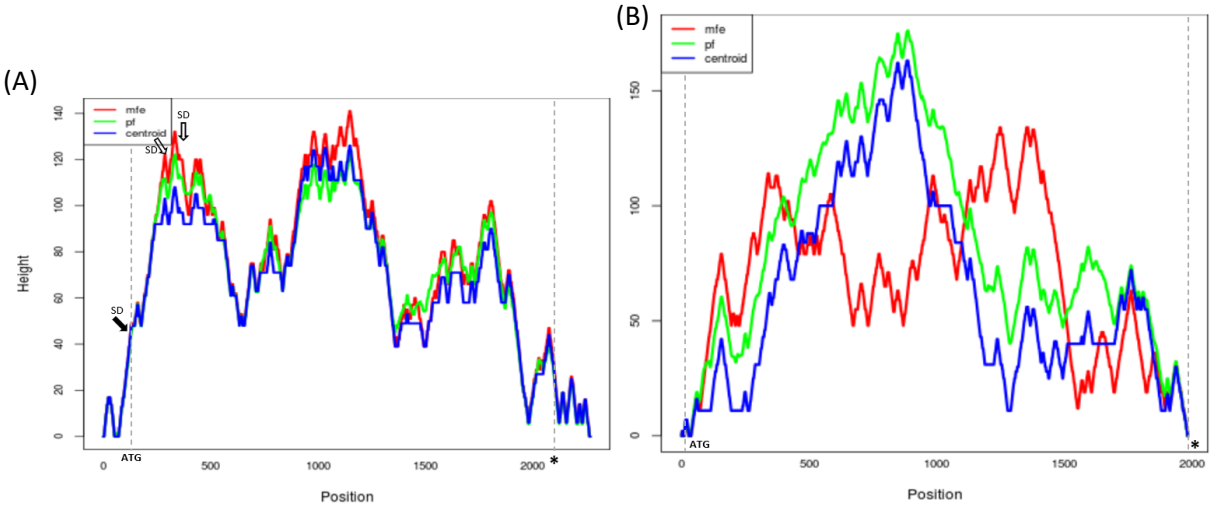


Figure 52: Predicting secondary structures in *N. gonorrhoeae* and *P. denitrificans nosZ* mRNA.
(**A**) Mountain plot predicting secondary structure in F1090 *nosZ* mRNA. (**B**) Mountain plot predicting secondary structure in PD1222 *nosZ* mRNA. Flanking regions from *nosR* stop codon to *nosZ* start codon and from *nosZ* stop codon to *nosD* start codon included. Hairpin loops are represented by high plateaus, stems by slopes, and internal loops and junctions by low plateaus.

Based on the idea proposed previously that hairpins closer to the ribosome binding site are more deleterious to translation (Espah Borujeni et al., 2017), we suggest the difference in the translation of the *nosZ* gene between the two species may be due to the occurrence of a large hairpin region early in the gonococcal mRNA.

We suggest that an alternative hypothesis to the rapid degradation of the gonococcal NosZ protein is that the gonococcal *nosZ* mRNA sequence may intrinsically hinder translation by the ribosome due to the presence of hairpins.

The *E. coli* SD sequence is thought to be an AG-rich region (GGAGG) in the 7–12 nt region upstream of an ATG start codon (Wen et al., 2020) while the gonococcal SD sequence is thought to be AAGGA (Callaghan et al., 2021), or AAGG (Goytia et al., 2015). SD sequences upstream of FA1090 denitrification genes are summarized in Table 33. Internally, the *specR* mRNA contains 3 AAGG sites (the first 87 bp downstream of the start codon) and 2 AAGGA sites (the first 362 bp downstream of the start codon) (Figure 51) while the *nosZ* mRNA contains 15 AAGG sites (the first 168 bp downstream of the start codon) and 3 AAGGA sites (the first 244 bp downstream of the start codon)

(Figure 51). The first of each of these internal AAGG and AAGGA SD sequences occur in the first *nosZ* hairpin region, suggesting ribosomes binding downstream of the *nosZ* start codon may be inhibited.

Table 33: Shine-Dalgarno sequences in *N. gonorrhoeae* FA1090

Gene	Sequence	Nucleotides upstream of gene start codon
aniA	AAGGA	8–12
accA	AAGGA	9-13
norB	AAGGA	10-14
nosR	AAGGA	8-12
nosZ	AAGGA	8–12
nosD	None	
nosF	None	
nosY	AAGG	8-12
nosL	None	

14.7 Validation of *nosZ* mutant strain genomes by whole-genome sequencing

To confirm mutants were generated successfully, and that there were no off-target mutations in our *nosZ* mutants, duplicate clones of *N. gonorrhoeae* WT strain and Δ*nosZ*, *strepII::nosZ* and *nosZ::strepII* mutant strains were sent for whole-genome sequencing (see Section 11.9.3).

Of the 269 genetic variants with identified by whole-genome sequencing wherein at least 90% of reads had the variant and the read depth was at least 10x, only a total of twenty-six were found to be discriminatory, i.e. there was nucleotide variation between the duplicate clones of the four strains (Table 34).

It should be noted that despite these nucleotide variants, all clones from each mutant strain had identical growth phenotypes (Figure 42A). As such, these variants are likely to be non-detrimental. Regardless, we give here a summary of each variant.

Table 34: Twenty-six discriminatory variants across eight sequenced gonococcal WT and mutant *nosZ* strains

FA1090 nt	Amino acid Substitution	FA1090 Ref Allele Bottom Strand	WT A	WT B	*ΔnosZ* A	*ΔnosZ* B	*strepII::nosZ* A	*strepII::nosZ* B	*nosZ::strepII* A	*nosZ::strepII* B	Mutation type	Notes
2,097,806	F98L	T		C	.	C	.				Missense*	Missense mutation in two clones.
1,693,242		CG	C	C		.	C	C	C	C	Deletion*	Deletion in six clones. Frameshift. A change in WT.
1,216,022		C		CCAAG CAAG	.						Intergenic insertion	Insertion in one clone. Frameshift.
1,064,763	E323DG VE	T		TTCCAC GCCG	TTCCAC GCCG	TTCCAC GCCG	TTCCAC GCCG	TTCCAC GCCG	TTCCAC GCCG		Insertion*	Insertion in six clones. No frameshift.
919,455	*60L?	T	TA	TA		TA		TA	TA	TA	Missense*	Insertion in six clones; stop codon lost. Frameshift. A change in WT.
868,832	D57G	T	C	C	C	C		C	C	C	Missense*	SNP in seven clones. A change in WT.
826,285		A	AC	AC	AC	AC		AC	AC	AC	Intergenic insertion	Insertion in seven clones. Frameshift. A change in WT.
789,110	G393	A			.	.		G	G		Synonymous	BsaI domesticated site in *nosZ*. Only occurs in two clones.
787,278	L221	C			A	A					Synonymous	Random CTG → CTT synonymous mutation in *ΔnosZ* clones *nosR*.
688,542	I4V	T		C	C				C	C	Missense*	SNP in four clones.
688,539	K3E	T		C	C					C	Missense*	SNP in three clones.
688,532		AC			A						Intergenic deletion	Deletion in one clone. Frameshift.
688,495		G			A						Intergenic SNP	SNP in one clone.
688,494		T			C						Intergenic SNP	SNP in one clone.
688,470		G			A						Intergenic SNP	SNP in one clone.
196,728	AA83A	GGCA	G			G				G	Deletion*	Deletion in three clones. No frameshift.
117,620		C			T	.	.				Missense*	SNP in one clone.
117,619		C			A	.	.			.	Missense*	SNP in one clone.
117,617		C			T	.	.				Missense*	SNP in one clone.
116,622		A				G					Intergenic SNP	SNP in one clone.
116,564		C			T	T		T		T	Intergenic SNP	SNP in four clones.
116,449		G						A			Intergenic SNP	SNP in one clone.
116,435		T			C	.		.		C	Intergenic SNP	SNP in two clones
116,423		A				.		G			Intergenic SNP	SNP in one clone.
116,185	V32	A	.		.	.	G	.	.	.	Synonymous	SNP in one clone.
116,156	Q23K	G	.	T	.	.	T	.		.	Missense*	SNP in two clones.

Variant key: Intergenic variants in grey text, *nos* operon region in grey highlight. * indicates variant of concern with possibly deleterious effect to protein-coding gene. A '.' in the sample column means that variant calling could not be performed at that location for that sample - this is usually due to the depth of reads being below the coverage threshold.

At the FA1090 nucleotide position 1,216,022, an 8-base insertion of the sequence CAAGCAAG into clone WT B occurs in an intergenic region without any annotated regulatory boxes. This position is

410 bp upstream of the adjacent NGO_05045, a hypothetical protein of unknown function, and 127 bp downstream of the adjacent NGO_0963 (arginyl-tRNA synthetase). As such, this variant appears unlikely to affect regulation of either gene. Aside from this large insertion, other variants occurring in intergenic regions were not investigated further.

Of the twenty-six variants, one was a planned mutation domesticating a BsaI recognition site occurring in *nosZ* and eleven variants occurred in intergenic regions, leaving only fourteen variants in genes. Of these fourteen variants, two were synonymous, leaving only twelve variants of concern with possibly deleterious effects to protein-coding genes.

A brief description of these remaining twelve variants of concern is given below (using annotations from the gonococcal NCBI GenBank record AE004969.1).

At the FA1090 nucleotide position 2,097,806, a missense 5' **T**TC 3' (Phe) → **C**TC (Leu) substitution in clones WT B and Δ*nosZ* B occurs in the 98th residue of NGO_00280, a hypothetical protein of unknown function with an alternate TTG start codon. This gene has homology to a gene in *N. meningitidis* only. The substitution Phe → Leu is a very highly conserved substitution between two hydrophobic residues, so would be expected to minimally effect protein function.

At the FA1090 nucleotide position 1,693,242, a single base G deletion in clones WT A, WT B, *strepII::nosZ* A, *strepII::nosZ* B, *nosZ::strepII* A and *nosZ::strepII* B occurs in the 39th residue of NGO_02520, a disrupted phage-associated protein annotated as a pseudogene. This gene has homology to a gene in *N. meningitidis, N. lactamica* and *Caudoviricetes* phage only. The deletion causes a frameshift for the C-terminal 56% of the protein, which would be expected to majorly disrupt protein function. However, an alternative hypothesis given the majority of the eight clones containing this variant is that our starting WT strain contained just the C base, and the Δ*nosZ* strains developed a CG insertion as was recorded in the ASM684v1 FA1090 genome. We predict this variant will have minimal effect on the fitness of these clones.

After the FA1090 nucleotide position 1,064,763, a 9 base insertion 5' CGGCGTGGA 3' in clones WT B, Δ*nosZ* A, Δ*nosZ* B and *strepII::nosZ* A, *strepII::nosZ* B and *nosZ::strepII* A causes the substitution of the 323rd residue of NGO_1149 GA**A** (Glu) → GA**C** (Asp) (a very highly conserved substitution) and the insertion of the following sequence: **GGC** (Gly) **GTG** (Val) **GAA** (Glu). NGO_1149 is a 386-residue O-succinylhomoserine (OSH) sulfhydrolase, which catalyses the formation of L-homocysteine (a non-proteinogenic α-amino acid) from O-succinyl-L-homoserine (OSHS) and hydrogen sulphide (Foglino et al., 1995). This is part of an alternative pathway of homocysteine biosynthesis, a step in methionine biosynthesis. *Neisseria* species have two different enzymes for methionine biosynthesis, a CGS that functions through transsulfuration and an OAH or an OSH sulfhydrylase that acts through direct sulfhydration (Hacham et al., 2003). As such, *N. gonorrhoeae* may be able to resist the loss of this enzyme by upregulating an alternative pathway. A more likely hypothesis given the number of clones containing this variant is that our starting WT strain contained the 5' CGGCGTGGA 3' sequence, and the WT A and *nosZ::strepII* B lost the 5' CGGCGTGGA 3' sequence as was recorded in the ASM684v1 FA1090 genome. Hence, we cannot predict an effect of this variant on the fitness of these clones.

At the FA1090 nucleotide position 919,455, a single base insertion cause a frameshift which removes the stop codon of NGO_06740 5' T**A**A 3' (*) → T**T**A (Leu) in clones WT A, WT B, Δ*nosZ* B, *strepII::nosZ* B, *nosZ::strepII* A and *nosZ::strepII* A. NGO_06740 is a hypothetical protein with a lipid A phosphoethanolamine transferase domain. Lipid A phosphoethanolamine transferase (EptA) is implicated in antibiotic resistance (Samantha and Vrielink, 2020). This insertion then makes the intergenic region between NGO_06740 and the following gene, NGO_06735 (annotated sulfatase with a GTG alternate start codon) in-frame. This suggests a EptA-sulfatase fusion protein may be translated, with a 12-residue SNFVFHVIALPF linker region. BLASTp analysis of NGO_06740, this linker region and NGO_06735 reveals homology to *N. meningitidis* EptA, suggesting the entire fusion protein is actually an EptA homolog which has been prematurely stopped in *N. gonorrhoeae*. An alternative hypothesis given the majority of the eight clones containing this variant is that our

starting WT strain contained the TA sequence, and that the Δ*nosZ* A and *strepII::nosZ* A clones lost the A base as was recorded in the ASM684v1 FA1090 genome. Hence, we cannot predict an effect of this variant on the fitness of these clones.

At the FA1090 nucleotide position 868,832, a missense 5' G**A**C 3' (Asp) → G**G**C (Gly) substitution in clones WT A, WT B, Δ*nosZ* A, Δ*nosZ* B, *strepII::nosZ* B, *nosZ::strepII* A and *nosZ::strepII* A occurs in the 57th residue of the 162-residue NGO_06995, a hypothetical protein with a TTG alternate start codon. BLASTp analysis reveals homology of NGO_06995 to the *N. meningitidis* IS630 family transposase. The Asp → Gly substitution is a non-conservative substitution from a charged to polar residue, so would be expected to impact protein function. An alternative hypothesis given the majority of the eight clones containing this variant is that our starting WT strain contained the C base, and that the *strepII::nosZ* A clones contained a missense 5' G**G**C 3' → G**A**C G57D substitution as was recorded in the ASM684v1 FA1090 reference genome. We cannot predict an effect of this variant on the fitness of these clones without a better understanding of the structure and function of this hypothetical protein.

At the FA1090 nucleotide position 688,542, a missense 5' **A**TT 3' (Ile) → **G**TT (Val) substitution in clones WT B, Δ*nosZ* A, *nosZ::strepII* A and *nosZ::strepII* B occurs in the 4th residue of the 47-residue NGO_1497, a hypothetical protein of unknown function with a TTG alternate start codon. BLASTp analysis reveals homology of NGO_1497 to a *N. meningitidis* hypothetical protein. The substitution Ile → Val is a very highly conserved substitution between two hydrophobic residues, so would be expected to minimally effect protein function. We predict this variant will have minimal effect on the fitness of these clones. This variant occurs in four out of eight clones, including one of the WT clones.

At the FA1090 nucleotide position 688,539, a 5' **A**AG 3' (Lys) → **G**AG (Glu) substitution in clones WT B, Δ*nosZ* A and *nosZ::strepII* B occurs in the 3rd residue of NGO_1497 (discussed above). The substitution Lys → Glu is a conservative substitution between two charged residues. We predict this

variant will have minimal effect on the fitness of these clones. This variant occurs in three out of eight clones, including one of the WT clones.

At the FA1090 nucleotide position 196,728, a 3 bp deletion 5' GC**TGC**C 3' (Ala Ala) → GCC (Ala) in clones WT A and Δ*nosZ* B removes the 84th Ala residue of the 187-residue NGO_1981, a putative adhesin component. BLASTp analysis reveals homology of NGO_1981 protein to a *N. meningitidis* putative adhesin component. We cannot predict an effect of this variant on the fitness of these clones without a better understanding of the structure and function of this hypothetical protein.

At the FA1090 nucleotide positions 117,617, 117,619 and 117,620, three SNPs in clone Δ*nosZ* A cause the variant 5' C**G**G**GG**A 3' (Arg Gly) → C**T**G**AT**A (Leu Ile) to occur in the 22-23rd residues of NGO_11095, a 66-residue disrupted hypothetical *Neisseria* protein of unknown function annotated as a pseudogene. These two substitutions are both non-conservative, so would be expected to impact protein function. BLASTn analysis reveals partial homology of NGO_11095 to four other intergenic regions in the gonococcal genome, suggesting the gene has been duplicated. We predict this variant will have minimal effect on the fitness of these clones.

At the FA1090 nucleotide position 116,156, a missense 5' **C**AA 3' (Gln) → **A**AA (Ala) non-conservative substitution in clones WT B and *strepII::nosZ* A occurs in the 23rd residue of NGO_11105, a 104-residue *pilS* cassette encoding Type IVb pilin. This non-conservative substitution may affect virulence in some clones. However, it is thought that the *pilS* cassette is duplicated at least three times in the *N. gonorrhoeae* genome (366 bp L-pilin; disrupted NGO_10990, 744 bp Large *pilS* cassette NGO_11155, 312 bp *pilS* cassette NGO_11105). Hence, one of these duplicates may compensate for a loss of the NGO_11105 *pilS*. Furthermore, examination of the sequence of the *N. gonorrhoeae pilS* crystal structure 2HI2 reveals high variation (71% identity and 22% positivity) between the C30 strain (Craig et al., 2006) and our ASM684v1 FA1090 reference genome, making evaluation of the impact of this variant challenging. We cannot predict an effect of this variant on the fitness of these clones without a better understanding of the structure and function of this protein.

Aside from the two variants in *nosR* and *nosZ*, no variant occurred in the *nos* operon or any other gene of interest to this study. We do not believe there were any genetic variants in our mutant strains which would have deleterious effects on the growth and/or gene expression of *N. gonorrhoeae*. A full list of all 269 discriminatory and non-discriminatory definite variants can be found in Appendix 11.

14.8 Discussion: Expression of the gonococcal NosZ protein

Growth of a gonococcal *nosZ* KO strain in static cultures was indistinguishable to the WT, suggesting loss of *nosZ* does not impair microaerobic growth. Loss of *nosZ* did not affect expression of *nosL*, *aniA*, *norB* or *copA*. Furthermore, we could not detect a N- or C-terminally StrepII-tagged NosZ protein in Western blots of static 8 hr or shaking 3 hr gonococcal cell cultures. We hypothesised a lack of Cu may cause NosZ misfolding and degradation. However, supplemented Cu concentrations of 1 µM (nutritional) and 10 µM (excess) did not stabilise the NosZ polypeptide such that it could be detected via Western blot. These findings suggest that WT gonococcal *nosZ* is a *napto*-potogene, i.e. a true pseudogene found to be without function (Brosius and Gould, 1992).

Previous work has shown that cellular availability of Cu is likely to be the most limited of all biologically relevant metals (Osman et al., 2019). Commensal Neisseria may have better access to Cu than the pathogens *N. gonorrhoeae* and *N. meningitidis* because the host reduces metal availability to them during initial infection. However, pathogenic bacteria are still thought to be exposed to the Cu overload via the host innate immune system phagosome ATP7A Cu transporter (Kim et al., 2012; White et al., 2009). While pathogenic *Neisseria* have no OprC porin homolog for Cu transport, they may acquire Cu via the ZnuD (TdfJ) transporter (Hecel et al., 2019; Maurakis et al., 2019) (FA1090 NGO_1205). As such, we cannot hypothesise whether *N. gonorrhoeae* evolved reductively to inactivate a Cu-rich enzyme such as NosZ in order to conserve Cu (e.g. for AniA or Cox).

However, we found a promoterless *specR* gene is translated from the gonococcal *nosZ* start codon. This is consistent with our finding of gonococcal *nos* operon transcription and suggests the WT *nos*

mRNA transcript must contain all the elements necessary for recognition by the ribosome to translate *nosZ*. This implies the nascent NosZ polypeptide is likely synthesized, but is then rapidly degraded by proteases, probably due to translation termination at the premature *nosZ* stop codon. Such a wasteful process would be expected to be selected against, yet the *nos* operon is highly conserved across gonococcal isolates (see Section .

15 Chapter 6: Discussion

The findings of this study suggest that *N. gonorrhoeae* has evolved multiple premature nonsense substitutions in the *nos* operon which lead to an absence of NosZ protein. Since there is no compensatory enzyme which can utilize N_2O, we may hypothesise that either 1) host provision minimises the need for complete denitrification so gonococci have evolved to lose NosZ (and so reduce proteome size), or 2) the *nos* premature stop codons confer some unknown benefit.

The gonococcal *nosZ* gene appears similar to *ggh*. The gonococcal *ggh* gene (a homolog of the meningococcal γ-glutamyl transpeptidase *ggt* gene) was the first bacterial pseudogene identified to be transcriptionally active but phenotypically silent (Takahashi and Watanabe, 2005). Takahashi and Watanabe (2005) found that, compared to the meningococcal *ggt* gene, the gonococcal *ggh* gene had various polymorphisms, including a 6-base insertion, a nonsense TAA mutation, a 7-base deletion, and a 46-base insertion. Hence, the gonococcal *nos* operon does not seem to be as disrupted as *ggh*. Similar to *nosZ*, the *ggh* gene is highly conserved amongst gonococcal isolates, is not thought to have arisen via duplication, and a *ggh* knockout strain exhibits no change in phenotype. Interestingly, a non-functional truncated Ggh protein is found in a minority of gonococcal isolates (Takahashi and Watanabe, 2005).

The gonococcal *napto*-potogene *porA* likewise is highly conserved across gonococcal isolates (Unemo et al., 2005). This finding of high levels of conservation in gonococcal pseudogenes corroborates research in other species which indicates pseudogenes are often evolutionarily conserved and transcriptionally active (Balakirev and Ayala, 2004; Pink et al., 2011). As such, we hesitate to use the high conservation of the *nos* operon across gonococcal isolates as an indicator of *nos* functionality.

Many transcribed pseudogenes have been found in *Mycobacterium leprae*; up to 43% of all pseudogenes in the genome (Williams et al., 2009). Factors causing pseudogene transcription are suggested to include: 1) co-orientation of the pseudogene with transcribed ORFs, 2) the dearth of

stem-loop transcriptional terminators between transcribed ORFs and downstream pseudogenes, and 3) retention of pseudogene promoters (Williams et al., 2009). The gonococcal *nosZ* gene appears to retain all the elements necessary for transcription, so finding it is transcribed is not surprising.

This study found gonococcal *nos* operon mRNA but no NosZ protein, suggesting either a lack of *nosZ* mRNA translation or total degradation of the NosZ polypeptide within 8 hr. In general, inhibition of transcript translation in pseudogenes is found to be associated with: 1) the lack of a strong SD site, 2) the lack of traditional translational start codons, 3) the presence of multiple in-frame stop codons, and 4) low functionality of putative protein products (Williams et al., 2009). However, the *nosZ* gene is downstream of a SD sequence, has a traditional ATG start codon, and only one in-frame premature stop codon. This leads us to consider the possible effect of premature truncation on the NosZ protein.

Based on the position of the NosZ C-terminal Cu_A site, adjacent to the N-terminal Cu_Z site on the opposite monomer, we hypothesise that loss of the NosZ C-terminus downstream of the premature stop codon will decrease stability of the NosZ homodimer, leading to dissociation of the two monomers and protein unfolding. It is known that metalloproteins are subject to faster degradation in low metal concentrations, suggesting apo-metalloproteins are less stable than holo-metalloproteins (Cain and Holt, 1979; González et al., 2016). Hence, unfolding of the NosZ protein likely causes the nascent polypeptide to be degraded rapidly *in vivo*. Dissociation of Cu from the NosZ N-terminus may then release Cu into the cytoplasm which will be instantly bound to other buffering components (rather than Cu exporter expression be induced). Alternatively, the NosZ polypeptide may be degraded before Cu insertion and assembly of the Cu sites.

15.1 The economics of denitrification: respiratory benefit vs metabolic cost

Despite previous suggestions that *N. gonorrhoeae* is undergoing reductive evolution (de Korne-Elenbaas et al., 2022), the inactivation of gonococcal NosZ is unexpected for two reasons: 1) *N. gonorrhoeae* inhabits a microaerobic niche, hence the loss of a part of denitrification may be

expected to confer a fitness cost, and 2) NorB is known to function in gonococci, meaning the substrate for NosZ, N_2O, is readily available in the periplasm. Considering the fact that *N. gonorrhoeae* does not possess a NO_3^- reductase, the question of why an organism which occupies a microaerobic niche could evolve to then lose a third of its existing denitrification pathway becomes apparent. Based on our finding that the gonococcal *nos* operon does not generate a stable NosZ protein, we discuss potential mechanisms by which NosZ may have been lost.

15.1.1 Concentrations of O_2 and NO_x

Considering hypothetical denitrifiers in a microaerobic environment, a species possessing the entire denitrification pathway from *nar* to *nosZ* could be expected to have an advantage over partial denitrifiers because more ATP would be generated per initial molecule of NOx. However, genomic surveys (Graf et al., 2014; Hester et al., 2019; Jones et al., 2008) and growth studies (Roco et al., 2017; I. H. Zhang et al., 2021) have revealed that many bacterial denitrifiers only possess part of the pathway - suggesting there is independent loss, gain, and horizontal transfer of these genes between species (I. H. Zhang et al., 2021). This corroborates the finding that the genomic organisation of denitrification operons and genes relative to each other is highly varied between different species (Jones et al., 2008; Zumft, 1997; Zumft and Körner, 1997). Interestingly, it has been shown that bacterial and archaeal genomes isolated from animal ecosystems contain mostly partial denitrifiers compared to other ecosystems (e.g. soil, marine, brackish), and this correlates with smaller genome sizes (Hester et al., 2019). We propose this reflects selective pressure to minimise metabolic costs of enzyme synthesis in a parasitic niche, driving loss of certain denitrification genes from the genome. An example of such denitrification loss within a species is the “bet hedging” seen in *Paracoccus denitrificans*, whereby all cells within a single culture synthesise Nos while only a minority of cells synthesise Nir (Lycus et al., 2018). This phenotypic diversity within a species is ascribed to stochastic initiation of *nir* transcription and is thought to have a selective advantage upon a sudden drop in O_2 level (Lycus et al., 2018; Morawska et al., 2022). Zhang et al (2021) suggest that the accumulation of denitrification intermediates within a culture indicates a spatial separation of denitrification steps

into separate cells, or temporal separation in transcription of denitrification genes or activity of certain enzymes. Zhang et al (2021) produced separate *Δnar* and *Δnir Pseudomonas aeruginosa* strains and compared them in co-culture to a culture of the WT parent. In the co-culture of *Δnar* and *Δnir* complementary partial denitrifiers (specialists), competition for NO_X^- was found to be reduced compared to a population of WT complete denitrifiers (generalists) (I. H. Zhang et al., 2021). Hence, the authors conclude that high NO_X^- environments may select for specialists, while low NO_X^- environments may select for generalists. Fitness benefits such as these may theoretically select for a gonococcus which only expresses NosZ stochastically or under certain conditions. Hence, we hypothesise that gonococcal NosZ is most likely to be expressed in a combination of low O_2 and low NO_2^-, where the benefit of increased AniA and NorB expression would be minimal. It is currently unknown whether the concentrations of O_2 and NO_2^- in the urogenital tract are low enough to meet this criteria (Table 2).

15.1.2 Respiratory contributions of denitrification enzymes

In addition to concentration of O_2 and NO_2^-, we had two alternative considerations as to possible evolutionary selection pressures on denitrification: 1) the relative anaerobic respiratory contribution of the AniA, NorB and NosZ enzymes, and 2) the requirement of each enzyme for amino acids and Cu cofactor. We hypothesise that AniA and NorB are sufficient for microaerobic gonococcal growth, and that translation termination of NosZ is beneficial for the gonococcus, possibly via conservation of amino acids and Cu.

The contribution each denitrification enzyme makes towards energy production in anaerobic respiration is thought to be equal. *Paracoccus denitrificans* oxidative phosphorylation is thought to occur to a similar extent in each of the electron transport chains associated with the reduction of NO_3^- to NO_2^-, NO_2^- to N_2O and N_2O to N_2 (Koike and Hattori, 1975b). In a review of denitrification focused on *Paracoccus denitrificans* and *Pseudomonas stutzeri*, van Spanning et al (2007) suggest that despite the different locations of the four denitrification enzymes across the bacterial membrane, the effective $2H^+/2e^-$ translocation stoichiometry for reduction of NO_2^-, NO, or N_2O is the

same as for the NO_3^- reductase reaction. Additional reviews corroborate that there is an equal contribution to respiration from each denitrification enzyme (Chen and Strous, 2013; Nicholls and Ferguson, 2003). This would suggest that NosZ will contribute as much to anaerobic respiration as AniA or NorB. However, the catalytic turnover rate of each denitrification enzyme should also be factored into consideration of contribution towards anaerobic respiration. While data on the catalytic rate of bacterial NarGHI is hard to find in the literature, studies have compared the catalytic rate of Gram-negative AniA, NorB and NosZ. In *Flavobacterium*, apparent K_m values for NO_3^- and NO_2^- reduction are ~15 µM, while the K_m value for N_2O reduction is only 0.5 µM (Betlach and Tiedje, 1981).

NarGHI and AniA are likely to achieve more catalytic turnovers in their lifetime than NosZ or NorB, since NarGHI and AniA have three catalytic sites per functional trimer (Bertero et al., 2003; Blasco et al., 2001; Boulanger and Murphy, 2002; Chen and Strous, 2013; Sikora et al., 2017), while NorB and NosZ have two active sites per functional homodimer (Jamali et al., 2020; Prudêncio et al., 2000).

Furthermore, degradation rate should also be factored into consideration of each enzyme's contribution towards anaerobic respiration. Gonococcal AniA is known to have high thermostability, which may be important for maintaining high activity in the extracellular space and make it less prone to denaturation and proteolysis (Barreiro et al., 2022). AniA is also likely to be more stable than NosZ in fluctuating O_2 conditions since NosZ in some bacterial species (e.g. *Paracoccus denitrificans*) is known to be inhibited by O_2 (Snyder and Hollocher, 1987; Wang et al., 2023).

15.1.3 Material requirement of denitrification enzymes

The material requirement of each denitrification enzyme is summarized in Table 35.

Table 35: The metabolic cost of different enzymes of denitrification

Enzyme	Enzyme size (residues/kDa)	Enzyme total metal requirement	Location	References
NO_3^- reduction; 5961 nt/448 kDa total (*E. coli*)				
NarGHI	2 x (140 + 58 + 26) = 448 kDa	2 x (1 x Mo + 11 x Fe)	Inner (trans) membrane	(Bertero et al., 2003; Blasco et al., 2001; Chen and Strous, 2013)
NO_2^- reduction; 1653 nt/174 kDa total (*N. gonorrhoeae*)				
AniA	3 x 41 kDa = 123 kDa	6 x Cu	Outer membrane	(Boulanger and Murphy, 2002; Sikora et al., 2017)
AccA	3 x 17 kDa = 51 kDa			(Firth, 2023)
$^{\bullet}NO$ reduction; 2256 nt/168 kDa total (*N. gonorrhoeae*)				
NorB	2 x 84 = 168 kDa	2 x Fe	Inner (trans) membrane	(Jamali et al., 2020)
N_2O reduction; 7908 nt/382 kDa total (*N. gonorrhoeae*)				
NosR	2 x 14 kDa = 28 kDa			
NosZ	2 x (68) = 136 kDa	12 x Cu	Periplasmic	(Zhang et al., 2019)
NosD	2 x 28 kDa = 56 kDa		Inner (trans) membrane	(Müller et al., 2022)
NosF	2 x 34 kDa = 68 kDa			
NosY	2 x 29 kDa = 58 kDa			
NosL	2 x 18 kDa = 36 kDa		Inner membrane	

NarGHI is a large enzyme complex (448 kDa) with a high metal requirement (1 x Mo + 11 x Fe), which is absent in all *Neisseria* except *N. mucosai*, *N. bacilliformis* and *N. sp. oral taxon 014 str. F0314*. Based on the lack of any trace of a vestigial *narGHIJ* operon in other *Neisserial* genomes, we suggest that the *Neisserial* ancestor never possessed a NO_3^- reductase, and *N. mucosa*, *N. bacilliformis* and *N. sp. oral taxon 014 str.* F0314 have acquired the *nar* operon from other clades via natural transformation.

While NosZ is a relatively small functional enzyme, it requires multiple other accessory proteins for functionality and has a high metal requirement compared to AniA and NorB. The entire NosZDFYL complex requires 382 kDa of amino acids and 12 Cu total, in addition to two sulfur atoms per Cu_Z site (L. Zhang et al., 2021; Zhang et al., 2019).

The AniA-AccA trimerdimer requires 174 kDa of amino acids and 6 Cu total, and NorB requires 168 kDa of amino acids and 2 heme sites. While NorB requires heme, the heme biosynthesis machinery is also required for other processes, such as the electron transport chain (Choby and Skaar, 2016). In summary, compared to NosZ, the other denitrification enzymes AniA and NorB have a reduced requirement for amino acids, metal ions and accessory proteins, which may explain their retention in the gonococcal genome while NosZ has been lost.

It is likely that transmembrane and periplasmic proteins are more metabolically costly to produce than cytoplasmic proteins due to the energy involved in translocation to and insertion into the crowded membrane (Cymer et al., 2015). This may place on organisms an additional selection pressure to inactivate N_2O reduction, since NosZ requires four sets of five NosY transmembrane helices to function (Müller et al., 2022). AniA is thought to possess an outer membrane-anchoring lipid domain (Hoehn and Clark, 1992) and NorB is thought to possess 12 transmembrane helices (Hino et al., 2012).

In addition to this consideration of metabolic cost to produce each different denitrification enzyme, an estimate of the energy cost to retain each denitrification gene should be made. In total, *E. coli* NO_3^- reduction requires 6 kb of nucleotides, while gonococcal NO_2^- reduction requires 1.7 kb, $^{\bullet}NO$ reduction 2.3 kb and N_2O reduction 8 kb. When these numbers are considered, it is clear that N_2O reduction requires by far the highest DNA retention of all denitrification steps.

We suggest that the pseudogenization of gonococcal Nos can be explained by enzyme metabolic cost, at the expense of a one third reduction in anaerobic respiration. Previous studies suggest that other denitrification enzymes can compensate for a loss of NosZ in other species; Felgate et al (2012) showed that loss of N_2O reduction makes minimal impact on the growth of *Paracoccus denitrificans* in an NO_3^--rich environment, since the bacteria can simply compensate by consuming more NO_3^-. Furthermore, the fact that so many soils emit large quantities of N_2O suggests that NosZ is either absent or non-functional in many species (Signor and Cerri, 2013).

Of course, our estimate of enzyme metabolic cost does not take into account the exogenous availability of all substrates in the pathway. It may be the case that NO_2^- and •NO are simply more readily available to gonococci than NO_3^- or N_2O. Based on our current knowledge, NO_3^- is thought to be the most abundant NO_x found in the human body, followed by NO_3^-, then •NO, then N_2O (Table 2). While the concentration of N_2O in the locations relating to the urogenital tract has not been measured, we think it is unlikely that N_2O is as abundant as upstream denitrification substrates.

It is known that in NO_3^--rich, low Cu conditions, *Paracoccus denitrificans* thrives by partially shutting down N_2O reduction via the synthesis of a Cu_Z-only NosZ with 40% efficiency. This loss of N_2O reduction is thought to have minimal impact on the growth of *Paracoccus denitrificans* in a NO_3^--rich environment (Felgate et al., 2012). Based on the large number of denitrifying bacteria isolated from NO_3^--supplemented agricultural areas which reduce NO_3^- to N_2O, but do not have a gene encoding a N_2O reductase, previous studies hypothesise that the abundance of NO_3^- has prompted reductive evolution (Felgate et al., 2012). In a study on 70 soil isolates classified as denitrifiers, only 17 could reduce N_2O, while 53 could reduce NO_3^-, 34 could reduce NO_2^-, and 24 could reduce •NO (Lycus et al., 2017). Another study analysing the genomes of denitrifying soil bacteria revealed that approximately one third lack the *nosZ* gene (Philippot et al., 2011).

15.1.4 NO_x toxicity

Another potential factor influencing retention of certain dentification enzymes is the question of substrate toxicity. While N_2O is an inert gas, NO_3^-, NO_2^- and •NO are toxic in high concentrations (NO_3^- and NO_2^- largely by virtue of reduction to •NO) (Moir, 2011b). Accordingly, the •NO-generating and •NO-consuming enzymes AniA and NorB are frequently transcriptionally co-regulated by the •NO-responsive NNR (Saunders et al., 1999). This has the result that concentration of free •NO during denitrification is maintained at a very low level to protect the bacteria from toxicity (Zumft, 1997). In the case of the urogenital tract, host-derived •NO during inflammation may add pressure on gonococci to retain NorB. In *N. gonorrhoeae*, *aniA* and *norB* are back-to-back adjacent, yet have their own FNR and NsrR regulatory boxes.

15.2 Potential degradation of the nascent NosZ polypeptide

An explanation for the lack of mature gonococcal NosZ is that the bacteria rapidly degrades the truncated polypeptide. It is known that misfolded proteins can be degraded within a few minutes in the cell (Goldberg, 2003). This finding is contrary to the findings of Felgate et al (2012) in *P. denitrificans* of a stable truncated N-terminal Cu_Z. Further research should aim to determine the effect of the different amino acid sequences from these two species on the stability of NosZ fragments.

15.2.1 Bacterial protein degradation

Proteolysis, the breakdown of proteins into smaller polypeptides and amino acids, is part of normal cellular functioning and is carried out by protease enzymes. The proteasome is a highly sophisticated protease complex which undertakes selective, efficient and processive hydrolysis of client proteins (Tanaka, 2009). While all eukaryotes and archaea use proteasomes for protein degradation, only certain orders of bacteria have proteasomes, not including *Neisseria* (Becker and Darwin, 2016). BLASTn reveals no FA1090 gonococcal homolog to the *Mycobacterium tuberculosis pcrA* or *pcrB* 20S proteasome α- and β-subunit genes. In the absence of a proteasome, bacteria contain various other AAA+ (ATPases associated with cellular activities) proteases which use ATP hydrolysis to recognize, unfold, translocate, and degrade proteins. These proteases include Lon, ClpXP, ClpAP, HslUV, and FtsH (Mahmoud and Chien, 2018).

While there is no gonococcal homolog to *E. coli* HslV, *N. gonorrhoeae* is known to possess genes encoding the proteases Lon, ClpP and FtsH (Knipfer et al., 1999). The FA1090 *lon* gene is annotated NGO_0775 “peptidase” and has high homology (77% query cover/69% identity) to the *E. coli lon* gene. The FA1090 *clpX* regulator and *clpP* protease genes (NGO_0645 and NGO_0593) have partial nucleotide homology (34% query cover/79% identity and 85% query cover/69% identity) to the *E. coli* genes. FA1090 also possesses the *clpA* regulator and *clpB* protease genes (NGO_0408 and NGO_0250). The FA1090 *clpA* gene has high homology (71% query cover/66% identity) to *E. coli* but the FA1090 *clpB* gene has no homolog in *E. coli*. There is a gene annotated FtsH in the FA1090

genome (NGO_0382) with homology to *E. coli ftsh* (64% query cover/69% identity). FtsH is known to degrade membrane-anchored proteins (Ito and Akiyama, 2005), so may be the protease responsible for NosZ degradation in gonococci.

Before an AAA+ protease can degrade a protein, there is initial recognition by the protease of some determinant on the substrate. Two determinants by which proteases recognise proteins for degradation are 1) when ribosomes are stalled and an SsrA (tmRNA) tag then added to the incomplete protein to signal it for degradation, and 2) when proteins are misfolded (Mahmoud and Chien, 2018).

Ribosomes stall at problematic mRNA sequences, typically at the 3'-end of a truncated mRNA without an in-frame stop codon (Sunohara et al., 2004). When this happens, the process of trans-translation occurs, whereby an 11-residue SsrA tag is appended to the incomplete polypeptide, the adaptor SspB then binds and tethers it to ClpXP for degradation. Some studies estimate that every 1 in 20 translation events results in a SsrA-tagged polypeptide (Lies and Maurizi, 2008). In *E. coli*, FtsH, ClpXP and ClpAP recognise SsrA RNA tags (Karzai et al., 2000). *N. gonorrhoeae* FA1090 possesses a ClpXP protease (NGO_0645 and NGO_0593) as well as the gene for the SsrA RNA tag, which has been found to be essential for gonococcal growth (Huang et al., 2000).

In the case of a premature stop codon, the ribosome will disassemble and release the polypeptide (Baggett et al., 2017; Svidritskiy et al., 2018). Hence, it is more likely that the mechanism of NosZ degradation occurs via recognition of protein misfolding after the loss of the C-terminus. In the case of terminally misfolded proteins, Lon recognizes hydrophobic motifs which are usually buried in the core of folded proteins. In *Escherichia coli*, Lon is thought to be responsible for the degradation of approximately 50% of misfolded proteins (Chung and Goldberg, 1981). *N. gonorrhoeae* FA1090 possesses a Lon protease (NGO_0775) with 66% identity and 14% similarity to the *E. coli* Lon protease. We hypothesise that Lon is the protease likely responsible for NosZ gonococcal degradation.

15.3 Possible functions of pseudogenes

It is possible that the gonococcal *nos* operon still has unknown function (i.e. it may be a *xapto*-potogene rather than a *napto*-potogene). Early studies describe pseudogenes as a paradigm of neutral evolution (Li et al., 1981). However, subsequent studies propose pseudogenes may instead incur energetic costs via continued transcription and translation of non-functional genes and/or through the production of proteins that are toxic to cells, leading to their deletion (Kuo and Ochman, 2010). For example, it is known that *Salmonella* actively expel pseudogenes, with the few pseudogenes remaining in *Salmonella* genomes having low expression and low connectivity in gene networks, such that any deleterious effects associated with their inactivation are minimal. This suggests that pseudogenes are often deleterious and there is selection pressure to remove them (Kuo and Ochman, 2010). However, other studies point to the evolutionary conservation of pseudogenes which suggests positive selection to retain them (Balakirev and Ayala, 2004). For instance, the mammalian *iglV* and *ighV* antibody pseudogenes contain significantly fewer stop codons generated by point mutation than would be expected under a model of random nucleotide change (Rothenfluh et al., 1995), and the rate of synonymous substitutions compared to nonsynonymous substitutions in the *Drosophila melanogaster ψEst-6* pseudogene is higher than the rate one would expect under neutral selection (Balakirev et al., 2003). The fact that the gonococcal *nos* operon has not been deleted suggest there is insufficient selection pressure to delete this supposedly non-functional operon. The high conservation of the *nos* operon across gonococcal isolates spanning three countries and 90 years (as well as 19,134 isolates from PubMLST) found in this study further suggests that there may be an evolutionary selection pressure acting to conserve the *nos* operon, suggesting it may still have some beneficial role.

15.3.1 Protein length diversity

A prematurely truncated protein variant may on occasion acquire a new function because of its novel structure. Alternatively, stochastic readthrough of a premature stop codon would lead to a mixed population of full-length and truncated protein from a single mRNA which could confer a

benefit to the organism. One example of a pseudogene generating a beneficial diversity of protein isoforms is in mouse IgV antibodies (Schiff et al., 1985). Previous studies have hypothesised premature stop codons in *N. gonorrhoeae* could be a mechanism of phase variation (Baarda et al., 2021; van der Woude and Bäumler, 2004). While this study found no evidence of any NosZ protein of any length being produced, it is possible that a truncated gonococcal NosR or NosD protein may be present with a novel function.

15.3.2 Regulation of gene and protein expression

Stop codons have been suggested to benefit organisms by providing a mechanism to downregulate gene expression (Belinky et al., 2021). Pseudogene transcripts can be processed into short interfering RNAs to regulate protein-coding genes via RNAi (Pink et al., 2011; Tutar, 2012). It is known that sRNAs are able to repress the expression of target mRNA transcripts with which they share similar complementarity by either decreasing mRNA stability or preventing translation (Gaimster et al., 2018). Previous studies have shown that duplicated pseudogene transcripts regulate the mRNA stability of their homologous coding genes (Hirotsune et al., 2003). Hence, it may be the case that the *nos* operon mRNA has regulatory function against another region of the gonococcal genome. The difficulty this study had in amplifying an entire *nos* operon mRNA without resorting to a series of overlapping fragments may hint at the possible degradation of the transcript; *nos* operon mRNA fragments may regulate a number of genes. This study did not attempt to identify possible target genes which may be regulated by the gonococcal *nos* operon mRNA. An avenue for future research should be to study if the knockout of gonococcal *nos* genes affects expression of any distant genes (i.e. whole-transcriptome sequencing).

It is known that the *Drosophila sechellia* pseudo-pseudogene *DsecIr75a* contains a premature stop codon yet encodes a functional receptor, due to efficient translational readthrough (Prieto-Godino et al., 2016). Readthrough occurs only in neurons and is independent of the type of termination codon but is dependent upon the sequence downstream of the premature stop codon. The authors

therefore speculate this may represent a regulatory mechanism whereby differences in tissue tRNA populations cause tissue-specific gene expression.

15.3.3 Translation of non-canonical amino acids

When stop codons are readthrough, non-canonical amino acids may be inserted in a TGA or TAG stop codon. Known readthrough mechanisms include reassignment of TGA to selenocysteine and cysteine (Gonzalez-Flores et al., 2013), reassignment of TGA or TAG to selenocysteine (Kotini et al., 2015) and reassignment of TAG to pyrrolysine (Wan et al., 2014). Translation of these non-canonical amino acids is thought to require certain *cis*-acting elements; the selenocysteine insertion sequence (SECIS) (Novoselov et al., 2007) and pyrrolysine insertion sequence (PYLIS) (Zhang et al., 2005). While this study found no evidence of a gonococcal NosZ protein, it remains a possibility that the TAG premature stop codon in gonococcal *nosD* may experience a stop codon reassignment which may provide some unknown benefit. However, it should be noted this is unlikely given the lack of matches of the SECIS and PYLIS sequences in the gonococcal *nos* operon.

15.4 Future work

Additional experiments could further test our core hypothesis that a gonococcal NosZ protein may be present under certain conditions.

Viability of 8 hr cultures without NO_2^-. This study assumed but did not confirm that 8 hr static *N. gonorrhoeae* cultures without NO_2^- were still viable. It is thought that only 20% of *N. gonorrhoeae* strain RUG7001 (F62) cells survive anaerobic incubation in the absence of NO_2^- (Householder et al., 2000). Plating out bacteria from FA1090 8 hr NO_2^- cultures grown without NO_2^- and comparing CFU count to 8 hr cultures grown with NO_2^- would determine viability.

Mutant strain *nosZ* transcription. This study assumed but did not confirm the *nosZ* gene is transcribed in *strepII::nosZ* and *nosZ::strepII* mutant strains. We did not think there was any reason to believe the *nosZ* gene would not be transcribed in *strepII::nosZ* and *nosZ::strepII* strains, given high genome homology of these strains to the WT (Table 34 and Appendix 11). However,

confirmation of *nosZ* transcription in our mutant strains would add confidence to our assessment that gonococcal *nosZ* is transcribed but not translated.

Functionality of *nos* operon mRNA transcripts. This study amplified the gonococcal *nos* operon mRNA as a series of seven overlapping amplicons in PCR (Figure 29). However, this study struggled to amplify the entire *nos* operon as a single transcript. Hence, it is unknown if the entire *nos* operon exists as one transcript; the *nos* mRNA may instead be subject to degradation such that there is a series of mRNA fragments which may go on to regulate other genes. Northern blot analysis would independently determine if the *nos* transcript is full-length or fragmented. Subsequent mRNA sequencing or whole-cell proteomics of WT vs KO strains and homology searches of *nos* operon fragments against the gonococcal genome using RNA target prediction software (Tjaden, 2023) may determine any possible regulatory targets of *nos* mRNA. qPCR could also be used to check if the *ΔnosZ* strain has altered expression of any target genes (Piehler et al., 2008).

Experiments "forcing" readthrough. *In vitro* evolution could be used to rescue NosZ. Cultures supplemented with only N_2O as a denitrification substrate may prompt expression of NosZ. Supplementing cultures with tryptophan (the rescued residue at the WT gonococcal *nos* operon premature stop codon) and cognate tRNA may also prompt expression of a full-length readthrough protein.

Measuring growth of WT vs *ΔnosZ* strains with NO_2^- limitation. A disadvantage of the method used in this study to phenotype the *ΔnosZ* mutant strain is that growth rate (OD_{600} over time) was measured in cultures supplemented with NO_2^- (Figure 42). With NO_2^-supplemntation, the impact of *nosZ* knockout would be minimal due to the activity of AniA and NorB. Repeating these growths under NO_2^- limitation would increase the likelihood of showing an effect of *nosZ* knockout. To further improve this method, a Griess assay could have been used to determine culture NO_2^- concentration during growth.

Furthermore, any impact of NosZ is likely to be on the growth *yield* under microaerobic conditions not the growth *rate* (J. Moir, personal communication). Due to the static cultures used in this study never reaching stationary phase, determination of growth yield with the current dataset is challenging. Longer growths would need to be attempted in order to reach stationary phase.

Measuring N_2O consumption of WT vs *ΔnosZ* strains. Future research should verify the finding of Lissenden et al (2000) that the end product of gonococcal denitrification is N_2O. Lissenden et al (2000) used RUG7001, a modified F62 strain carrying a chromosomal *aniA::lacZ* fusion integrated into the non-essential *pro* region of strain F62 (Silver and Clark, 1995). However, the RUG7001 genome sequence is not available. Determining similarly low N_2O reduction rates in FA1090 WT and *ΔnosZ* strains (e.g. using a microsensor (Andersen et al., 2001) or electrode chamber (Rapson and Dacres, 2014; Tzeng et al., 2023)) would give further confidence that WT gonococci do not produce NosZ.

Determination of culture O_2 content. One of the most puzzling findings of this study was the fact that FNR-induced genes are not expressed until 8 hr in static gonococcal cultures and are maximally expressed in 3 hr shaking cultures. Finding expression of FNR-regulated genes does not necessarily indicate microaerobic conditions; determination of O_2 concentration would help ascertain if there is a sharp drop in O_2 in our static culture setup at 8 hr, or in shaking cultures at 3 hr. One could modify our culture setup using a Clark electrode (Renger and Hanssum, 2009) inserted through a hole in the lid of a Falcon tube and supplement NO_2^- through an airtight septa (Householder et al., 1999).

Shaking to static growth switch. In order to increase the microaerobicity of gonococcal cultures (to potentially increase expression of NosZ), cultures with initial shaking could be used to generate significant cell density and then shaking ended to generate microaerobic conditions. This may have the effect of decreasing O_2 concentration further than the static culture setup used in this study.

Comparison of gonococci to other bacteria. Knocking out the *nosZ* gene in commensal *Neisseria* species (thought to possess NosZ) and comparing their growth to the gonococcal WT and *ΔnosZ*

strains (thought to lack NosZ) may provide insight into the contribution of NosZ towards microaerobic growth in this genus. Including *N. meningitidis* and *N. mucosa* may help to test the hypothesis that pathogenic bacteria have lost NosZ. *N. lactamica* has the highest homology to gonococcal NosZ, so will provide a useful control to study the effect of the gonococcal premature stop codon.

Comparing growth of *P. denitrificans* WT and *ΔnosZ* strains to *N. gonorrhoeae* would provide a positive control for the impact of functional NosZ knockout, since *P. denitrificans ΔnosZ* is known to have a reduced growth rate in O_2-limited conditions (Bergaust et al., 2012).

Unpublished evidence suggests a minority of gonococcal isolates exist which possess an intact *nosZ* and *nosD*. In addition to the lack of a premature stop codon, they are also quite dissimilar to the other isolate *nosZ* and *nosD* sequences. However, they are still more homologous to *N. gonorrhoeae* than other *Neisseria* (T. Mortimer, personal communication). Western blotting against these isolates could help determine if the premature stop codons in the majority of isolates do cause NosZ truncation.

Pathogenicity assays. Growth of *N. gonorrhoeae* in liquid medium is thought to not be representative of all environmental stressors encountered in the host (Baarda et al., 2018). It may be the case that gonococcal *nosZ* is only functional while in the human host niche. Comparison of virulence between gonococcal WT and *ΔnosZ* strains inside a host cell survival model could help test the hypothesis that *nos* is a *napto*-potogene (i.e. non-beneficial). Growth methods which better simulate the gonococcal niche have been developed, such as human immune cell culture (Edwards and Apicella, 2004), primary human cervical epithelial (Pex) cell culture (Day et al., 2022; Edwards et al., 2001), and 3D mucosa culture (Heydarian et al., 2022, 2019). Isolation of cell extracts for Western blotting from such alternative growth methods, while more challenging, may prove more robust than the liquid media employed by this study.

Endogenous periplasmic control for Western blotting. The GFP-StrepII positive control protein used in our Western blots is not an endogenous control. Hence, it is possible that all periplasmic proteins were degraded in our sample prep without our knowledge. An antibody against a periplasmic protein known to be expressed in gonococci (e.g. NorB) would provide a more robust positive control.

Further *in-silico* bioinformatics studies. It is unknown if there is any precedent for so many nonsense substitutions in a single operon. Further analyses of the incidence of nonsense substitutions within an operon and their effect may shed light onto this least deleterious of nonsense mutations.

15.5 Conclusion

The past assumption that gonococcal *nosZ* is a non-functional *napto*-potogene (i.e. true pseudogene) due to premature stop codons in the *nos* operon may likely be correct. This study found transcription of the *nos* operon into mRNA, but no evidence of a stable NosZ protein. Whether this is the result of the premature stop codon in *nosR*, *nosZ*, *nosD* (or a combination of multiple genes) remains uncertain. However, we discuss the possible effect of the premature truncation of NosZ, which likely leads to an unstable truncated NosZ protein that is rapidly degraded even in excess Cu. Contrary to studies which suggest stop codon readthrough provides a means of downregulating protein expression (Belinky et al., 2021), we have shown in this instance that stop codon readthrough does not occur and protein expression is totally abolished. The loss of the *nosZ* gene does not affect growth phenotype in static microaerobic cultures, suggesting a lack of WT NosZ function.

Our finding that a series of three nonsense substitutions in the *nos* operon can cause complete loss of any stable protein contributes to our understanding of NosZ protein assembly and folding. These nonsense mutations are thought to have arisen after the speciation of *N. gonorrhoeae* from *N. meningitis*. However, independent disruption of the *nos* operon in other *Neisseria* associated with

infection (primarily *N. meningitidis* and *N. mucosa*) leads us to hypothesise that inactivation of N_2O reduction may provide a selective advantage in the human host niche.

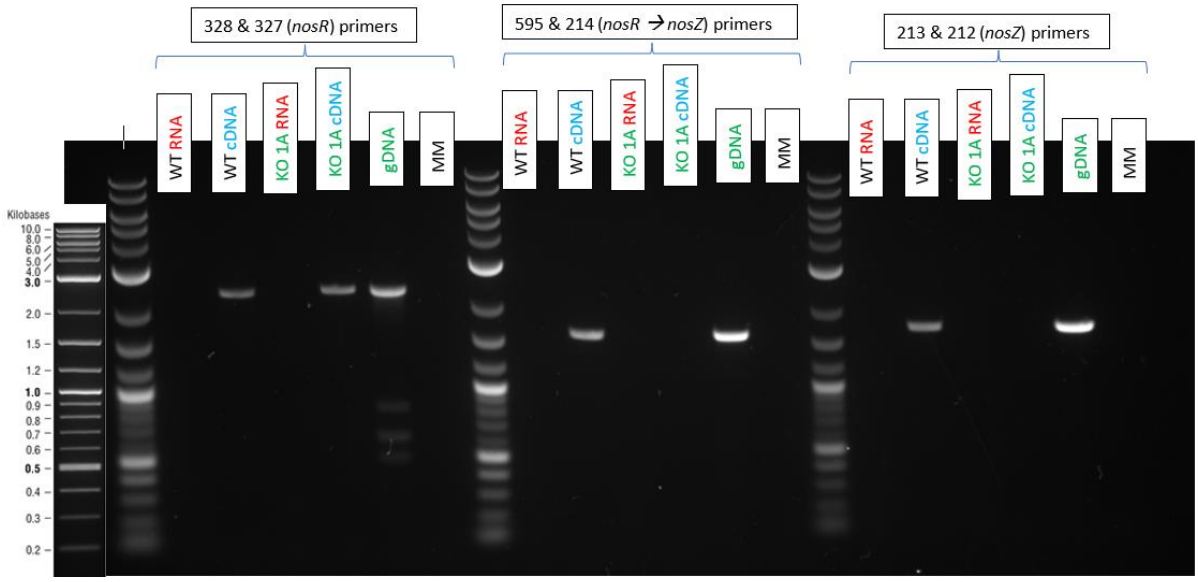
The transcription of the gonococcal *nos* operon into mRNA, only for the nascent NosZ polypeptide to be rapidly degraded appears an inefficient use of cellular resources. While Feavers & Maiden (2002) suggest that the selective advantage of deleting the 1kb *porA* pseudogene is insufficient for such a mutation to spread in the gonococcal population, the *nos* operon is a large 8 kb operon, accounting for 0.4% of the gonococcal genome. As such, it seems that deletion of the entire *nos* operon (or at least the transcriptional promoter) would be advantageous if *nos* has no functional role.

If gonococcal *nos* were a true non-functional pseudo-operon, one may expect a variety of random mutations to accumulate in the promoter region over time, as is the case for *porA* (Feavers and Maiden, 1998). The high conservation of the *nos* coding region and preceding FNR and NsrR promoter regions across gonococcal isolates may suggest that the *nos* operon mRNA have a regulatory role, or the NosRDFYL proteins (which are presumably still translated even partially) may have unknown benefits. Alternatively, in low concentrations of NO_2^-/$^\bullet$NO and/or high concentration of N_2O, NosZ may be expressed through translational readthrough to supplement the low rate of anaerobic respiration facilitated by these more metabolically efficient denitrification enzymes. Further investigation of these hypotheses may explain why the large *nos* operon is not inactivated by more serious mutations so as to prevent unnecessary transcription.

Despite absence of NosZ protein, we are unwilling to label gonococcal *nos* as a true pseudo-operon due to the high conservation of *nos* promoter and coding regions across isolates. However, we have found no evidence to suggest there is a function for gonococcal *nosZ*, so it cannot be identified as a *xapto*-potogene. We suggest gonococcal *nosZ* be simply considered a potogene, a gene with *potential* function, until further research has been carried out.

16 Appendices

16.1 Appendix 1: Transcription of *nosZ* gene was confirmed to be abolished in *ΔnosZ* strains



NEB Q5 PCR

NB: Primer 595 was renumbered 612. MM; PCR master mix.

16.2 Appendix 2: GenScript synthetic gene sequences used in Golden Gate Assembly

BsaI recognition sites in bolt italics, ***4-base annealing overhangs highlighted in bold italics***. Start and stop codons underlined. StrepII tag double underlined. TAT signal peptide dashed underline. Linker regions wavy underlined. Domesticated BsaI recognition site in bold underline. BsaI-removed regions in lowercase.

16.2.1 new *specR* (for *strepII::nosZ* and *nosZ::strepII* assemblies)

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16.2.2 strepII::nosZ

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16.2.3 nosZ::strepII

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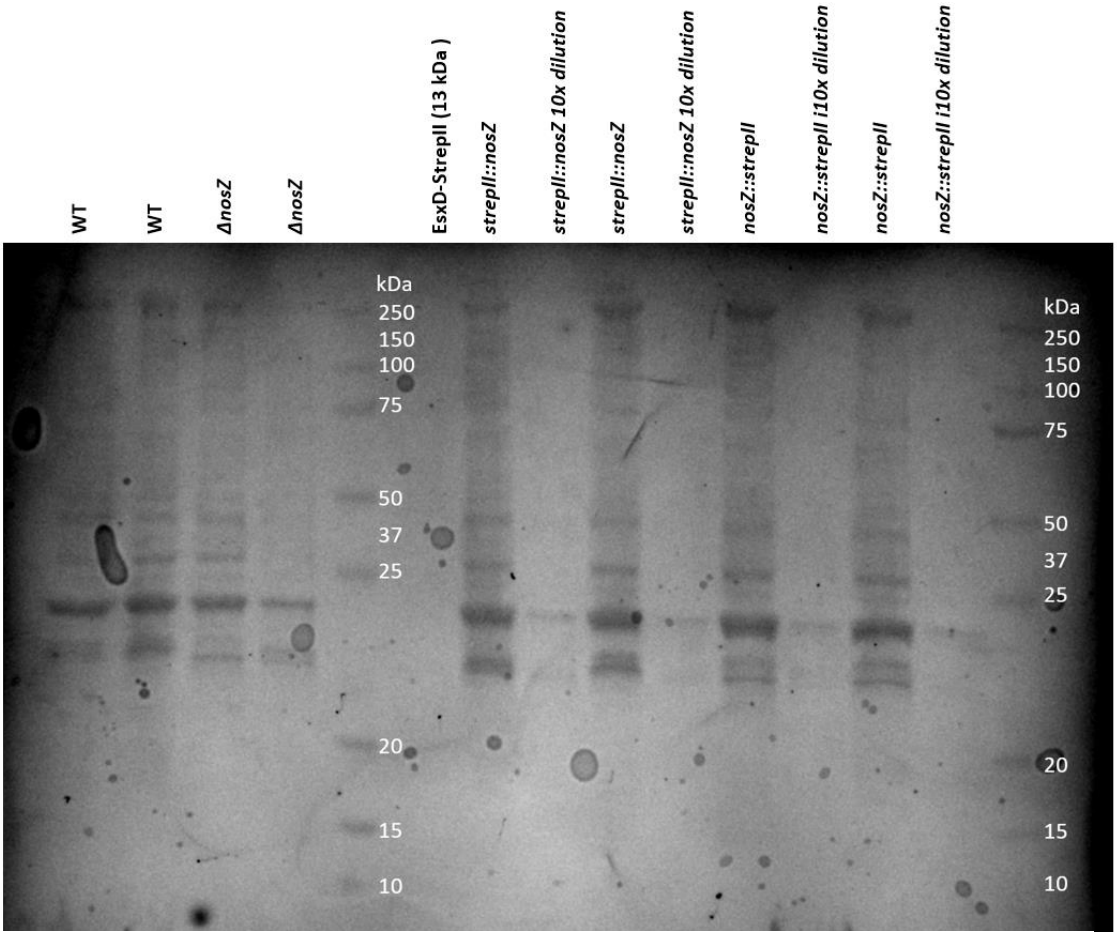
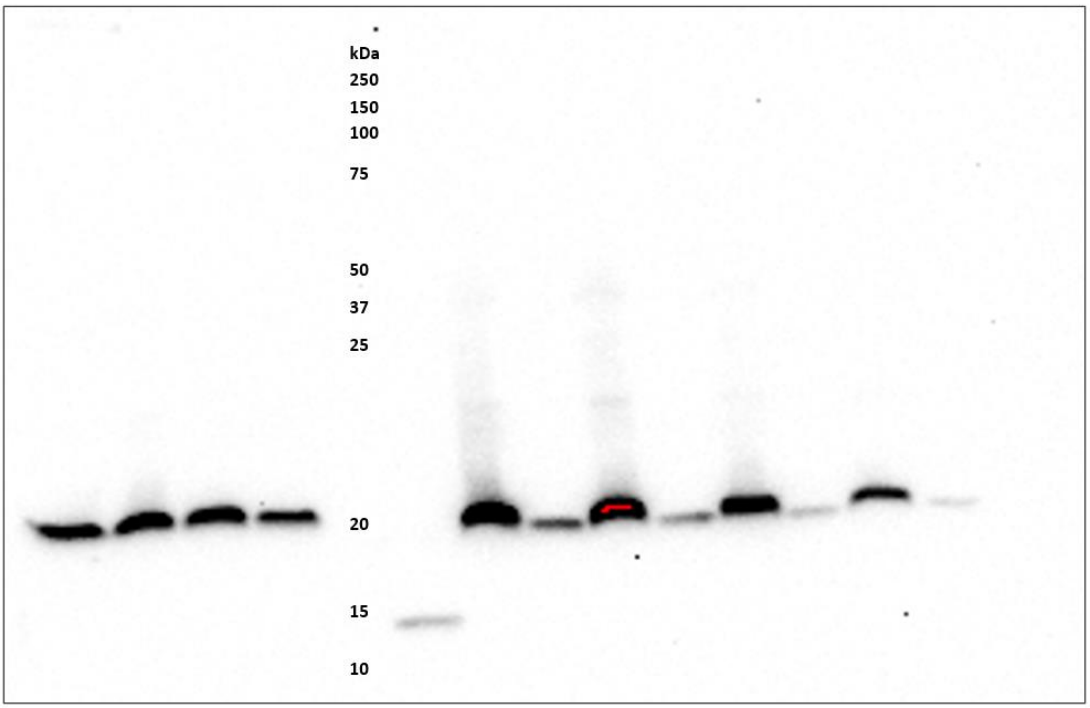
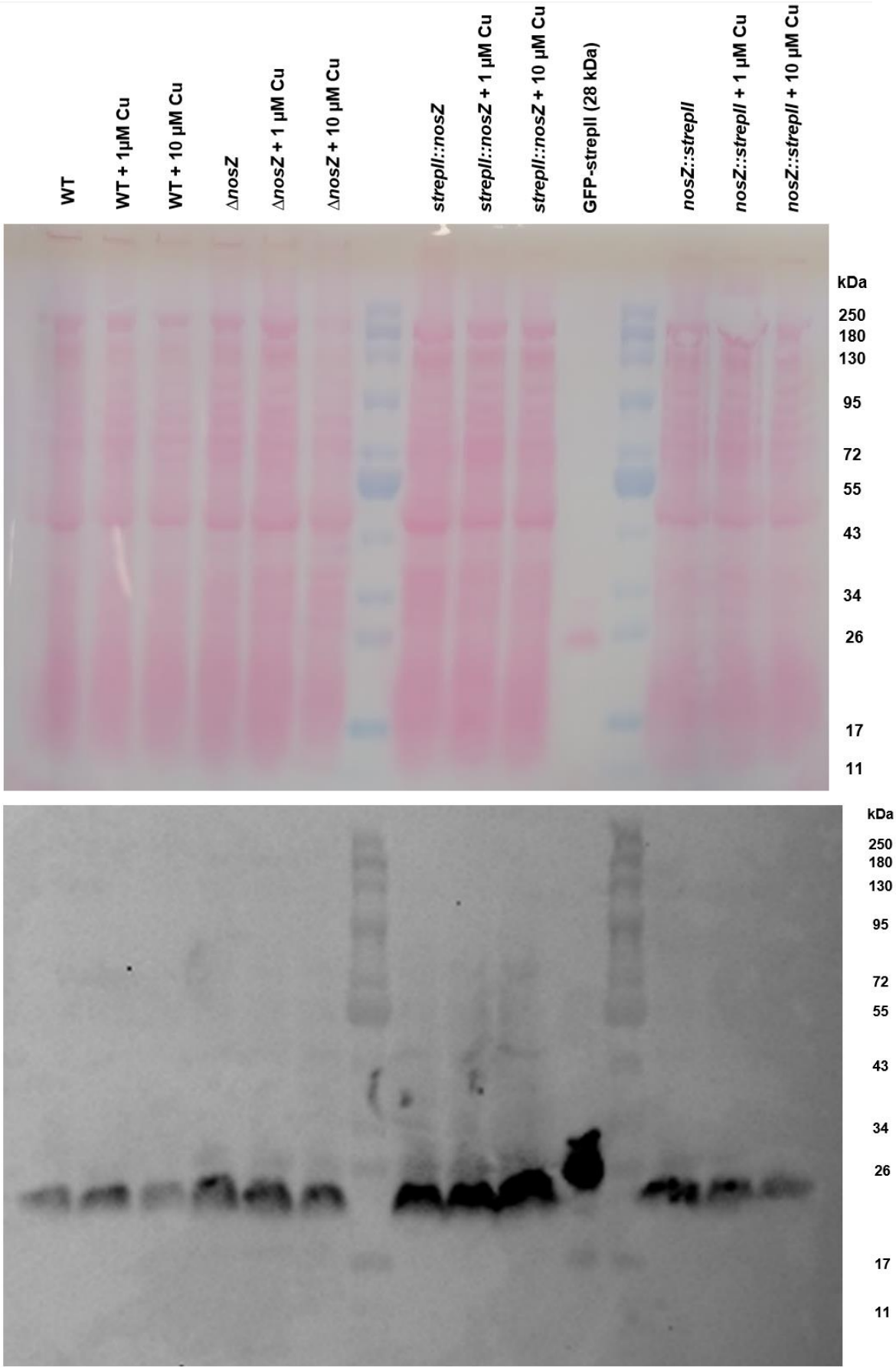
16.3 Appendix 3: Example Ponceau S stains with Western blots

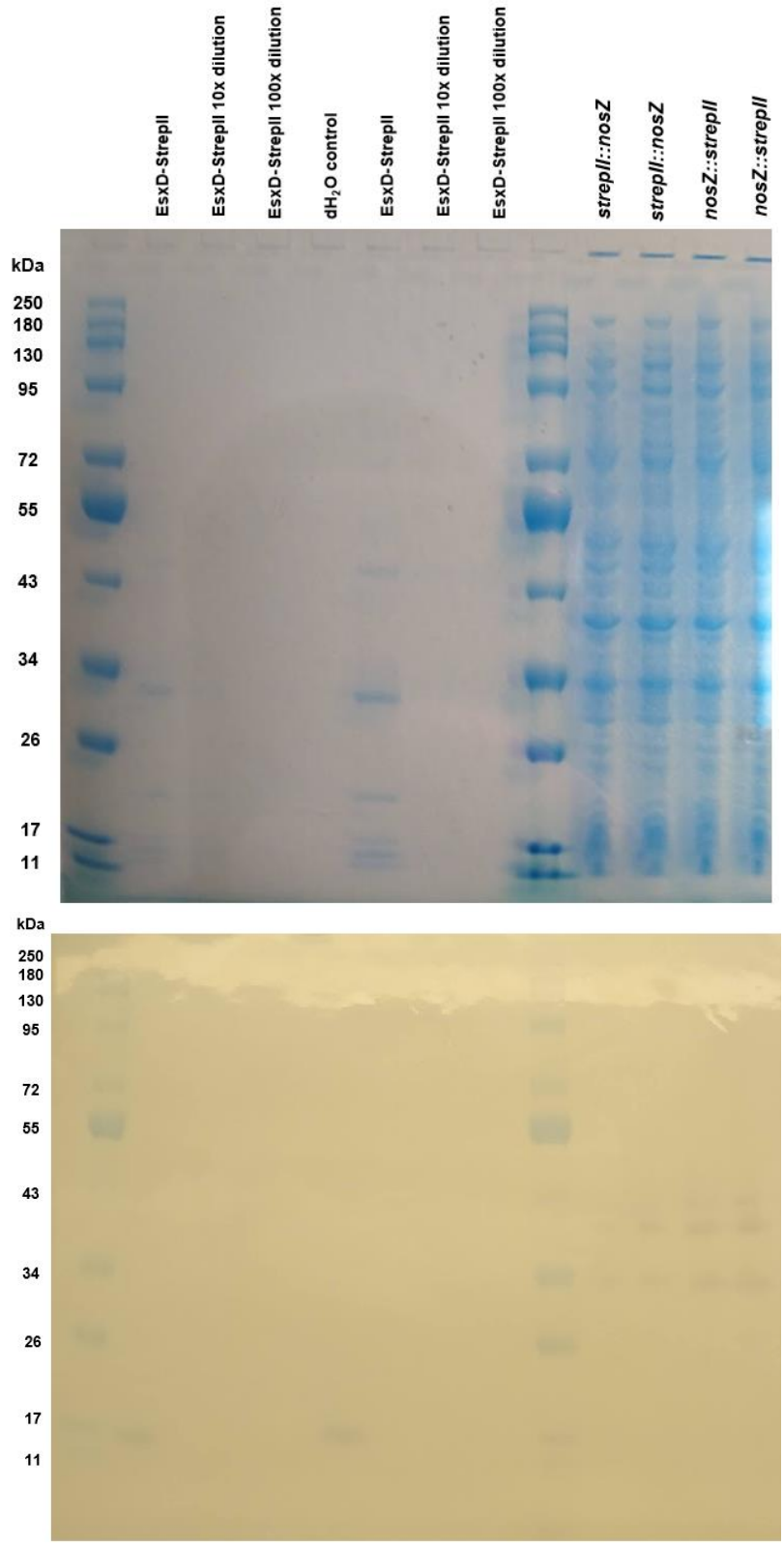




Ponceau S stain and Western blot. NB: ~20 kDa protein must be very low concentration, as it hardly shows up as a band in the Ponceau S stain. Modifications to subsequent Western blot protocol: used transfer buffer with added 0.05% SDS and used EsxD-StrepII positive control protein (~13 kDa) rather than GFP-StrepII.



Ponceau S stain and Western blot with 0, 1 and 10 µM Cu.



SDS-PAGE gel with Coomassie stain and Western blot.

16.4 Appendix 4: *Neisseria gonorrhoeae* FA1090 *nos* operon sequence

Start and stop codons in bold. Alternative start codons underlined. The FNR box (O_2 sensor), NsrR ($^{\bullet}NO$ sensor) box, possible Shine-Dalgarno sequences, and the terminator stem loop highlighted in green, yellow, blue and pink respectively.

NosR sequence in red (nt 118-2448), NosZ sequence in blue (nt 2577-4544), NosD sequence in yellow (nt 4747-6072), NosF sequence in green (nt 6100-7026), NosY sequence underlined (nt 7023-7853), NosL sequence in brown (nt 7850-8344), and NosX sequence in purple (nt 8470-9069).

>FA_1090_nos_operon

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NosZ = 1968 nt, 656 aa

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GTTACCGATTGGACGAATCCTAATGCCGACACGGAGTGGATAGATGCGAAAAAAGCCTTTTACGTCATCGACAGCGGCTTTAT
CGGCGGTATGGGCGCGGAAGACGCGCTGCCGTTCGGCAACAAGGAGCAGGCTGAAAAATTTGCAAAGGATAAAGGCGGCAAGG
TCGTCGGTTTTGACGATATGCCCGATGCTTACATTTTCAAG**TAA**ATCCAAACAGAAATGCCGTCTGAACGTTGAAGCCAAGGT
TCAGACGGCATTTTTTTGTCATAGGTTCAAACGGGGCAGGTTCGAACAACTGCCCCA

…

GAAGGCGCGTTCGATCCGACGGTTCAGGCG**TTG**TGGAAACTGTATGCCGATTATTTTGCCGCCCGTCCCGATGCGGAAACGCC
GCCGGAGAATAGCGTGAAGGAAACCCTGAAACGGGTCGGCTTCGGCAAAGTCTCGTTTGACGACCGTGAAATCCGTTTTGCCG
AAAAGGGTATGGGTTTGTCCTTGAACGGCATCGCGCAAGGCTATATCACGGACAAGGTTGTTGCGCTGCTTAAAGCAAACGGA
GTGCCTGCCGCGCTGGTCGATATGGGCGAAATCAGGGGGTTTGATACAAACGGCAGGCGGATGTGGAATGTCGGCATCCGCAA
TCCGGACGACGAAGAAGGCGTGTTGGCAAATATCACGATGAAAGACAAGGCGTTTGCCACTTCGGGCGGCTATGGGACGGTGA
TGGACAAAGCGGGCAGGCTTACCCATTTGTTCGATCCGCGAACCGGCGTGAGTACGCCGCGTTATAAAAGCATGAGCGTAATG
GCGGATGATGCGGCGGTTGCCGATGCCTTGTCGACGGCGTTTTCCGTGATGGATTTGCCGCTGATACGGTCGGTTGCCGAAAG
CAGGCGGCTTAAAGTTAGGCTGGCCATGCCGGACAATATAGTGGAT**TAA**CAAAAACCGGTACGGCGTTGCCTCGCCTTGCCGT
ACTATCTGTACTGTCTGCGACTTCGTCGCCTTGTCCTGATTTAAATTTAATCCACTATACTTTGAACGAAATAGGTTGAATCT
GAAGCAAAATGCCGTCTGAAAGGCTTTCAGACGGCATTTTGCCGTCGGGCGGTATGT

16.5 Appendix 5: *Neisseria gonorrhoeae nos* operon gene translations

Signal peptide regions calculated with SignalP highlighted

>FA_1090_nosR
MSCFSIRRMSAFRARITAFFTAFVFLTAALPAYAERLPDFLAKIQPSEIFPGADRYGKPE
GKPMVARVYKGDEQLGLVYITTDAVNTRGYSSKPIDTLMALANDGTIAGAKLVDHHEPIM
LIGIPQSRVDKFIDKYIGLNFIKNPPTPSVAPGDIISGATVTLMVVNDSIQRSYKVIANQ
YRLGSDKALQTASASDVREAAPASETRPRRMANPDKQDILSWDELLKQKAVGHLHITLDQ
INKLFEKGGKAGVADHAEQGDPDDTFIDLYVALVSQPSIGKSLLGEDGWAHLQKRLKPGQ
QAVLVAGEGRYSWKGSGYVRGGIFDRIEMIQGENSFRFTDAQHERVVELSAADAPRFKEV
SWFTIPEGVAFDGAEPWRL*LMVQRVLSVNDKAFVTADLDYELPQAYYVDDPKAPPVEIS
APVEAVPAAASDTASDGIAEDASAENGVSNQLWKQIWKAKQGQIVVVGIALTILLLVFLF
QDWIVRYEKWYDRFRFAFLTFTLFYIGWYAQAQLSVVNTLTLFSAILTEFHWEFFLMDPI
VFILWLFTAATMLLWNRGTFCGWLCPFGSLQELTNRIAKKLGVKQITVPHMLHTRLNVIK
YLILFGFLAISLYDLGTAEKFAEVEPFKTAIILKFMCDWWFVAFAVALLIAGLFIERFFC
RYLCPLGAGIALPGRFRVFDWLRRYKMCGNPCQICTHECPVQAIAPEGDIHPNECIQCLH
CQVMYHHDTRCPQVVAENKKKQKQAAAKSGELENVSKQPQEQVVRFVKPETAQSEK*

>FA_1090_nosZ
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ESKPGQLSSEVGPGELDQYYGFLSGGQSGEMRLIGLPSMRELMRIPVFNMDSATGWGRTN
ESLKVLNGNITEETRKFLKDSGLRCYPNGDLHHPHLSFTDQTYDGRYAYANDKANNRVCR
VRLDVMKADKIIDIPNDSGIHGLRPQRYPKTGYVFANGEHITPVSGVGKLDDAKTWNAVY
TAIDGETMEIAWQVLVDGNLDNGDADYQGKYSFATCYNSERALTVQGASSNEQDWCVVFD
LKAIEEGIKAGDFKEVNGVKMLDGRAEAKSKYTRYIPVPNSPHGCNASPDGKYIMPNGKL
PPTVTVLDVSKLDDLFAGKIKERDVVVAEPQLGLGPLHTAFDGRGNAYTTLFIDSQMVKW
NIDDAIKAYKGEKIDPIKQKLDVHYQPGHNHTTMGETKEADGQWLVSLNKFSKDRFLNAG
PLKPECDQLIGISGDEMRLVHDNPTFAEPHDLCLVAASKLNPGKTWDRKDPWF*QDALEQ
AKKDGVELEKAAKAVREGNKVRVYMTAVAPAYSIPQFEVNQGDEVTVYVTNVETIEDLTH
GFTLEGCGIAMEIGPQATSSVTFKAVRPGVHWYYCQWFCHALHMEMSGQMIVHPK*

>FA_1090_nosD
MHTSALRIWLKAVLILAAGSIFQTASAAVVHVSPQDNLAEIFARARAGDTIKLASGVYQT
KLYIDKPITIEGPADRSATIEGDKSGRTIAVHAPDVTLRNLTVTRSGMSLPAMDAGIYLE
KAAPRALVEHNNIFDNSFGVYLHGSADAMVRENKIVGDATLRVNERGNGVTVWNAPGAQV
VGNDISKGRDGIFSNTSTHNTYKNNRFSDLRFAVHYMYTNDSEVSGNISVGNNMGYVLMF
SERLKVFDNIAVGSRD*GIMLNYVNYSDIHDNIINKAGKCVFAYNANYDKLSANHFENCQ
IGMHFTAAIEGTSLHDNSFINNGSQVKYVSTRFLDWSEGGHGNYWSDNSPFDLNGDGFGD
SAYRPDGIIDQIIWRAPVSRLLMNSPAISIVKWAQAQFPAVLPGGVVDSKPLMKPYAPKI
QTRYQAMKDELLKEAETRQSERGRAENGSLN*

>FA_1090_nosF
MPSENTKGTTMTTHHVELRKVTKRFGAQKAVSQVDLVLKAGESVGLAGHNGAGKSTIMKL
ILGLITPTEGEVMLLGERTGSKAGARLRSQIGYLPETVALHPSLTGIETLDFYAKLKKQP
LTQNRGLLERVGISQTAHRRVGTYSKGMRQRLALAQALLGEPKVLLFDEPTTGPDPASRQ
MFYEVVRELNGRGATILLSTHALAELDGHADRIVVMKNGVKVADGSMDELHVQSGLPLTV
NIRLNAPRTLSSRWQPLSDGISYRAQCQAEERMELLGELGSLSDLAYFDIHTPTLDEMYA
RFLKREDV*

>FA_1090_nosY
MNPVWIITGKEARDSLRNRWVLAAVLLLAALALSLGFLGSSPTGSVKVDPLTVTVVSLSS
LSIFLIPLIAMLLSYDALIGEIERGTMALLLSYPIWRNQILAGKFVGHLIILALATTAGY
GLAGITLQLANGGFDIAAWKPFALLIAASVILGAAFLSMGYLISAKVKERGTAAGISIGV
WLFFVVIFDMALLGILVADSKQVITAPVVETVLLFNPTDIYRLLNLTGYENTAMYAGMAG
LSGQIGLTVPVLLTAQVLWVIIPLVLAAGIFRKRRI*

>FA_1090_nosL
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DQPVWFSTVKQMFGYTKLPEEPKGIRVIYVTDMGNVTDWTNPNADTEWIDAKKAFYVIDS
GFIGGMGAEDALPFGNKEQAEKFAKDKGGKVVGFDDMPDAYIFK*

>FA_1090_NosX
LWKLYADYFAARPDAETPPENSVKETLKRVGFGKVSFDDREIRFAEKGMGLSLNGIAQGY
ITDKVVALLKANGVPAALVDMGEIRGFDTNGRRMWNVGIRNPDDEEGVLANITMKDKAFA
TSGGYGTVMDKAGRLTHLFDPRTGVSTPRYKSMSVMADDAAVADALSTAFSVMDLPLIRS
VAESRRLKVRLAMPDNIVD*

16.6 Appendix 6: Protein sequences of NosZ crystal structures

Proposed Cu_A and Cu_Z sites highlighted in purple and blue respectively. Signal peptides highlighted in grey. Four amino-acid deletion site from *Neisseria gonorrhoeae* FA1090 NosZ underlined.

>Shewanella denitrificans (PDB: 5I5M)
MSENSENKSLELQDSSRRSFMGRSALIGAGAVAAPMTAAMFASMAKAQTQTQGASAVVHPGELDEYYGFWSGGHSGEVRI
LGIPSMRELMRIPVFNIDSATGWGITNESKRIKGDSAHLMTGDSHHPHMSMTDGSYNGKYVFINDKANSRVARIRCDVMK
TDKMITIPNVQAIHGLRVQKVPYTKYVICNGEFEIPMNNDGKASLEDVSTYRSLFNVIDAEKMEVAFQVMVDGNLDNTDA
DYDGKYFFSTCYNSEMGMNLGEMITAERDHVVVFSLERCLAALKAGKFTNYNGNKVPVLDGRKGSDLTRYIPVPKSPHGI
NTAPDGKYFVANGKLSPTVSVVEIARLDDVFSGKIQPRDAIVAEPELGLGPLHTAFDNKGNAFTTLFLDSQIAKWNIQDA
IKAYNGEKVNYLRQKLDVHYQPGHNHTSQGETRDTDGKWLVVLCKFSKDRFLPVGPLRPENDQLIDISGDEMKLVHDGPT
FAEPHDCMIVHRSKVKPQKLWTRDDPMFADTVAMAKQDGVTLEMDNKVIRDGNKVRVYMTSIAPNFGMNEFKVKLGDEVT
VVVTNLDQVEDVTHGFCMTNHGVQMEVAPQATASVTFIANKPGVQWYYCNWFCHALHMEMRGRMLVEALEWSHPQFEK

>Paracoccus denitrificans (PDB: 1FWX)
ASADGSVAPGQLDDYYGFWSSGQSGEMRILGIPSMRELMRVPVFNRCSATGWGQTNESVRIHERTMSERTKKFLAANGKRIHD
NGDLHHVHMSFTEGKYDGRFLFMNDKANTRVARVRCDVMKCDAILEIPNAKGIHGLRPQKWPRSNYVFCNGEDETPLVNDGTN
MEDVANYVNVFTAVDADKWEVAWQVLVSGNLDNCDADYEGKWAFSTSYNSEKGMTLPEMTAAEMDHIVVFNIAEIEKAIAAGD
YQELNGVKVVDGRKEASSLFTRYIPIANNPHGCNMAPDKKHLCVAGKLSPTVTVLDVTRFDAVFYENADPRSAVVAEPELGLG
PLHTAFDGRGNAYTSLFLDSQVVKWNIEDAIRAYAGEKVDPIKDKLDVHYQPGHLKTVMGETLDATNDWLVCLSKFSKDRFLN
VGPLKPENDQLIDISGDKMVLVHDGPTFAEPHDAIAVHPSILSDIKSVWDRNDPMWAETRAQAEADGVDIDNWTEEVIRDGNK
VRVYMSSVAPSFSIESFTVKEGDEVTVIVTNLDEIDDLTHGFTMGNYGVAMEIGPQMTSSVTFVAANPGVYWYYCQWFCHALH
MEMRGRMLVEPKEA

>Marinobacter hydrocarbonoclasticus (PDB: 1QNI)
AAAEEARNKAHVAPGELDEYYGFWSGGHQGEVRVLGVPSMRELMRIPVFNVDSATGWGITNESKEILGGDQQYLNGDCHH
PHISMTDGRYDGKYLFINDKANTRVARIRLDIMKTDKITHIPNVQAIHGLRLQKVPKTNYVFCNAEFVIPQPNDGTDFSL
DNSYTMFTAIDAETMDVAWQVIVDGNLDNTDADYTGKYATSTCYNSERAVDLAGTMRNDRDWVVVFNVERIAAAVKAGNF
KTIGDSKVPVVDGRGESEFTRYIPVPKNPHGLNTSPDGKYFIANGKLSPTVSVIAIDKLDDLFEDKIELRDTIVAEPELG
LGPLHTTFDGRGNAYTTLFIDSQVCKWNIADAIKHYNGDRVNYIRQKLDVQYQPGHNHASLTESRDADGKWLVVLSKFSK
DRFLPVGPLHPENDQLIDISGEEMKLVHDGPTYAEPHDCILVRRDQIKTKKIYERNDPYFASCRAQAEKDGVTLESDNKV
IRDGNKVRVYMTSVAPQYGMTDFKVKEGDEVTVYITNLDMVEDVTHGFCMVNHGVSMEISPQQTASVTFTAGKPGVYWYYCNW
FCHALHMEMVGRMLVEAA

>Achromobacter cycloclastes (PDB: 2IWK)
MESKEHKGLSRRALFSATAGSAILAGTVGPAALSLGAAGLATPARAATGADGSVAPGKLDDYYGFWSSGQTGEMRILGIP
SMRELMRVPVFNRCSATGWGQTNESIRIHQRTMTEKTKKQLAANGKKIHDNGDLHHVHMSFTDGKYDGRYLFMNDKANTR
VARVRCDVMKTDAILEIPNAKGIHGMRPQKWPRSNYVFCNGEDEAPLVNDGSTMTDVATYVNIFTAVDADKWEVAWQVKV
SGNLDNCDADYEGKWAFSTSYNSEMGMTLEEMTKSEMDHVVVFNIAEIEKAIKAGQYEEINGVKVVDGRKEAKSLFTRYI
PIANNPHGCNMAPDRKHLCVAGKLSPTVTVLDVTKFDALFYDNAEPRSAVVAEPELGLGPLHTAFDGRGNAYTSLFLDSQ
VVKWNIDEAIRAYAGEKINPIKDKLDVQYQPGHLKTVMGETLDAANDWLVCLCKFSKDRFLNVGPLKPENDQLIDISGDK
MVLVHDGPTFAEPHDAIAVSPSILPNIRSVWDRNDPLWAETRKQAEADEVDIDEWTEAVIRDGNKVRVYMTSVAPSFSQP
SFTVKEGDEVTVIVTNLDEIDDLTHGFTMGNHGVAMEVGPQQTSSVTFVAANPGVYWYYCQWFCHALHMEMRGRMFVEPK
GA

>Pseudomonas stutzeri (PDB: 3SBQ)
MSDKDSKNTPQVPEKLGLSRRGFLGASAVTGAAVAATALGGAVMTRESWAQAVKESKQKIHVGPGELDDYYGFWSGGHQG
EVRVLGVPSMRELMRIPVFNVDSATGWGLTNESRHIMGDSAKFLNGDCHHPHISMTDGKYDGKYLFINDKANSRVARIRL
DIMKCDKMITVPNVQAIHGLRLQKVPHTKYVFANAEFIIPHPNDGKVFDLQDENSYTMYNAIDAETMEMAFQVIVDGNLD
NTDADYTGRFAAATCYNSEKAFDLGGMMRNERDWVVVFDIHAVEAAVKAGDFITLGDSKTPVLDGRKKDGKDSKFTRYVP
VPKNPHGCNTSSDGKYFIAAGKLSPTCSMIAIDKLPDLFAGKLADPRDVIVGEPELGLGPLHTTFDGRGNAYTTLFIDSQ
VVKWNMEEAVRAYKGEKVNYIKQKLDVHYQPGHLHASLCETNEADGKWLVALSKFSKDRFLPVGPLHPENDQLIDISGDE
MKLVHDGPTFAEPHDCIMARRDQIKTKKIWDRNDPFFAPTVEMAKKDGINLDTDNKVIRDGNKVRVYMTSMAPAFGVQEF
TVKQGDEVTVTITNIDQIEDVSHGFVVVNHGVSMEISPQQTSSITFVADKPGLHWYYCSWFCHALHMEMVGRMMVEPA

>Neisseria gonorrhoeae (no crystal, predicted copper binding residues)
MSDEKLEQNGLSRRSFLGTAAASGAGIAGAGLLGLAGCSKDGEQAAANASGAAPVAKAQGESKPGQLSSEVGPGELDQYYGFL
SGGQSGEMRLIGLPSMRELMRIPVFNMDSATGWGRTNESLKVLNGNITEETRKFLKDSGLRCYPNGDLHHPHLSFTDQTYDGR
YAYANDKANNRVCRVRLDVMKADKIIDIPNDSGIHGLRPQRYPKTGYVFANGEHITPVSGVGKLDDAKTWNAVYTAIDGETME
IAWQVLVDGNLDNGDADYQGKYSFATCYNSERALTVQGASSNEQDWCVVFDLKAIEEGIKAGDFKEVNGVKMLDGRAEAKSKY
TRYIPVPNSPHGCNASPDGKYIMPNGKLPPTVTVLDVSKLDDLFAGKIKERDVVVAEPQLGLGPLHTAFDGRGNAYTTLFIDS
QMVKWNIDDAIKAYKGEKIDPIKQKLDVHYQPGHNHTTMGETKEADGQWLVSLNKFSKDRFLNAGPLKPECDQLIGISGDEMR

LVHDNPTFAEPHDLCLVAASKLNPGKTWDRKDPWF*QDALEQAKKDGVELEKAAKAVREGNKVRVYMTAVAPAYSIPQFEVNQ
GDEVTVYVTNVETIEDLTHGFTLEGCGIAMEIGPQATSSVTFKAVRPGVHWYYCQWFCHALHMEMSGQMIVHPK*

16.7 Appendix 7: *nosZ* gene nucleotide sequences from species which have produced NosZ crystals

>Achromobacter_cycloclastes_ATCC_21921_IAM_1013_nosZ_AF047429.2
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AGGGCCGGCCGCACTCAGCCTCGGCGCTGCAGGATTGGCGACACCGGCCCGTGCGGCCACGGGTGCCGACGGCTCGGTCGCAC
CGGGCAAGCTCGACGATTACTACGGCTTCTGGTCCTCCGGTCAGACCGGCGAGATGCGCATTCTCGGCATTCCCTCGATGCGC
GAACTGATGCGCGTGCCGGTGTTCAACCGTTGCTCGGCCACCGGTTGGGGGCAGACCAATGAATCGATCCGGATCCATCAGCG
CACGATGACGGAAAAGACGAAGAAGCAGCTTGCCGCCAACGGCAAGAAAATCCACGACAACGGCGACCTGCATCACGTCCATA
TGTCCTTCACCGACGGTAAATATGACGGTCGCTACCTGTTCATGAACGACAAGGCCAATACGCGCGTGGCGCGCGTGCGCTGC
GACGTGATGAAGACCGACGCCATCCTGGAGATCCCCAACGCCAAGGGCATCCACGGCATGCGTCCGCAGAAATGGCCGCGTTC
AAACTATGTGTTCTGCAACGGCGAGGACGAGGCCCCGCTCGTCAACGACGGCTCGACGATGACGGACGTGGCGACCTACGTGA
ACATCTTCACCGCCGTCGATGCCGACAAGTGGGAAGTGGCGTGGCAGGTGAAGGTCTCGGGCAACCTCGACAATTGCGATGCC
GACTACGAGGGCAAGTGGGCGTTCTCCACCAGCTACAACTCCGAAATGGGCATGACGCTGGAGGAGATGACCAAGTCCGAGAT
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TGGTGGACGGGCGCAAGGAGGCAAAGTCGCTCTTCACGCGCTACATCCCGATCGCCAACAACCCCCACGGCTGCAACATGGCG
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TGCCGCCAACGACTGGCTCGTTTGCCTGTGCAAATTCTCCAAGGACCGGTTCCTGAATGTCGGCCCGCTGAAGCCGGAAAACG
ATCAGTTGATCGACATTTCCGGTGACAAGATGGTGCTGGTCCATGACGGCCCGACCTTCGCCGAGCCGCATGACGCCATCGCC
GTCTCCCCCTCGATCCTGCCGAACATCCGCTCGGTCTGGGATCGCAAGGATCCGCTGTGGGCCGAAACCCGCAAGCAGGCCGA
GGCCGATGAGGTCGACATCGACGAATGGACCGAGGCCGTGATCCGCGACGGCAACAAGGTTCGCGTCTACATGACCTCGGTCG
CACCCAGCTTCAGCCAGCCGAGCTTTACGGTGAAGGAGGGCGACGAAGTCACGGTCATCGTCACCAATCTCGATGAAATCGAT
GACCTTACCCATGGCTTCACCATGGGCAATCACGGCGTGGCGATGGAGGTCGGCCCGCAACAGACGAGCTCCGTTACCTTCGT
TGCGGCCAATCCTGGCGTCTACTGGTACTATTGCCAATGGTTCTGCCATGCCCTGCACATGGAAATGCGCGGCCGCATGTTCG
TGGAACCGAAGGGCGCC**TGA**

>Achromobacter_cycloclastes_ATCC_21921_IAM_1013_nosZ_AF047429.2
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ELMRVPVFNRCSATGWGQTNESIRIHQRTMTEKTKKQLAANGKKIHDNGDLHHVHMSFTDGKYDGRYLFMNDKANTRVARVRC
DVMKTDAILEIPNAKGIHGMRPQKWPRSNYVFCNGEDEAPLVNDGSTMTDVATYVNIFTAVDADKWEVAWQVKVSGNLDNCDA
DYEGKWAFSTSYNSEMGMTLEEMTKSEMDHVVVFNIAEIEKAIKAGQYEEINGVKVVDGRKEAKSLFTRYIPIANNPHGCNMA
PDRKHLCVAGKLSPTVTVLDVTKFDALFYDNAEPRSAVVAEPELGLGPLHTAFDGRGNAYTSLFLDSQVVKWNIDEAIRAYAG
EKINPIKDKLDVQYQPGHLKTVMGETLDAANDWLVCLCKFSKDRFLNVGPLKPENDQLIDISGDKMVLVHDGPTFAEPHDAIA
VSPSILPNIRSVWDRKDPLWAETRKQAEADEVDIDEWTEAVIRDGNKVRVYMTSVAPSFSQPSFTVKEGDEVTVIVTNLDEID
DLTHGFTMGNHGVAMEVGPQQTSSVTFVAANPGVYWYYCQWFCHALHMEMRGRMFVEPKGA*

>Paracoccus_denitrificans_NL1B8944_nosZ_X74792.1
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GCCAACAACCCGCATGGCTGCAACATGGCGCCGGACAAGAAGCACCTGTGCGTCGCGGGCAAGCTGTCGCCCACGGCGACGGT
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CCTCAAGACCGTGATGGGCGAGACGCTGGACGCCACCAACGACTGGCTGGTCTGCCTGTCCAAGTTCTCGAAGGACCGCTTCC
TGAACGTCGGGCCGCTGAAGCCGGAGAACGACCAGCTGATCGACATCTCGGGCGACAAGATGGTGCTGGTCCATGACGGCCCC
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GTGATCGTCACCAACCTGGACGAGATCGACGATCTGACGCATGGCTTCACCATGGGCAACTACGGCGTCGCCATGGAGATCGG

GCCGCAGATGACCAGCTCGGTCACCTTCGTCGCCGCCAATCCCGGGGTCTATTGGTATTATTGCCAATGGTTCTGCCATGCGC
TGCACATGGAGATGCGCGGCCGCATGCTGGTCGAGCCGAAGGAGGCC**TGA**

>Paracoccus_denitrificans_NL1B8944_nosZ_X74792.1
MESKQEKGLSRRALLGATAGGAAVAGAFGGRLALGPAALGLGTAGVATVAGSGAALAASGDGSVAPGQLDDYYGFWSSGQSGE
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ANTRVARVRCDVMKCDAILEIPNAKGIHGLRPQKWPRSNYVFCNGEDETPLVNDGTNMEDVANYVNVFTAVDADKWEVAWQVL
VSGNLDNCDADYEGKWAFSTSYNSEKGMTLPEMTAAEMDHIVVFNIAEIEKAIAAGDYQELNGVKVVDGRKEASSLFTRYIPI
ANNPHGCNMAPDKKHLCVAGKLSPTATVLDVTRFDAVFYENADPRSAVVAEPELGLGPLHTAFDGRGNAYTSLFLDSQVVKWN
IEDAIRAYAGEKVDPIKDKLDVHYQPGHLKTVMGETLDATNDWLVCLSKFSKDRFLNVGPLKPENDQLIDISGDKMVLVHDGP
TFAEPHDAIAVHPSILSDIKSVWDRNDPMWAETRAQAEADGVDIDNWTEEVIRDGNKVRVYMSSVAPSFSIESFTVKEGDEVT
VIVTNLDEIDDLTHGFTMGNYGVAMEIGPQMTSSVTFVAANPGVYWYYCQWFCHALHMEMRGRMLVEPKEA*

>Marinobacter_hydrocarbonoclasticus_617_NosZ_DQ504302.1
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CCCGCAACAAGGCTCACGTCGCCCCGGGCGAGTTGGATGAGTACTACGGCTTCTGGAGTGGTGGCCACCAGGGCGAAGTGCGC
GTGCTTGGCGTGCCCTCCATGCGTGAACTGATGCGTATTCCGGTATTTAACGTGGATTCCGCAACGGGTTGGGGTATTACCAA
TGAAAGTAAGGAAATCCTGGGCGGTGATCAGCAGTACCTGAACGGCGACTGCCACCACCCGCACATTTCCATGACTGACGGTC
GTTACGATGGTAAGTACCTGTTTATCAACGACAAGGCCAACACCCGGGTTGCCCGTATCCGTCTGGATATCATGAAGACGGAC
AAGATTACCCACATCCCGAACGTTCAGGCCATCCATGGTCTGCGCCTGCAGAAAGTACCCAAGACCAACTACGTGTTCTGTAA
CGCCGAGTTCGTCATCCCGCAGCCCAACGACGGTACGGATTTCAGCCTGGACAACAGCTACACCATGTTCACCGCCATCGACG
CAGAAACCATGGATGTAGCCTGGCAGGTCATCGTTGACGGTAACCTGGATAACACCGATGCCGACTACACCGGCAAGTACGCT
ACTTCTACCTGTTACAACTCAGAGCGTGCCGTTGATCTTGCGGGCACCATGCGTAACGACCGCGACTGGGTGGTTGTGTTCAA
TGTGGAGCGCATTGCTGCAGCGGTAAAAGCCGGCAACTTCAAGACCATTGGCGATTCCAAGGTGCCGGTTGTCGACGGCCGAG
GCGAATCCGAATTCACCCGCTATATTCCTGTGCCGAAGAACCCGCACGGTCTGAACACCTCTCCCGATGGCAAGTACTTCATT
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CGACACCATCGTGGCCGAGCCGGAGCTGGGTCTCGGACCCTTGCACACTACCTTCGACGGCCGTGGCAATGCCTACACCACGC
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>Marinobacter_hydrocarbonoclasticus_617_NosZ_DQ504302.1
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>Pseudomonas_stutzeri_A1501_NosZ_AY957390.1
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CC**TGA**

>Pseudomonas_stutzeri_A1501_NosZ_AY957390.1
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>Shewanella_denitrificans_OS217_NosZ_WP_011496652.1
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>Shewanella_denitrificans_OS217_NosZ_WP_011496652.1
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GKLSPTVSVVEIARLDDVFSGKIQPRDAIVAEPELGLGPLHTAFDNKGNAFTTLFLDSQIAKWNIQDAIKAYNGEKVNYLRQK
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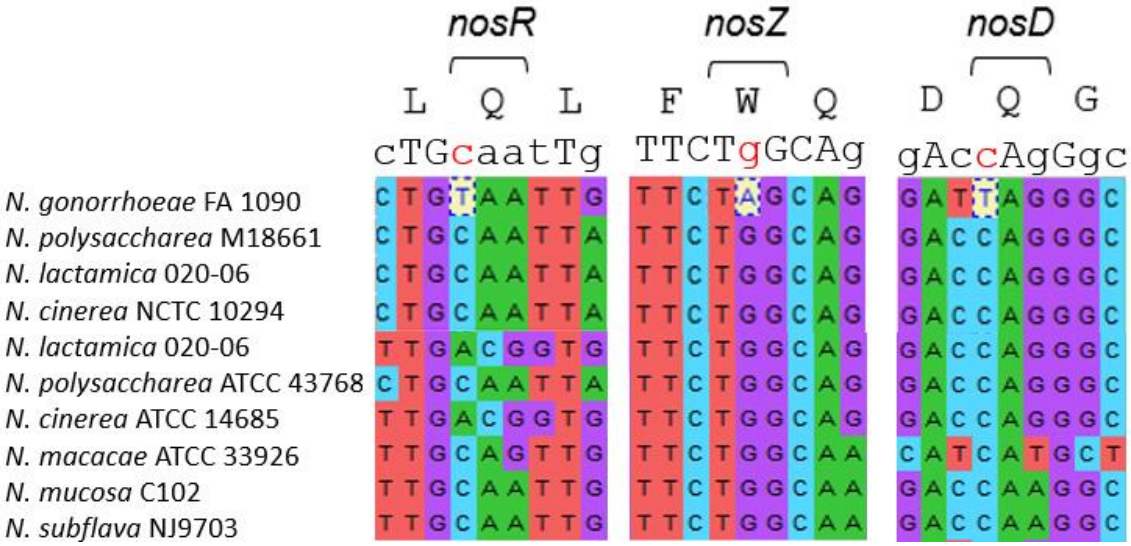
KLWTRDDPMFADTVAMAKQDGVTLEMDNKVIRDGNKVRVYMTSIAPNFGMNEFKVKLGDEVTVVVTNLDQVEDVTHGFCMTNH
GVQMEVAPQATASVTFIANKPGVQWYYCNWFCHALHMEMRGRMLVEA*

16.8 Appendix 8: Sanger sequencing *N. gonorrhoeae* strain F62 *nos* operon

Overton et al (2006) suggest that mutations in *N. gonorrhoeae nos* are frameshifts in the F62 strain – i.e. *indels*.

Here are the FA1090 sequences compared to other strains:



In FA1090, the *nosR* nonsense mutation is a substitution: C→A.

In FA1090, the *nosZ* nonsense mutation is a substitution: G→A.

In FA1090, the *nosD* nonsense mutation is a substitution: C→T.

16.8.1 NosR in F62 is the same as in FA1090

FA1090 n*osR* stop codon is:

TGACGGT**GCG**GAG**CCG**TGG**CGG**CTG**TAA**TTG**ATG**GTT**CAG**CGC**GTC**TTGAGTG

nosR-int-F sequencing:

TGACGGTGCGGAGCCGTGGCGGCTG**TAA**TTGATGGTTCAGCGCGTATTGAGTG

Confirms substitution; no frameshift

diluted *nosR-int-F* seq:

TGACGGTGCGGAGCCGTGGCGGCTG**TAA**TTGATGGTTCAGCGCGTATTGAGTG

Confirms substitution; no frameshift

nosR-int-Rv2 seq:

TGACGGTGCGGAGCCGTGGCGGCTG**TAA**TTGATGGTTCAGCGCGTATTGAGTG

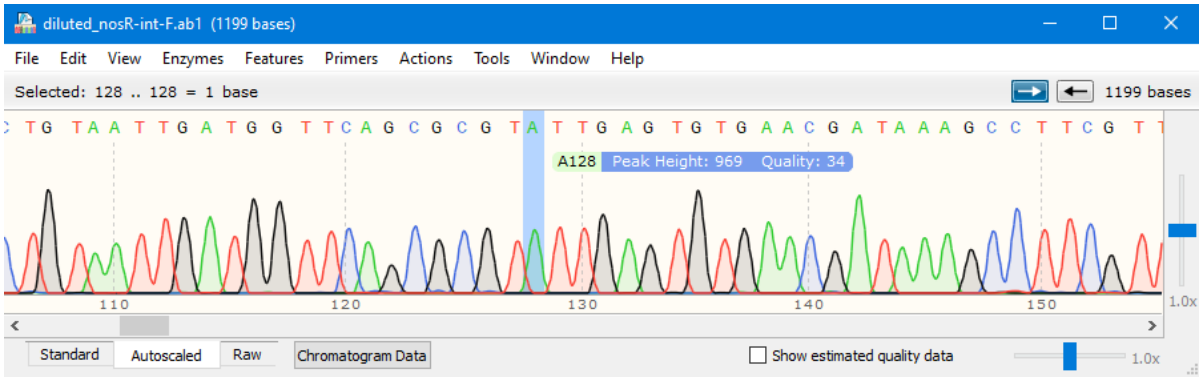
Confirms substitution; no frameshift

diluted *nosR-int-Rv2* seq:

TGACGGTGCGGAGCCGTGGCGGCTG**TAA**TTGATGGTTCAGCGCGTATTGAGTG

Confirms substitution; no frameshift

The is a substitution downstream which does not affect frame or codon (Val → Val). The signal peaks for this are quite convincing:



16.8.2 NosZ in F62 is the same as in FA1090

FA1090 *nosZ* stop codon is:
T**TGG**GAC**CGC**AAA**GAC**CCG**TGG**TTC**TAG**CAG**GAT**GCT**TTG**GAA**CAG**GCG**AAA**A

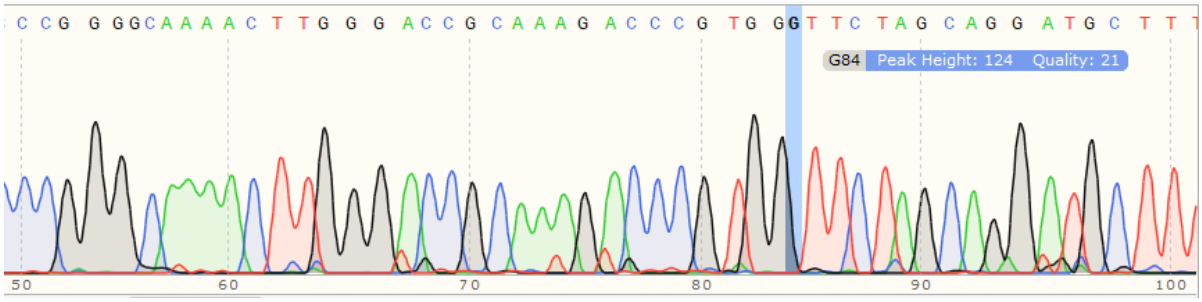
nosZ-int-F sequencing:
T**TGG**GAC**CGC**AAA**GAC**CCG**TGG**GTT**CTA**GCA**GGA**TGC**TTT**GGA**ACA**GGC**GAA**A

TGGTTC**TAG**CAGGAT

TGG**G**TTC**TAG**CAGGA

Insertion.

DBS Genomics said: "Your sequencing results are attached. Some have produced fairly high signals so we will dilute and rerun them." NosR and NosZ (but not NosD) samples were diluted.



It is clear when we look at the sequencing signals, that this is not a peak at all!

Overall verdict: substitution, not indel.

diluted *nosZ-int-F* seq:
T**TGG**GAC**CGC**AAA**GAC**CCG**TGG**TTC**TAG**CAG**GAT**GCT**TTG**GAA**CAG**GCG**AAA**A

Confirms substitution; no framshift

nosZ-int-Rv2 seq:
T**TGG**GAC**CGC**AAA**GAC**CCG**TGG**TTC**TAG**CAG**GAT**GCT**TTG**GAA**CAG**GCG**AAA**A

Confirms substitution; no framshift

16.8.3 NosD in F62 is the same as in FA1090

FA1090 *nosD* stop codon is
CAATATCGCCGTCGGCAGC**CGC**GAT**TAG**GGC**ATC**ATGCTCAACTATGTCAACT

nosD-int-Fv2 seq:
CAATATCGCCGTCGGCAGC**CGC**GAT**TAG**GGC**ATC**ATGCTCAACTATGTCAACT

Confirms substitution; no framshift

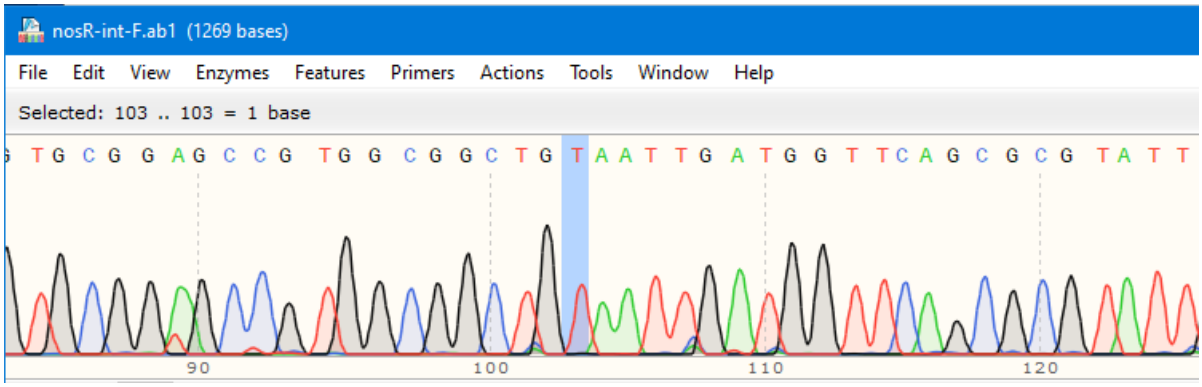
nosD-int-R seq:
CAATATCGCCGTCGGCAGC**CGC**GAT**TAG**GGC**ATC**ATGCTCAACTATGTCAACT

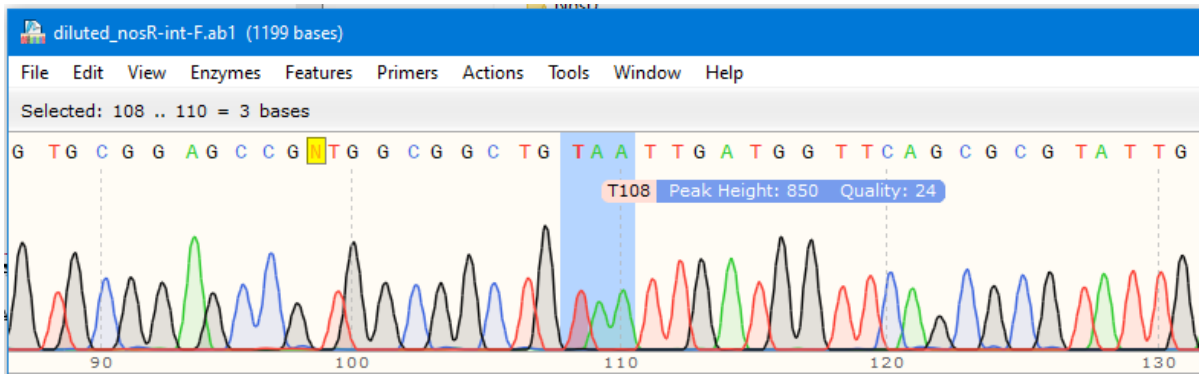
Confirms substitution; no framshift

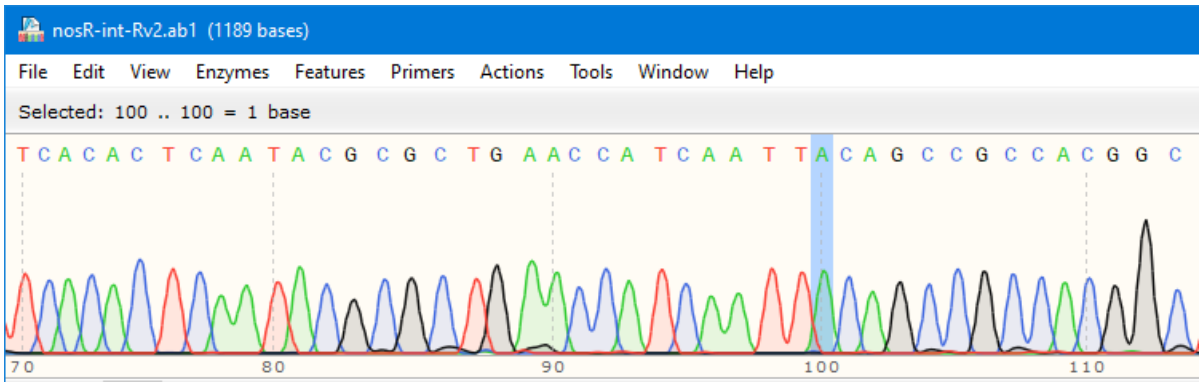
16.8.4 Conclusions

Neisseria gonorrhoeae F62 has *substitution* nonsense mutations (like F1090), not indel-frameshift nonsense mutations.

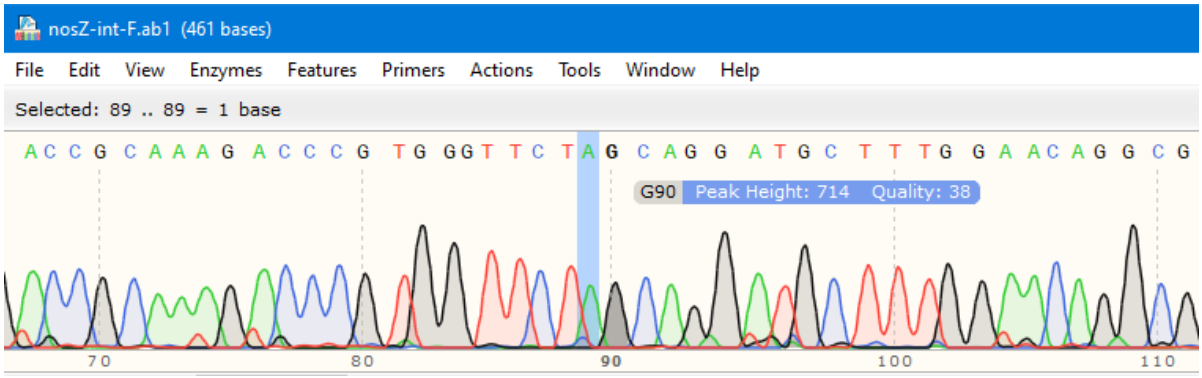
It should be noted that the F62 *nosRZD* stop codon regions are sometimes poorly amplified (see below) – which might explain why previous authors believed there had been a single-base deletion.

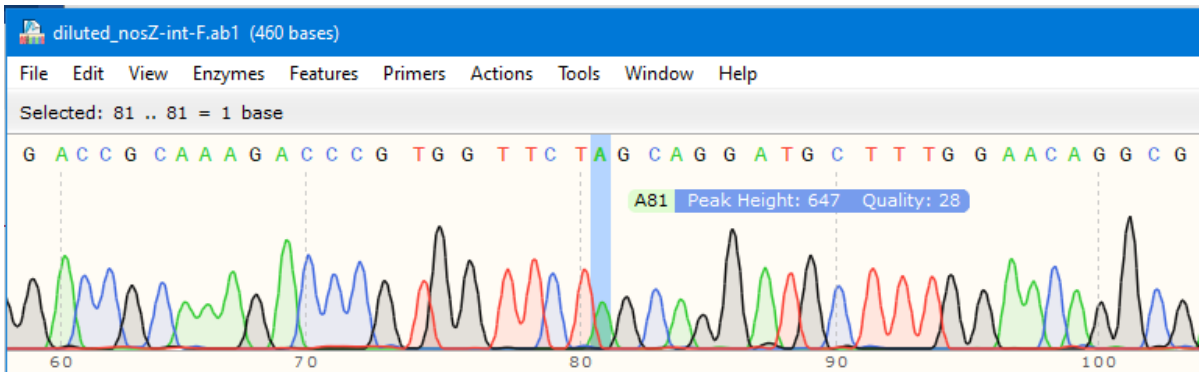


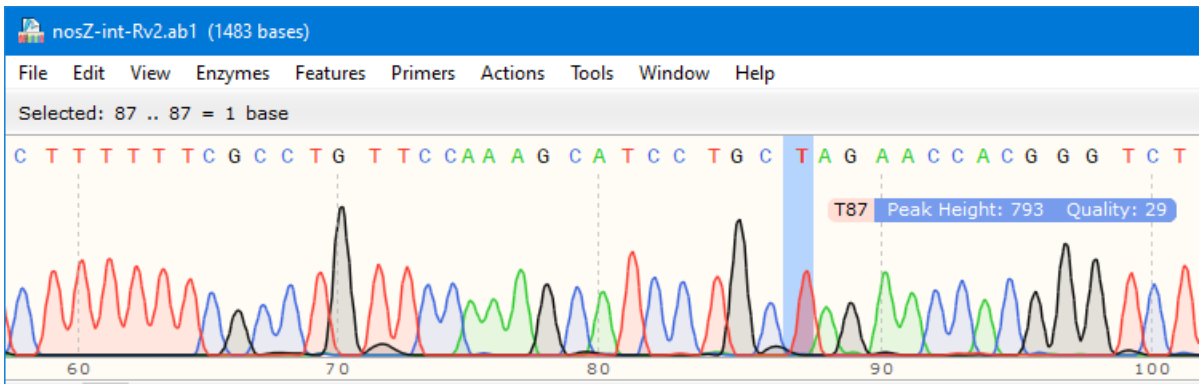





diluted_nosR-int-Rv2.ab1 (1206 bases)
File Edit View Enzymes Features Primers Actions Tools Window Help
Selected: 88 .. 88 = 1 base
A88 Peak Height: 917 Quality: 46
60 70 80 90 100


nosZ-int-F.ab1 (461 bases)
File Edit View Enzymes Features Primers Actions Tools Window Help
Selected: 89 .. 89 = 1 base
G90 Peak Height: 714 Quality: 38
70 80 90 100 110


diluted_nosZ-int-F.ab1 (460 bases)
File Edit View Enzymes Features Primers Actions Tools Window Help
Selected: 81 .. 81 = 1 base
A81 Peak Height: 647 Quality: 28
60 70 80 90 100


nosZ-int-Rv2.ab1 (1483 bases)
File Edit View Enzymes Features Primers Actions Tools Window Help
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T87 Peak Height: 793 Quality: 29
60 70 80 90 100


nosD-int-Fv2.ab1 (684 bases)
File Edit View Enzymes Features Primers Actions Tools Window Help
Selected: 86 .. 87 = 2 bases
A T A T C G C C G T C G G C A G C C G C G A T T A G G G C A T C A T G C T C A A C T A T G
T87 Peak Height: 788 Quality: 40
70
80
90
100
nosD-int-R.ab1 (840 bases)
File Edit View Enzymes Features Primers Actions Tools Window Help
Selected: 74 .. 75 = 2 bases
A A T A G T T G A C A T A G T T G A G C A T G A T G C C C T A A T C G C G G C T G C C G A C
A74 Peak Height: 543 Quality: 45
50
60
70
80

16.9 Appendix 9: BLASTn of FA1090 and TUM19854 *nosZ*

```
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>Ngono_TUM19854_ASM1303007v1_nosZ
Sequence ID: Query_445519 Length: 1968
Range 1: 1 to 1968

Score:3624 bits(1962), Expect:0.0,
Identities:1966/1968(99%),  Gaps:0/1968(0%), Strand: Plus/Plus

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Sbjct  1     ATGTCAGACGAAAAATTAGAACAAAACGGCTTGAGCCGTCGTTCGTTCTTAGGTACGGCC  60

Query  61    GCCGCTTCCGGTGCAGGTATTGCCGGTGCGGGCCTGTTGGGTTTGGCGGGTTGCTCTAAA  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61    GCCGCTTCCGGTGCAGGTATTGCCGGTGCGGGCCTGTTGGGTTTGGCGGGTTGCTCTAAA  120

Query  121   GACGGCGAACAGGCTGCCGCTAACGCTTCCGGCGCGGCTCCCGTCGCCAAGGCGCAAGGG  180
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Sbjct  121   GACGGCGAACAGGCTGCCGCTAACGCTTCCGGCGCGGCTCCCGTCGCCAAGGCGCAAGGG  180

Query  181   GAATCCAAACCCGGCCAACTGTCTTCCGAAGTCGGTCCGGGCGAACTCGATCAATATTAC  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181   GAATCCAAACCCGGCCAACTGTCTTCCGAAGTCGGTCCGGGCGAACTCGATCAATATTAC  240

Query  241   GGTTTCCTTTCCGGCGGCCAGTCCGGCGAGATGCGCCTGATCGGTCTGCCTTCTATGCGC  300
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Query  301   GAACTGATGCGTATCCCCGTGTTCAATATGGACAGCGCGACCGGTTGGGGACGCACCAAT  360
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Sbjct  301   GAACTGATGCGTATCCCCGTGTTCAATATGGACAGCGCGACCGGTTGGGGACGCACCAAT  360

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Query  961   ATGCTGGACGGCCGCGCTGAGGCCAAATCCAAATACACGCGTTATATCCCTGTGCCGAAC  1020
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  961   ATGCTGGACGGCCGCGCTGAGGCCAAATCCAAATACACGCGTTATATCCCTGTGCCGAAC  1020

Query  1021  TCTCCTCACGGCTGTAACGCAAGCCCTGACGGTAAATACATCATGCCCAACGGTAAACTG  1080
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1021  TCTCCTCACGGCTGTAACGCAAGCCCTGACGGTAAATACATCATGCCCAACGGTAAACTG  1080

Query  1081  CCTCCAACCGTTACCGTATTGGATGTCAGCAAACTGGACGATTTGTTCGCCGGCAAAATC  1140
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1081  CCTCCAACCGTTACCGTATTGGATGTCAGCAAACTGGACGATTTGTTCGCCGGCAAAATC  1140

Query  1141  AAAGAGCGCGATGTGGTCGTAGCCGAACCGCAACTGGGTCTCGGCCCGTTACACACTGCA  1200
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1141  AAAGAGCGCGATGTGGTCGTAGCCGAACCGCAACTGGGTCTCGGCCCGTTACACACTGCA  1200

Query  1201  TTCGACGGTCGCGGCAATGCTTATACGACATTGTTTATCGACAGCCAAATGGTGAAATGG  1260
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1201  TTCGACGGTCGCGGCAATGCTTATACGACATTGTTTATCGACAGCCAAATGGTGAAATGG  1260

Query  1261  AATATTGACGATGCGATCAAAGCCTACAAAGGCGAGAAAATCGATCCGATCAAACAAAAA  1320
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1261  AATATTGACGATGCGATCAAAGCCTACAAAGGCGAGAAAATCGATCCGATCAAACAAAAA  1320

Query  1321  CTCGACGTTCACTATCAACCGGGTCACAACCATACGACCATGGGCGAAACCAAAGAAGCC  1380
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1321  CTCGACGTTCACTATCAACCGGGTCACAACCATACGACCATGGGCGAAACCAAAGAAGCC  1380

Query  1381  GACGGTCAATGGCTGGTGTCCTTGAACAAATTCTCCAAAGACCGCTTCTTGAATGCCGGT  1440
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1381  GACGGTCAATGGCTGGTGTCCTTGAACAAATTCTCCAAAGACCGCTTCTTGAATGCCGGT  1440

Query  1441  CCTTTGAAACCCGAGTGCGACCAATTGATCGGCATCTCCGGCGACGAAATGCGTCTGGTA  1500
             |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct  1441  CCTTTGAAACCCGAGTGCGACCAATTGATCGACATCTCCGGCGACGAAATGCGTCTGGTA  1500

Query  1501  CACGACAACCCGACTTTTGCCGAACCGCACGACTTGTGTTTGGTTGCCGCGTCCAAACTG  1560
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1501  CACGACAACCCGACTTTTGCCGAACCGCACGACTTGTGTTTGGTTGCCGCGTCCAAACTG  1560

Query  1561  AACCCGGGCAAAACTTGGGACCGCAAAGACCCGTGGTTCTAGCAGGATGCTTTGGAACAG  1620
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1561  AACCCGGGCAAAACTTGGGACCGCAAAGACCCGTGGTTCTAGCAGGATGCTTTGGAACAG  1620

Query  1621  GCGAAAAAAGACGGTGTCGAGCTGGAAAAAGCCGCCAAAGCCGTACGCGAAGGCAACAAA  1680
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1621  GCGAAAAAAGACGGTGTCGAGCTGGAAAAAGCCGCCAAAGCCGTACGCGAAGGCAACAAA  1680

Query  1681  GTACGCGTGTATATGACGGCCGTCGCGCCTGCTTACAGTATCCCGCAGTTTGAAGTGAAC  1740
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1681  GTACGCGTGTATATGACGGCCGTCGCGCCTGCTTACAGTATCCCGCAGTTTGAAGTGAAC  1740

Query  1741  CAAGGCGACGAAGTAACCGTTTACGTTACCAACGTCGAAACCATCGAGGACCTGACCCAC  1800
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1741  CAAGGCGACGAAGTAACCGTTTACGTTACCAACGTCGAAACCATCGAGGACCTGACCCAC  1800

Query  1801  GGCTTCACTTTGGAAGGCTGCGGCATCGCTATGGAAATCGGCCCGCAAGCCACTTCTTCC  1860
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1801  GGCTTCACTTTGGAAGGCTGCGGCATCGCTATGGAAATCGGCCCGCAAGCCACTTCTTCC  1860

Query  1861  GTAACCTTCAAAGCCGTCCGCCCGGGTGTGCACTGGTACTACTGCCAATGGTTCTGCCAC  1920
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1861  GTAACCTTCAAAGCCGTCCGCCCGGGTGTGCACTGGTACTACTGCCAATGGTTCTGCCAC  1920

Query  1921  GCATTGCATATGGAAATGTCCGGTCAGATGATTGTTCATCCCAAATAA  1968
             ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1921  GCATTGCATATGGAAATGTCCGGTCAGATGATTGTTCATCCCAAATAA  1968
```

16.10 Appendix 10: Static 8 hr and shaking 3 hr gonococcal culture fold change data

	Gene expression ($2^{-\Delta\Delta Cq}$) in this study						
	Static expression			Shaking expression			
Gene	**Fold change 0 vs 8 hr (+NO2- cultures)**	**Fold change +NO_2^- (8 hr cultures)**		**Fold change 0 vs 3 hr (+NO2- cultures)**	**Fold change +NO_2^-(3 hr cultures)**	**FNR site**	**Chromosome location (bp)**
aniA †	11.7	8.1		46.3	1.8	TTGACTTAAATTAA	924,020
norB	10.6	24.5		10.6	8.0	TGAATTTTAAACAA	925,572
nosZ(N)	5.7	5.8		4.6	1.9	TTGATTTGCATCAA	789,543
nosZ(C)	4.2	2.7		1.5	1.3	TTGATTTGCATCAA	789,543
laz	1.1	3.6		0.8	0.6	TTGATTTCCGTAAA	1,188,745
sco	0.7	3.5		1.6	0.6	-	964,295
nosL	1.2	2.4		1.0	0.7	-	793,205
accA †	1.1	2.7		4.0	0.48	TTAATCTACATCAA	987,781
aph	1.1	2.3		0.9	0.8	TTGAGTGAAAATAA	476,597
copA2	1.7	2.2		0.7	1.2	-	1,476,918
ccp	1.2	1.2		5.4	0.4	TTGATTTTCCGCAA	419,185
copA	1	1.4		0.9	0.9	-	1,585,805
ompU †	1.6	1.8		0.8	0.6	-	511,238
res †	0.6	0.49		0.4	1.1	TTGAATGTGATTAT	1,630,754

16.11 Appendix 11: Mutant *nos* strain whole-genome sequencing SNPs

Whole-genome sequencing (WGS) found there to be 269 definite SNPs in the WT, Δ*nosZ*, *nosZ**534W, *strepII::nosZ* or *nosZ::strepII* strains compared to the FA1090 strain genome assembly ASM684v1, wherein at least 90% of reads had the variant and the read depth was at least 10x. Of these 269 definite SNPs, 243 were non-discriminatory (i.e. there was no variation between the five different strains sent for WGS). A '.' in the sample column means that variant calling could not be performed at that location for that sample - this is usually due to the depth of reads being below the coverage threshold.

Locus Tag	FA1090 position	Amino acid Substitution	FA1090 nt	WT A	WT B	KO A	KO B	strepII::nosZ A	strepII::nosZ B	nosZ::strepII A	nosZ::strepII B	Discriminatory
NGO_0001	2,153,543	F74	T	C	C	C	C	C	C	C	C	N
NGO_0002	2,152,077	F18V	T	G	G	G	G	G	G	G	G	N
NGO_0002	2,152,055	L25R	T	G	G	G	G	G	G	G	G	N
NGO_0037	2,119,396	V405A	A	G	G	G	G	G	G	G	G	N
NGO_00280	2,097,806	F98L	T		C	.	C	.				Y
Intron	2,097,612		T	C	C	.	C	C	C	C	C	N
NGO_0095	2,047,999	T70	C	G	G	G	G	G	G	G	G	N
Intron	2,032,559		C	CG	CG	CG	CG	CG	CG	CG	CG	N
NGO_00715	2,012,114		C	T	T	T	T	T	T	T	T	N
NGO_00715	2,012,109		GC	G	G	G	G	G	G	G	G	N
NGO_0138	2,007,954	A458	C	G	G	G	G	G	G	G	G	N
NGO_0139	2,006,120	Q103E	G	C	C	C	C	C	C	C	C	N
NGO_0139	2,006,099	V96L	C	G	G	G	G	G	G	G	G	N
NGO_0168	1,982,273	T158A	T	C	C	C	C	C	C	C	C	N
NGO_0168	1,982,072	E91K	C	T	T	T	T	T	T	T	T	N
NGO_0187	1,965,594	P349R	G	C	C	C	C	C	C	C	C	N
NGO_0235	1,917,239	T803A	T	C	C	C	C	C	C	C	C	N
NGO_0238	1,911,766		G	C	C	C	C	C	C	C	C	N
NGO_0238	1,911,762		T	C	C	C	C	C	C	C	C	N
NGO_0238	1,911,723		A	C	C	C	C	C	C	C	C	N

NGO_0238	1,911,721		G	C	C	C	C	C	C	C	C	N
NGO_0238	1,911,704		G	C	C	C	C	C	C	C	C	N
NGO_0238	1,911,703		T	G	G	G	G	G	G	G	G	N
NGO_0238	1,911,697		CG	C	C	C	C	C	C	C	C	N
NGO_01405	1,894,817		AT	A	A	A	A	A	A	A	A	N
Intron	1,865,663		C	T	T	T	T	T	T	T	T	N
NGO_0318	1,837,550	F212L	T	C	C	C	C	C	C	C	C	N
NGO_0318	1,837,053	D377E	C	A	A	A	A	A	A	A	A	N
NGO_0340	1,818,594	V190	G	A	A	A	A	A	A	A	A	N
NGO_0340	1,818,572	I198V	A	G	G	G	G	G	G	G	G	N
NGO_0340	1,818,569	E199K	G	A	A	A	A	A	A	A	A	N
NGO_0340	1,818,563	C201G	T	G	G	G	G	G	G	G	G	N
NGO_0340	1,818,562	C201F	G	T	T	T	T	T	T	T	T	N
NGO_0340	1,818,552	E204	A	G	G	G	G	G	G	G	G	N
NGO_0340	1,818,551	A205P	G	C	C	C	C	C	C	C	C	N
NGO_0340	1,818,547	G206D	G	A	A	A	A	A	A	A	A	N
NGO_0340	1,818,546	G206	C	G	G	G	G	G	G	G	G	N
NGO_0385	1,775,630	S117P	T	C	C	C	C	C	C	C	C	N
NGO_0414	1,742,214	*567	A	G	G	G	G	G	G	G	G	N
NGO_0420	1,737,535	P26	TC	T	T	T	T	T	T	T	T	N
NGO_02330	1,721,573		C	G	G	G	G	G	G	G	G	N
NGO_02330	1,721,536		C	G	G	G	G	G	G	G	G	N
NGO_02330	1,721,482		C	CG	CG	CG	CG	CG	CG	CG	CG	N
NGO_02520	1,693,242		CG	C	C		.	C	C	C	C	Y
Intron	1,680,983		C	A	A	A	A	A	A	A	A	N
NGO_0509	1,669,165	H10	TG	T	T	T	T	T	T	T	T	N
NGO_0509	1,669,147	R4Q	C	T	.	T	.	.	.	.	T	N
Intron	1,669,051		C	T	T	T	T	T	T	T	T	N
NGO_0513	1,664,173	I31S	T	G	G	G	G	G	G	G	G	N
Intron	1,663,418		CA	C	C	C	C	C	C	C	C	N
NGO_0545	1,634,264	S548	C	G	G	G	G	G	G	G	G	N
NGO_0559	1,616,624	K121	A	G	G	G	G	G	G	G	G	N
NGO_0566	1,603,839	H181Y	C	T	T	T	T	T	T	T	T	N
NGO_0571	1,598,837	K406	A	G	G	G	G	G	G	G	G	N
NGO_0571	1,598,831	K408	A	G	G	G	G	G	G	G	G	N
NGO_0571	1,598,778	K426R	A	G	G	G	G	G	G	G	G	N
Intron	1,590,198		TA	T	T	T	T	T	T	T	T	N
Intron	1,527,999		A	G	G	G	G	G	G	G	G	N
Intron	1,527,972		C	A	A	A	A	A	A	A	A	N
Intron	1,527,937		G	A	A	A	A	A	A	A	A	N
lldD	1,527,900	I11	T	C	C	C	C	C	C	C	C	N
lldD	1,527,889	H15R	A	G	G	G	G	G	G	G	G	N
NGO_0662	1,499,588	F98S	A	G	G	G	G	G	G	G	G	N
Intron	1,484,951		G	A	A	A	A	A	A	A	A	N
Intron	1,465,348		C	G	G	G	G	G	G	G	G	N
NGO_0719	1,433,674	L218	G	A	A	A	A	A	A	A	A	N
NGO_03890	1,423,315	W188R	A	G	G	G	G	G	G	G	G	N
Intron	1,398,928		A	C	C	C	C	C	C	C	C	N
Intron	1,398,927		C	G	G	G	G	G	G	G	G	N
Intron	1,398,908		C	G	G	G	G	G	G	G	G	N
Intron	1,398,889		CG	C	C	C	C	C	C	C	C	N
recR	1,391,239	Q192	A	G	G	G	G	G	G	G	G	N
NGO_04070	1,391,048		GC	G	G	G	.	G	G	G	G	N
NGO_04090	1,386,590		GT	G	G	G	G	G	G	G	G	N
NGO_0805	1,356,752	N120D	A	G	G	G	G	G	G	G	G	N
NGO_0831	1,333,184		T	G	G	G	G	G	G	G	G	N
NGO_0831	1,333,141		A	G	G	G	G	G	G	G	G	N
NGO_0831	1,333,097		CT	C	C	C	C	C	C	C	C	N
NGO_04510	1,312,425		CG	C	C	C	C	C	C	C	C	N
Intron	1,307,596		TG	T	T	T	T	T	T	T	T	N
NGO_0868	1,302,134	E100K	G	A	A	A	A	A	A	A	A	N
NGO_0874	1,295,104	I272K	T	A	A	A	A	A	A	A	A	N
NGO_0874	1,295,063	F286V	T	G	G	G	G	G	G	G	G	N
NGO_0892	1,283,076	Q68P	A	C	C	C	C	C	C	C	C	N
NGO_0892	1,283,075	Q68H	A	T	T	T	T	T	T	T	T	N
NGO_0900	1,273,795	L322	G	C	C	C	C	C	C	C	C	N
NGO_0900	1,273,721	G347D	G	A	A	A	A	A	A	A	A	N
NGO_0903	1,270,032	G96C	C	A	A	A	A	A	A	A	A	N
NGO_0929	1,244,205	A222	A	T	T	T	T	T	T	T	T	N
Intron	1,216,022		C		CCAAGCAAG	.						Y
Intron	1,211,396		G	C	C	C	C	C	C	C	C	N
Intron	1,211,394		G	T	T	T	T	T	T	T	T	N
Intron	1,211,338		G	C	C	C	C	C	C	C	C	N
NGO_1041	1,151,992	S26N	G	A	A	A	A	A	A	A	A	N
NGO_1058	1,136,717	V45L	C	G	G	G	G	G	G	G	G	N
NGO_1058	1,136,716	S44	G	C	C	C	C	C	C	C	C	N
NGO_05615	1,120,999		A	G	G	G	G	G	G	G	G	N

NGO_1086	1,108,589	R49	CG	C	C	C	C	C	C	C	C	N
NGO_05685	1,108,030		T	C	C	C	C	C	C	C	C	N
NGO_05685	1,108,029		C	T	T	T	T	T	T	T	T	N
NGO_05685	1,108,028		A	T	T	T	T	T	T	T	T	N
NGO_1093	1,098,691	S405L	G	A	A	A	A	A	A	A	A	N
NGO_1093	1,098,664	E396A	T	G	G	G	G	G	G	G	G	N
NGO_1093	1,098,528	P350	CG	C	C	C	C	C	C	C	C	N
NGO_1124	1,079,577		CG	C	C	C	.	C	C	C	C	N
NGO_1128	1,078,078	G16A	G	C	.	.	.	.	.	C	C	N
Intron	1,067,745		G	A	A	A	A	A	A	A	A	N
NGO_1149	1,064,763	E323DGVE	T		TTCCACGCCG	TTCCACGCCG	TTCCACGCCG	TTCCACGCCG	Y			
NGO_1189	1,020,445	H34Q	G	C	C	C	C	C	C	C	C	N
NGO_1215	987,950	V57G	A	C	C	C	C	C	C	C	C	N
NGO_1215	987,917	P46H	G	T	T	T	T	T	T	T	T	N
NGO_1226	977,792	G39	T	C	C	C	C	C	C	C	C	N
NGO_1229	974,461	T154N	C	A	A	A	A	A	A	A	A	N
NGO_1241	960,398	R281	A	G	G	G	G	G	G	G	G	N
NGO_1246	956,238	V199	G	A	A	A	A	A	A	A	A	N
NGO_1251	951,808	P35	G	A	A	A	A	A	A	A	A	N
NGO_1251	951,760	G19	C	G	G	G	G	G	G	G	G	N
NGO_06740	919,455	*60L?	T	TA	TA		TA		TA	TA	TA	Y
nusA	916,486	G154	G	C	C	C	C	C	C	C	C	N
NGO_1286	914,281	M385V	A	G	G	G	G	G	G	G	G	N
NGO_1286	914,261	K391	A	G	G	G	G	G	G	G	G	N
NGO_1286	914,259	K392M	A	T	T	T	T	T	T	T	T	N
NGO_1286	914,200	K412E	A	G	G	G	G	G	G	G	G	N
NGO_1286	914,197	K413E	A	G	G	G	G	G	G	G	G	N
NGO_1286	914,124	E437V	A	T	T	T	T	T	T	T	T	N
NGO_1290	908,525	M364V	T	C	C	C	C	C	C	C	C	N
NGO_06860	896,834	K90E	A	G	G	G	G	G	G	G	G	N
NGO_06995	868,832	D57G	T	C	C	C	C		C	C	C	Y
NGO_1333	861,962	V485I	C	T	T	T	T	T	T	T	T	N
Intron	833,198		A	G	G	G	G	G	G	G	G	N
NGO_1363	831,421	K181E	T	C	C	C	C	C	C	C	C	N
NGO_1365	827,199	E275D	C	G	G	G	G	G	G	G	G	N
Intron	826,285		A	AC	AC	AC	AC		AC	AC	AC	Y
NGO_07410	789,110	G393	A			.	.		G	G		Y
NGO_07415	787,278	L221	C			A	A					Y
NGO_1418	771,590	N236D	A	G	G	G	G	G	G	G	G	N
NGO_1418	771,585	A237	A	G	G	G	G	G	G	G	G	N
Intron	765,792		C	G	G	G	G	G	G	G	G	N
Intron	765,783		T	A	A	A	A	A	A	A	A	N
NGO_07535	765,741	P129	C	G	G	G	G	G	G	G	G	N
NGO_07535	765,724	G124R	C	G	G	G	G	G	G	G	G	N
NGO_07535	765,721	V123L	C	G	G	G	G	G	G	G	G	N
NGO_07535	765,718	Y122H	A	G	G	G	G	G	G	G	G	N
NGO_1440	747,686	T37A	T	C	C	C	C	C	C	C	C	N
Intron	747,522		G	A	A	A	A	A	A	A	A	N
Intron	747,486		T	A	A	A	A	A	A	A	A	N
Intron	747,471		G	T	T	T	T	T	T	T	T	N
Intron	747,462		G	T	T	T	T	T	T	T	T	N
NGO_1456	729,420	S471G	A	G	G	G	G	G	G	G	G	N
pntA	719,353	R418G	C	G	G	G	G	G	G	G	G	N
NGO_1489	699,466		G	GC	GC	GC	GC	GC	GC	GC	GC	N
NGO_1489	699,462		C	CG	CG	CG	CG	CG	CG	CG	CG	N
NGO_1489	699,268		A	C	C	C	C	C	C	C	C	N
NGO_1496	688,872	P38	G	C	C	C	C	C	C	C	C	N
NGO_1497	688,542	I4V	T		C	C				C	C	Y
NGO_1497	688,539	K3E	T		C	C					C	Y
Intron	688,532		AC			A						Y
Intron	688,495		G			A						Y
Intron	688,494		T			C						Y
Intron	688,470		G			A						Y
NGO_1540	640,557	A175	A	G	G	G	G	G	G	G	G	N
NGO_08295	612,319	R40	T	A	A	A	A	A	A	A	A	N
Intron	578,343		T	G	G	G	G	G	G	G	G	N
NGO_08695	555,841		G	.	.	.	.	.	.	.	C	N
NGO_1641	553,888	R102	T	C	.	.	.	.	C	C	C	N
NGO_1641	553,887	R102K	C	T	.	.	.		T	T	T	N
emrB	519,773	G493V	C	A	A	A	A	A	A	A	A	N
NGO_1685	516,033	S83	A	C	C	C	C	C	C	C	C	N
NGO_1688	511,346	C37R	A	G	G	G	G	G	G	G	G	N
NGO_1714	480,359	V60A	A	G	G	G	G	G	G	G	G	N
NGO_1726	465,947	N124D	A	G	G	G	G	G	G	G	G	N
NGO_1751	441,810	D64A	T	G	G	G	G	G	G	G	G	N
NGO_1751	441,761	S48A	A	C	C	C	C	C	C	C	C	N
NGO_1751	441,746	K43E	T	C	C	C	C	C	C	C	C	N

NGO_1767	421,695	V491A	T	C	C	C	C	C	C	C	C	N
NGO_19026	410,668		A	C	C	C	C	C	C	C	C	N
NGO_19041	410,346		A	G	G	G	G	G	G	G	G	N
NGO_19046	388,446		T	G	G	G	G	G	G	G	G	N
NGO_1793	388,246	T34A	A	G	G	G	G	G	G	G	G	N
NGO_1801	378,549	S585L	C	T	T	T	T	T	T	T	T	N
NGO_09675	362,354	T235A	T	C	C	C	C	C	C	C	C	N
Intron	354,690		T	G	G	G	G	G	G	G	G	N
NGO_1825	354,415	M90I	C	T	T	T	T	T	T	T	T	N
NGO_1875	308,867	T213A	T	C	C	C	C	C	C	C	C	N
NGO_1890	296,481	G177	T	G	G	G	G	G	G	G	G	N
NGO_1890	296,447	V166A	A	G	G	G	G	G	G	G	G	N
NGO_1896	289,309	L160	G	A	A	A	A	A	A	A	A	N
NGO_1900	284,620	I206M	T	C	C	C	C	C	C	C	C	N
NGO_1900	284,543	S181A	A	C	C	C	C	C	C	C	C	N
NGO_1908	271,903	D275N	G	A	A	A	A	A	A	A	A	N
NGO_1910	269,029	C383G	T	G	G	G	G	G	G	G	G	N
NGO_1917	258,679	Q116	G	A	A	A	A	A	A	A	A	N
NGO_1917	258,592	F145	T	C	C	C	C	C	C	C	C	N
NGO_1917	258,571	F152	T	C	C	C	C	C	C	C	C	N
NGO_1917	258,550	T159	T	C	C	C	C	C	C	C	C	N
NGO_1917	258,547	G160	T	C	C	C	C	C	C	C	C	N
NGO_1926	249,913	K457E	A	G	G	G	G	G	G	G	G	N
NGO_1981	196,824	G116	G	A	A	A	A	A	A	A	A	N
NGO_1981	196,812	F112	A	G	G	G	G	G	G	G	G	N
NGO_1981	196,735	N87D	T	C	C	C	C	C	C	C	C	N
NGO_1981	196,728	AA83A	GGCA	G			G				G	Y
NGO_1981	196,680	T68	G	A	A	A	A	A	A	A	A	N
NGO_1981	196,646	P57L	G	A	A	A	A	A	A	A	A	N
NGO_1981	196,620	T48	G	T	T	T	T	T	T	T	T	N
NGO_1981	196,611	E45D	T	G	G	G	G	G	G	G	G	N
NGO_1981	196,553	P26L	G	A	A	A	A	A	A	A	A	N
Intron	196,469		G	A	A	A	A	A	A	A	A	N
Intron	196,447		T	C	C	C	C	C	C	C	C	N
NGO_1982	196,420	S99G	T	C	C	C	C	C	C	C	C	N
NGO_1982	196,402	S93A	A	C	C	C	C	C	C	C	C	N
NGO_1982	196,383	E86	C	T	T	T	T	T	T	T	T	N
NGO_1982	196,251	R42	A	G	G	G	G	G	G	G	G	N
NGO_2001	179,753	E310	A	G	G	G	G	G	G	G	G	N
NGO_2001	179,707	N326D	A	G	G	G	G	G	G	G	G	N
NGO_2001	179,679	P335R	C	G	G	G	G	G	G	G	G	N
NGO_2001	179,651	E344D	A	C	C	C	C	C	C	C	C	N
NGO_2001	179,594	V363	G	C	C	C	C	C	C	C	C	N
Intron	175,875		C	A	A	A	A	A	A	A	A	N
Intron	167,672		C	CG	CG	CG	CG	CG	CG	CG	CG	N
NGO_11095	117,693		A	.	.	.	.	.	.	G	G	N
NGO_11095	117,692		A	.	.	.	.	.	.	G	G	N
NGO_11095	117,676		G	.	.	.	.		.	A	.	N
NGO_11095	117,620		C			T	.	.				Y
NGO_11095	117,619		C			A	.	.			.	Y
NGO_11095	117,617		C			T	.	.				Y
Intron	116,622		A				G					Y
Intron	116,564		C			T	T		T		T	Y
Intron	116,449		G						A			Y
Intron	116,435		T			C	.		.		C	Y
Intron	116,423		A		.		.		G			Y
NGO_11105	116,205	D39A	T	.	G	.	.	.	.	.	.	N
NGO_11105	116,204	D39N	C	.	T	.	.	.	.	.	.	N
NGO_11105	116,200	K37N	T	.	A	.	.	.	.	.	.	N
NGO_11105	116,199	K37T	T	.	G	.	.	.	.	.	.	N
NGO_11105	116,198	K37E	T	.	C	.	.	.	.	.	.	N
NGO_11105	116,185	V32	A	.		.	.	G	.	.	.	Y
NGO_11105	116,176	N29K	G	.	T	.	.	T	.	.	.	N
NGO_11105	116,156	Q23K	G	.	T	.	.	T	.		.	Y
NGO_11105	116,132	S15T	A	.	T	.	.	T	.	.	.	N
NGO_11105	116,097	G3E	C	T	T	T	T	T	.	T	T	N
Intron	116,068		C	T	T	T	T	T	.	T	T	N
Intron	116,063		A	G	G	G	G	G	.	G	G	N
Intron	115,540		C	.	.	.	.		.	T	.	N
NGO_2135	38,236	D202	A	G	G	G	G	G	G	G	G	N
NGO_2135	38,199	G190A	C	G	G	G	G	G	G	G	G	N
NGO_2135	38,187	P186Q	G	T	T	T	T	T	T	T	T	N
NGO_2135	38,159	G177R	C	G	G	G	G	G	G	G	G	N
NGO_2135	38,139	G170D	C	T	T	T	T	T	T	T	T	N
NGO_2135	38,120	T164A	T	C	C	C	C	C	C	C	C	N
NGO_2135	38,000	G124R	C	G	G	G	G	G	G	G	G	N
NGO_2135	37,969	A113	A	T	T	T	T	T	T	T	T	N

NGO_2135	37,951	S107R	A	T	T	T	T	T	T	T	T	N
NGO_2135	37,944	V105A	A	G	G	G	G	G	G	G	G	N
NGO_2135	37,939	Y103*	A	C	C	C	C	C	C	C	C	N
NGO_2135	37,938	Y103F	T	A	A	A	A	A	A	A	A	N
NGO_2135	37,936	Y102	G	A	A	A	A	A	A	A	A	N
NGO_2135	37,935	Y102S	T	G	G	G	G	G	G	G	G	N
NGO_2135	37,932	M101K	A	T	T	T	T	T	T	T	T	N
NGO_2135	37,921	K97N	C	G	G	G	G	G	G	G	G	N
NGO_2135	37,891	G87	T	G	G	G	G	G	G	G	G	N
NGO_2135	37,888	A86	T	G	G	G	G	G	G	G	G	N
NGO_2144	31,842	K99R	A	G	G	G	G	G	G	G	G	N
NGO_2144	31,446	V231G	T	G	G	G	G	G	G	G	G	N
NGO_2171	5,873	L15	G	A	A	A	A	A	A	A	A	N
NGO_2171	5,850	S8A	A	C	C	C	C	C	C	C	C	N
NGO_2171	5,849	E7D	T	A	A	A	A	A	A	A	A	N
NGO_2172	5,730	D173	A	G	G	G	G	G	G	G	G	N
NGO_2172	5,729	D173G	T	C	C	C	C	C	C	C	C	N
NGO_2174	4,451	N43T	T	G	G	G	G	G	G	G	G	N

16.12 Appendix 12: PubMLST gonococcal isolates with polymorphisms to FA1090 *nos*

id	isolate	aliases	country	town_or_city	year	age_yr	age_range	sex	disease	source	epidemiology
47804	SRR3349576		UK [England]	Brighton	2014						
48522	SRR3357307		UK [England]	Brighton	2015						
49075	SRR3361210		UK [England]	Brighton	2012						
56783	NG10b		Spain	Madrid	2016	41	25-44	male	uncomplicated gonorrhoea	throat swab	MSM
56784	NG11a		Spain	Madrid	2016	23	15-24	male	uncomplicated gonorrhoea	rectal swab	MSM
61635	DRR117965	SAMD00105885	Unknown								
78427	H160940039	2016-02_48	UK [England]	Brighton	2016						
87787	ECDC_PT13_087		Portugal		2013						
87793	ECDC_PT13_093		Portugal		2013						
107318	ERR3578023		UK		2016			male			MSW
119267	16AM109666		Sweden	Stockholm	2016	25	25-44	male		throat swab	MSM
130439	ECDC_UK18_SCOT-11		UK		2018						

bioproject_accession	biosample_accession	run_accession	sender	curator	date_entered	datestamp	PorB_profile	strain_designation
PRJNA315363	SAMN04623845; SRS1382139	SRR3349576	264	-1	01/11/2016	21/04/2024		ND: P1.18-10,43: F3-14: ST-11864 ()
PRJNA315363	SAMN04625248; SRS1384669	SRR3357307	264	-1	01/11/2016	22/04/2024		ND: P1.ND,ND: F-ND: ST-ND (-)
PRJNA315363	SAMN04624194; SRS1386314	SRR3361210	264	-1	01/11/2016	22/04/2024		ND: P1.18-10,43: F3-14: ST-ND (-)
			420	-1	01/12/2017	24/04/2024		ND: P1.18-10,43: F3-14: ST-13148 ()
			420	206	04/12/2017	26/04/2024		ND: P1.18-10,43: F3-14: ST-9363 ()
PRJDB6504	SAMD00105885	DRR117965	264	-1	28/08/2018	25/04/2024		ND: P1.18-10,43: F1-26: ST-7363 ()

PRJEB23008	ERS1972888; SAMEA104347910	ERR2172259	206	206	04/07/2020	02/07/2024	PIB:5:4:7:1:7	ND: P1.18-10,43: F4-7: ST-1580 ()
PRJEB9227	ERS792927; SAMEA3485644	ERR1560855	206	206	14/05/2019	02/07/2024	PIB:5:13:4:6:7	ND: P1.18-10,43: F1-211: ST-1901 ()
PRJEB9227	ERS792933; SAMEA3485650	ERR1560861	206	206	14/05/2019	02/07/2024	PIB:5:13:4:6:7	ND: P1.18-10,43: F1-211: ST-1901 ()
PRJEB19989	ERS1806363; SAMEA104147345	ERR3578023	206	206	19/08/2021	02/07/2024	PIB:5:4:7:1:7	ND: P1.18-10,43: F4-7: ST-1580 ()
PRJEB47922	ERS9876433; SAMEA12279579	ERR7986882	206	206	20/07/2022	02/07/2024	PIA:1:1:1:7:1	ND: P1.18-10,43: F3-14: ST-9363 ()
PRJEB34068	ERS3906627; SAMEA6105948	ERR9838047	206	-1	21/04/2023	11/05/2024		ND: P1.18-10,43: F3-14: ST-9363 ()

azithromycin_mic_sign	azithromycin_mic	cefixime_mic_sign	cefixime_mic	cefixime_SIR	ceftriaxone_mic_sign	ceftriaxone_mic	ceftriaxone_SIR	ciprofloxacin_mic_sign	ciprofloxacin_mic	ciprofloxacin_SIR	penicillin_mic_sign	penicillin_mic	penicillin_SIR
					<	0.016	S	=	0.003	S	=	0.25	I
					<	0.016	S	=	0.008	S	=	0.19	I
=	0.5	=	0.032	S	=	0.012	S	=	32	R			
=	0.5	=	0.032	S	=	0.023	S	=	32	R			
=	8	=	0.03	S	=	0.008	S				=	0.5	I
=	0.06	<	0.015	S	<	0.015	S	>	1	R			

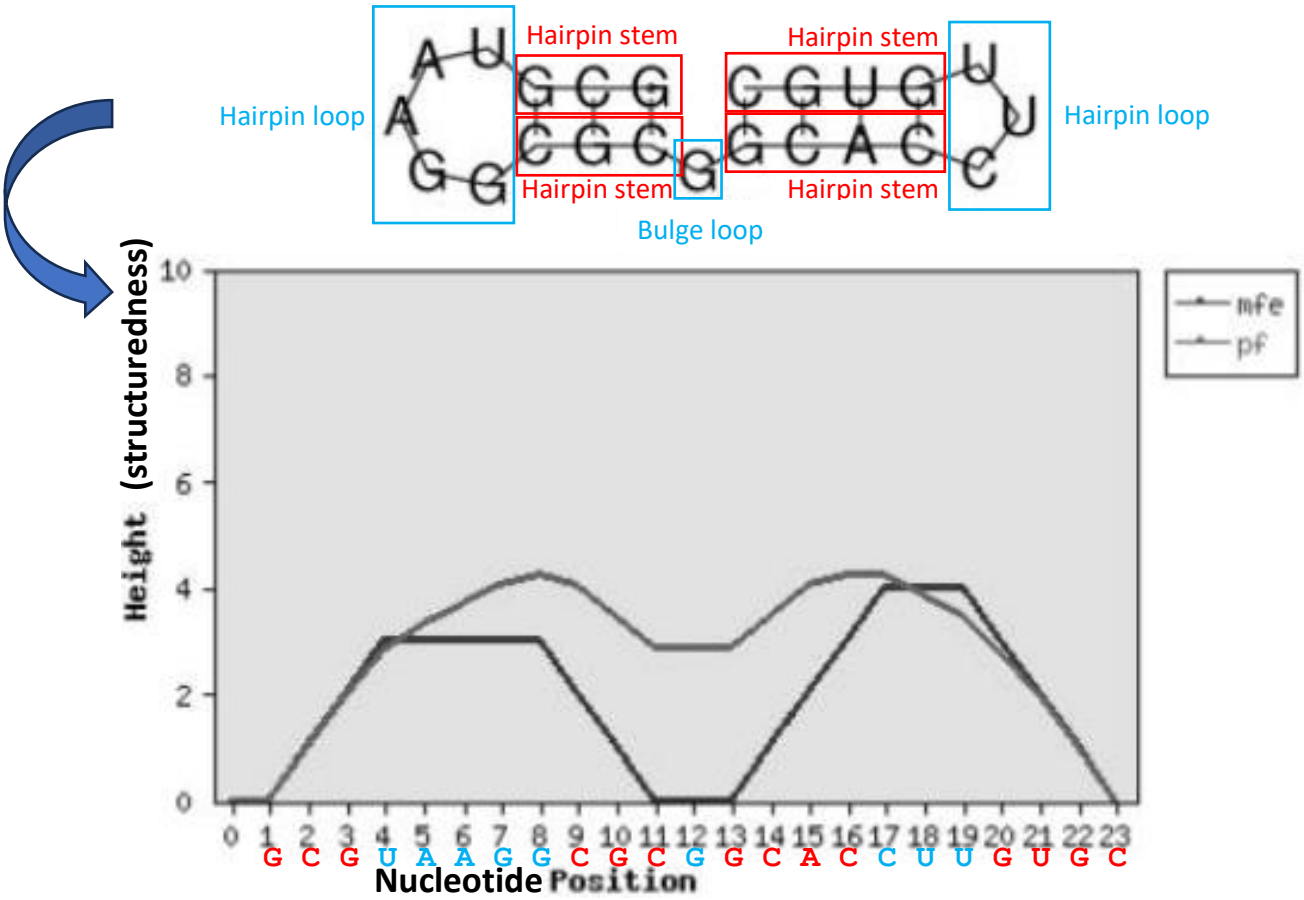
Red are isolates with polymorphisms in *nosZ* PSC

Blue are isolates with polymorphisms in *nosD* PSC

Purple are isolates with polymorphisms in both *nosZ* and *nosD* PSCs

16.13 Appendix 13: Mountain plot example

The following example of a mountain plot describing RNA secondary structure is taken from a review of the Vienna RNA secondary structure server (Hofacker, 2003).



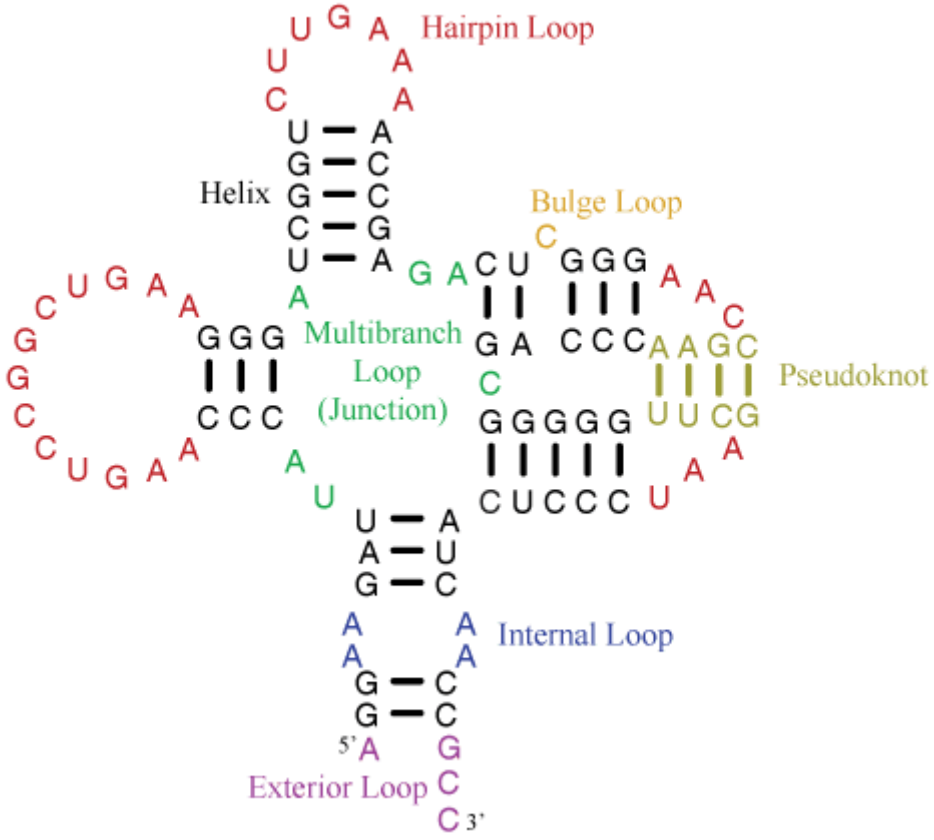
Two representations for secondary structure and structure ensembles as produced by the RNAfold service: structure graph and mountain plot.

The *x* axis represents the nucleotide position whereas the *y* axis is the number of nucleotides enclosing the base. Loops are plateaus where there is no change of enclosing nucleotides; helices correspond to slopes with an increasing or decreasing number of enclosing bases; stem-loops are obtuse peaks composed of at least two slopes and a plateau.

16.14 Appendix 14: Examples of RNA secondary structures

In a mountain plot, structured regions of RNA secondary structure (i.e. helices) will form mountain slopes. Unstructured regions (i.e. loops) will form flat mountaintop plateaus and plains surrounding the mountains (valley floors).

Such unstructured loops include internal loops, bulge loops and multibranch loops (junctions). The figure below illustrates the motifs that appear in RNA secondary structures (https://rna.urmc.rochester.edu/NNDB/help.html). See the Turner group for more on RNA secondary structure. NB: (hairpin) stems are also known as helices.



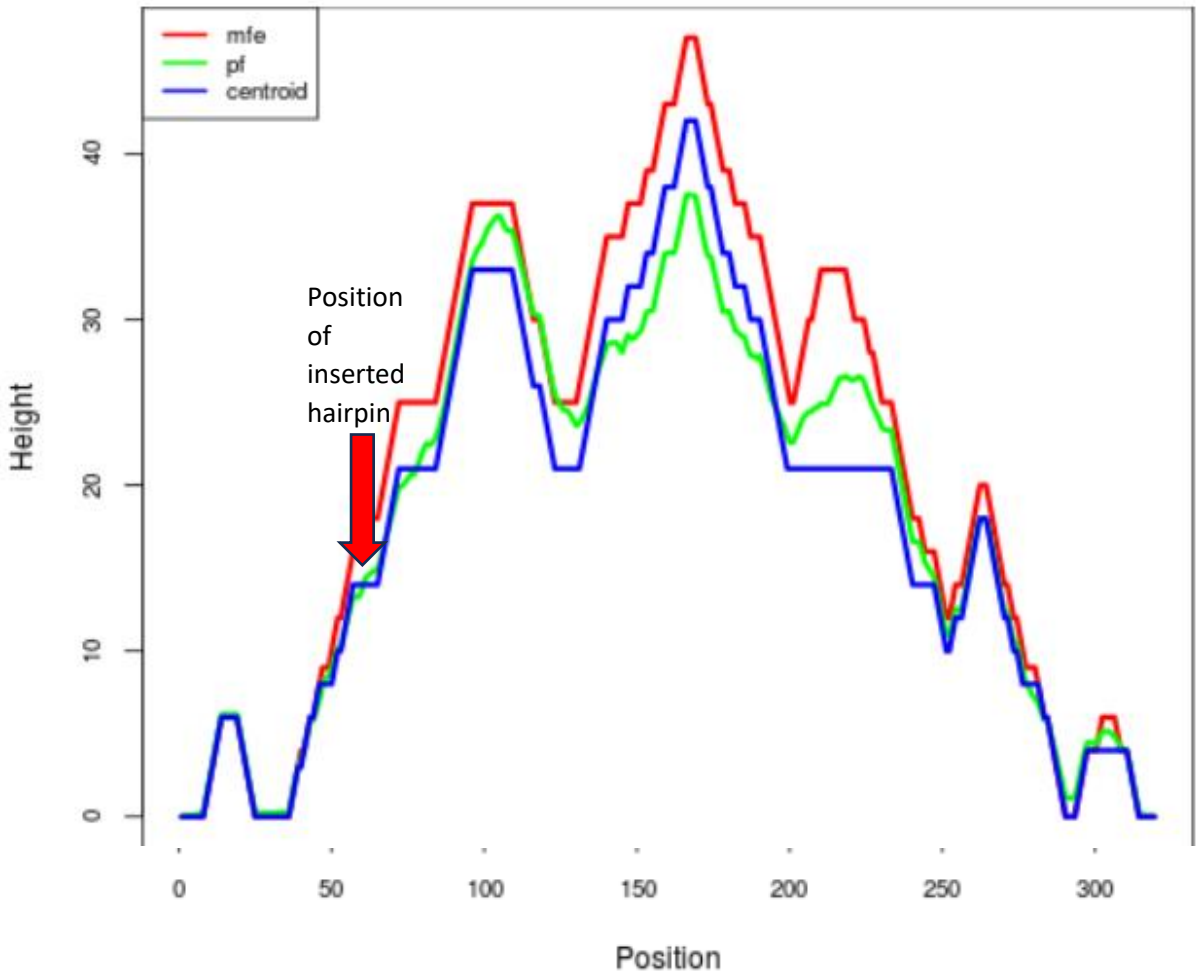
16.15 Appendix 15: Modelling hairpins in *E. coli mRFP1* mRNA using RNAfold

RNAfold predicts two secondary structures: optimal minimum free energy (ME) and centroid structures. A mountain plot shows the similarity between the optimal secondary structure (red line), the centroid structure (green line), and pair probabilities (blue line).

A previous study described how insertion of the 13-GG hairpin-loop sequence into the WT mRNA sequence of *E. coli mRFP1* inhibits translation (Espah Boruheni et al., 2017, Figure 4A). Running the sequences from that paper through RNAfold shows how the hairpin-loop sequence creates a mountain from area of the plot which had previously been a slope in the WT.

>EcherichiacolimRFP1_WT
TCTAGAacccgccatatacggcgggacacacacAAGGAGacCATATGaaacagaacaaagaacagatcaccaaacagaac
atcaacgcGAGCTCTgaagacgttatcaaagagttcatgcgtttcaaagttcgtatggaaggttccgttaacggtcacgagttcgaaatcg
aaggtgaaggtgaaggtcgtccgtacgaaggtacccagaccgctaaactgaaagttaccaaaggtggtccgctgccgttcgcttgggacatc
ctgtccccgcagttccagtacggttccaaagcttacgttaaacacccggctgacat

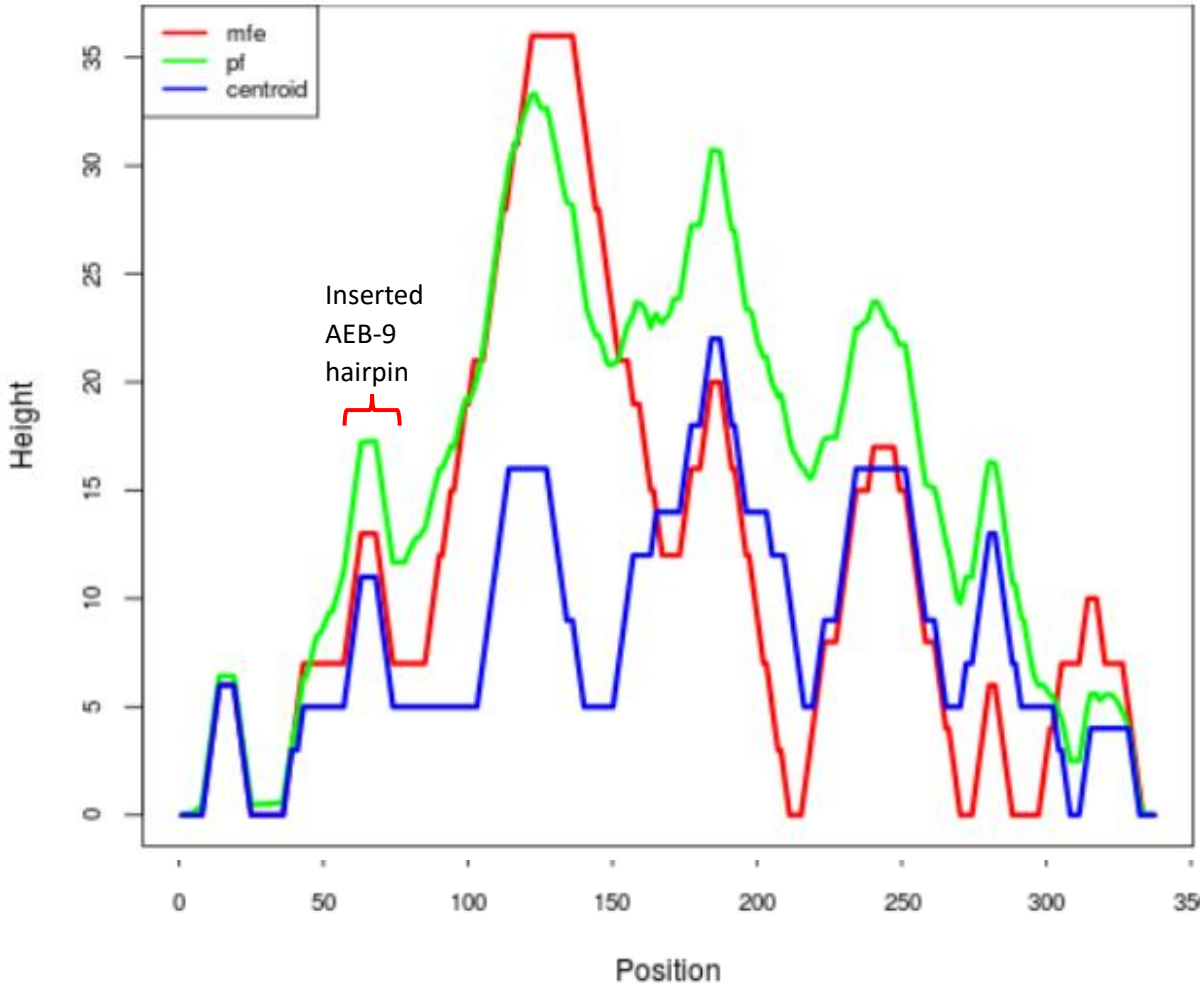
RNAfold mountain plot:



>EcherichiacolimRFP1_AEB-9_hairpin
TCTAGAacccgccatatacggcgggacacacacAAGGAGacCATATGaaacagaac**GGCGCGattatcCGCGCC**aa
agaacagatcaccaaacagaacatcaacgcGAGCTCTgaagacgttatcaaagagttcatgcgtttcaaagttcgtatggaaggttccgt
taacggtcacgagttcgaaatcgaaggtgaaggtgaaggtcgtccgtacgaaggtacccagaccgctaaactgaaagttaccaaaggtggt
ccgctgccgttcgcttgggacatcctgtccccgcagttccagtacggttccaaagcttacgttaaacacccggctgacat

Hairpin is nt 57-74

RNA fold mountain plot:



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