The Role of FNR-Regulated sRNA in Controlling Bacterial Denitrification

Daniel James Seagrove

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Abstract

Nitrous Oxide (N_2O) is a long-lived, ozone-depleting greenhouse gas of which emissions are increasing at an alarming rate, largely due to modern agricultural practices. Biological N_2O primarily originates from a truncated denitrification pathway which normally allows the sequential reduction of nitrate (NO_3) to nitrogen (N_2) under anaerobic conditions by four reductases: nitrate reductase, nitrite reductase, nitric oxide reductase and nitrous oxide reductase. The denitrification pathway is tightly regulated by transcriptional regulators belonging to the FNR family. A number of environmental signals have significant impact on these transcriptional regulators and the activity of denitrification. A relatively new area of study has looked at regulation of denitrification by small RNAs (sRNAs), which have previously been found to have a wide range of physiological functions and are largely controlled themselves by transcriptional regulators. Previous studies have identified the sRNAs in the model bacterial denitrifier *Paracoccus denitrificans.* Here it was hypothesised that as FNR transcriptional regulators play a key role regulating the denitrification pathway that sRNAs under the control of FNR transcriptional regulators may also play a key role in regulating denitrification. This study aimed to identify sRNAs with a putative FNR binding motif upstream of their promoter region. 7 sRNAs with a putative FNR binding motif within 200 bp of their promoter regions were discovered on the *P. denitrificans* genome and these sRNAs were subsequently computationally characterised. Their secondary structures, mRNA targets and sequence conservation in other species of bacteria were investigated. 3 of these candidate sRNAs, sRNA18, sRNA36 and sRNA79 showed the most likely characteristics to be involved in denitrification. Overexpression of sRNA36 impacted on the rate of N_2O reduction and regulation by FNR was experimentally confirmed.

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Chapter 1: Introduction

1.1 The Nitrogen Cycle

1.1.1 The Global Nitrogen Cycle

Nitrogen (N_2) is the most abundant gas in the Earth's atmosphere, accounting for just over 78% of the air that we breathe (Galloway *et al*., 2004). It is fundamental to the survival of all organisms being a key component of nucleotides and amino acids, the 'building blocks' of life (Stein & Klotz, 2016). However, N_2 is inaccessible to most organisms as gas meaning it is often a limiting resource. Therefore, N_2 must be converted into 'reactive nitrogen' (N_r) that is readily available to organisms and can take many forms (Galloway & Cowling, 2002).

The nitrogen cycle comprises a number of different processes, each responsible for the transformation of nitrogen compounds from one form to another (Figure 1). A diverse array of microorganisms have evolved over millions of years that are capable of performing these processes and many microorganisms are still being discovered to this day (Cabello et al., 2009). The following sections will highlight the main pathways of the nitrogen cycle with an in-depth insight into the key enzymes and microorganisms that are involved in each stage.

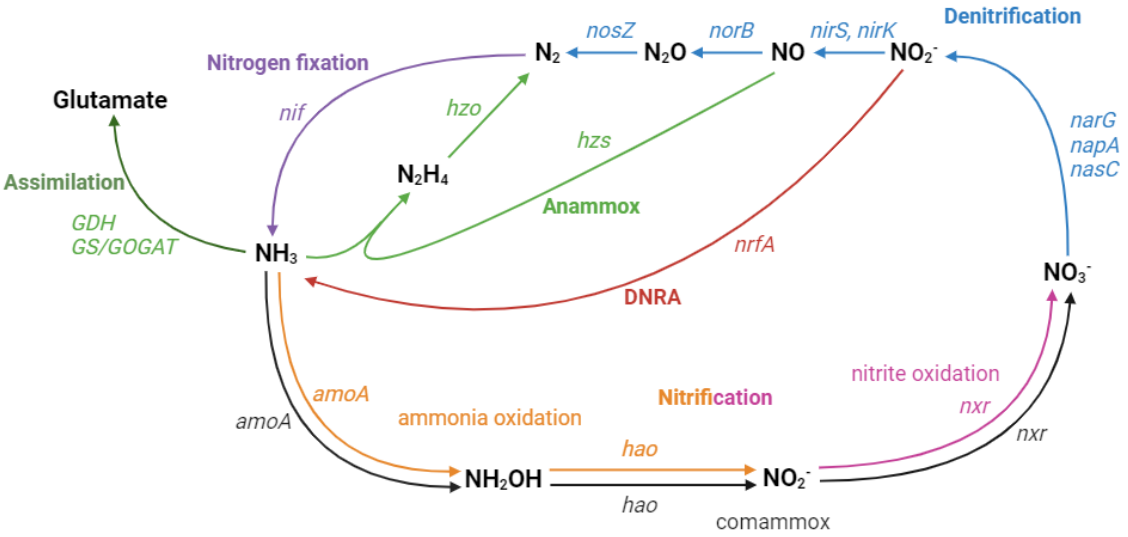


Figure 1 : The nitrogen cycle and key biological processes involved. These processes include: nitrogen fixation (purple), nitrification (ammonia oxidation & nitrite oxidation) (orange & pink), comammox (black), assimilation (dark green), denitrification (blue), dissimilatory nitrate reduction to ammonia (red) and anammox (light green). The key enzymes that regulate these processes are also indicated in the corresponding colours.

1.1.2 Nitrogen fixation

N_2 is inaccessible to most organisms due its chemical composition whereby two nitrogen atoms are joined by a strong triple bond which is difficult to break (Galloway *et al*., 2008). To

break this bond requires one of the most “metabolically expensive” biological processes and the presence of 8 electrons and 16 ATP molecules (Equation 1) (Swanson *et al*., 1982). N_2-fixation is the process whereby N_2 is converted into ammonia (NH_3) which can subsequently be transformed into other forms of N_r (Bernhard *et al*., 2010). N_2-fixation can occur naturally by way of bacterial N_2-fixation (BNF) or through lightning strikes (Galloway & Cowling, 2002).

Equation 1:

$$N_2 + 8H^+ + 8e^- \rightarrow 2NH_3 + H_2$$

BNF is a process whereby a limited number of microorganisms, commonly known as diazotrophs, are able to convert N_2 into NH_3 compounds which can then be transformed into essential amino acids and oxidised by soil microorganisms (Fowler *et al*., 2013). N_2-fixing bacteria and archaea are ubiquitous in nature, being found in a wide range of habitats including both marine and terrestrial environments (Robertson & Groffman, 2006). The enzyme nitrogenase (Nif) is the only enzyme known to naturally fix N_2 and is therefore a necessary component of all N_2-fixing microorganisms (Hoffman *et al*., 2014). The enzyme complex of Nif consists of dinitrogenase reductase, a molybdenum iron protein encoded by the genes *nifD* and *nifK,* that is responsible for binding with N_2; and nitrogenase reductase, a smaller iron protein encoded by *nifH,* that acts as an electron donor to dinitrogenase reductase (Berges & Mulholland, 2008). The genes *nifBEN* work by synthesising the iron-molybdenum cofactor found in Nif. *nifA* is a regulatory gene, and *nifFJ* encode electron transport proteins. These genes are all part of the *nif* gene operon and are essential to its function (Aber *et al*., 2014). Nif is highly sensitive to Oxygen (O_2) and will only function in anoxic environments (Lehnert *et al*., 2021). Nif therefore requires protection from host microorganisms such as by compartmentalisation and spatial decoupling (Stein & Klotz, 2016).

Diazotrophs are phylogenetically diverse, falling under several genera such as *Rhizobium, Pseudomonas* and *Azorhizobium* (Imran *et al*., 2021). The majority of diazotrophs found in soil are part of a subset of plant growth-promoting rhizobacteria (PGPR) which promote plant growth. Endophytic diazotrophs, found in the root/shoot of the plant phyllosphere are advantageous over associative diazotrophs found on the root surface due to a more stable environment in the phyllosphere (Bashan *et al*., 2004). As these diazotrophs are found in such close proximity to the roots, nutrient acquisition by the plants through these diazotrophs is greatly improved leading to increased growth rate (Imran *et al*., 2021). An example can be found in *Herbaspirillum* and *Burkholderia* that fix nitrogen in sugar cane (van Deynze *et al*., 2018). These diazotrophs benefit as they position themselves in areas where Nif is well-protected from O_2. Some diazotrophs share a symbiotic relationship with plant hosts in which a carbon molecule is donated from the plant to the microorganism in exchange for N_2

(Schwember *et al*., 2019). This is thought to be the most efficient N_2-fixation process fixing around 170-300 kg N_2 per hectare per year (Imran *et al*., 2021). A well-known example of this is the *Rhizobium*-Legume Symbiosis whereby *Rhizobia* attach themselves to the roots and occasionally the stem of a number of different legume hosts. The *Rhizobia* species can be found in 18 genera of bacteria indicating their great evolutionary success in symbiosis (Maróti & Kondorosi, 2014).

Free-living diazotrophs named cyanobacteria are key contributors to N_2-fixation in marine environments. Cyanobacteria, which have existed for 2.7 – 2.9 billion years, are oxygenic and were key to the change from an anoxic to an oxic Planet Earth (Latysheva *et al*., 2012). Cyanobacteria belonging to the genus *Trichodesmium* are responsible for fixing 240 Tg $N_2\,y^{-1}$ equating to approximately 42% of global N_2-fixation each year (Berman-Frank *et al*., 2003). Due to their ability to fix N_2, they have been used as biofertilisers, responsible for more than 70% of the N_2 fixed in agriculture (Berman-Frank *et al*., 2003).

Aside from BNF, N_2-fixation can also take place naturally through lightning strikes. Lightning strikes connect with N_2 atoms in the stratosphere, breaking their triple bond and leaving a singular N atom. This singular N atom bonds with O_2 atoms to produce atmospheric NO which is then converted to nitrogen dioxide (NO_2) and nitric acid (HNO_3). These compounds are then deposited as N_r into ecosystems by wet and dry deposition (Galloway *et al*., 2004).

N_2-fixation is an essential stage in the nitrogen cycle, as without it, organisms would not be able to utilise necessary nitrogen compounds needed for growth and other key physiological processes. N_2 is critical to plant growth as agricultural crop yield can largely depend on the abundance of N_r in soils available to plants (Bloom, 2015). Therefore, in recent times, as the world population has seen rapid exponential growth, an increasing demand for food has led to an over-exploitation of nitrogen-based additives in agricultural practices. This in turn has led to a disruption of biochemical processes involved in the nitrogen cycle (Fowler *et al*., 2013). Until the early 1900s, crop yield was entirely dependent on natural N_2-fixation to supply plants with enough nutrients for growth. However, the discovery of the Haber-Bosch process in 1909 meant that humans no longer had to rely on BNF and lightning to naturally fix N_2 to improve crop yield. The Haber Bosch process involves industrially heating N_2 molecules to high enough temperatures to break the triple bond held between the two N atoms. The N atoms are then fixed with hydrogen at high pressure and temperature to form NH_3 ($N_2 + 3H_2 \rightarrow 2NH_3$) (Darmawan *et al*., 2021). Industrially produced NH_3 can then be used as a nitrogen source in synthetic fertilisers. The Haber-Bosch process has been highly successful in increasing agricultural crop yield and, since its discovery, it is thought that ~40% of the human population are dependent on the Haber-Bosch process for survival. In fact, it is thought that almost 80%

of nitrogen found in the protein of human tissues originated from the Haber-Bosch process, showing the extent of its successful use in food production globally (Howarth, 2008). In 2010, N_2-fixation by the Haber Bosch process (120 tg N yr^{-1}) was almost double natural terrestrial sources of N_r (63 Tg N yr^{-1}) (Fowler *et al*., 2013). The use of nitrogen-containing fertilisers in agricultural practices has become very common, ensuring there is no nitrogen limitation acting upon crop yield. However, it is thought that only 30-50% of nitrogen additives are assimilated by crops when using these fertilisers, with the rest being converted to nitrogen oxides (NO_x) such as nitric oxide (NO) and nitrous oxide (N_2O) by microbes found in the soil which are harmful to the environment (Lehnert *et al*., 2021).

1.1.3 Nitrification

Once N_2-fixation has taken place and N_2 has been converted to NH_3, NH_3 must then be converted to nitrate (NO_3) or nitrite (NO_2), as NH_3 can be toxic to plants. Nitrification is the oxidation of NH_3 to NO_2 and then subsequently to NO_3 in aerobic conditions (Ward, 2008). Prokaryotes involved in nitrification are either ammonia-oxidising bacteria/archaea (AOB/AOA) which are responsible for the oxidation of NH_3 to NO_2; or nitrite oxidising bacteria (NOB) that are responsible for the further oxidation of NO_2 to NO_3 (Casciotti *et al*., 2011).

Ammonia oxidisers are ubiquitous in nature and are found in a wide range of environments (Leininger *et al*., 2006; Mußmann *et al*., 2011). AOB are present in two phylogenetic clades, *Betaproteobacteria* and *Gammaproteobacteria* (Purkhold *et al*., 2000); whereas AOA are found in the phylogenetic clades *Thaumarcheota* and *Nitrospira* (Myrold, 2021). Studies have found that AOA are the dominant ammonia-oxidising microbe in marine and soil environments (Leininger *et al*., 2006; Wuchter *et al*., 2006). Ammonia oxidation is a two stage, six-electron oxidation process. It begins with the hydroxylation of NH_3 to hydroxylamine (NH_2OH) catalysed by the enzyme ammonia monooxygenase (AMO), a copper-containing enzyme belonging to the membrane-bound monooxygenase family of enzymes (Equation 2). NH_2OH is then subsequently oxidised to NO_2 by the homotrimeric enzyme hydroxylamine oxidoreductase (HAO) (Equation 3) (Girkin & Cooper, 2022). Interestingly, as well as the primary product of ammonia oxidation being NO_2, it has been found that ammonia oxidisers also contain the enzyme nitrite reductase (Nir) which is responsible for the reduction of NO_2 to nitric oxide (NO) (Myrold, 2021). Although, NO emitted from ammonia oxidation is normally at a rate of less than 1%. However, if soil O_2 availability is reduced in such environments such as waterlogged soil, AMO and HAO activity is inhibited and NO_2 will be used as an electron acceptor, increasing NO output (Zhu *et al*., 2013). It has also been discovered that ammonia oxidisers release one mole of H_2 for every mole of NH_3 that they oxidise. This can lead to very acidic soils which for some nitrifiers can inhibit function, whereas others have been found to be acidophilic (Myrold, 2021).

Equation 2:

$$NH_3 + O_2 + 2e^- \rightarrow NH_2OH + H_2O$$

Equation 3:

$$NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e^-$$

NOB are responsible for the oxidation of NO_2 to NO_3 (Equation 4). Once ammonia oxidisers have converted NH_3 to NO_2, the conversion to NO_3 by nitrite oxidisers is rapid meaning there is little accumulation of NO_2. However, environmental conditions may limit this process such as in conditions of high urea concentrations and/or fluctuations from optimal soil temperature (Girkin & Cooper, 2022). Nitrite oxidation to NO_3 is the second stage of nitrification and is a two-electron process carried out by NOB. Nitrifiers include the genera: *Nitrospina, Nitrococcus* and *Nitrospira.* Nitrite oxidation is catalysed by the enzyme nitrite oxidoreductase, a multiprotein enzyme of vital importance to the generation of NO_3 despite little knowledge of its structure and function being found in literature (Chicano *et al*., 2021).

Equation 4:

$$NO_2^- + \frac{1}{2}O_2 \rightarrow NO_3^-$$

Interestingly, ammonia oxidisers and nitrite oxidisers are not closely related even though they both work to reduce NH_3 to NO_3. They must therefore work closely together and interact to ensure the nitrification pathway is completed (Daims *et al*., 2015). The energy generated by both ammonia oxidisers ($\Delta G°'$ – 275 kJ mol^{-1} NH_3) and nitrite oxidisers ($\Delta G°'$ – 74 kJ mol^{-1} NO_2) is relatively low meaning the growth rate of both is slow. They are both autotrophic and generate organic carbon through fixing carbon dioxide (CO_2). Due to the low levels of energy generated by each, they must oxidise many molecules of NH_3 and NO_2 respectively to be able to fix enough CO_2. Prior to 2015, the functional separation of ammonia oxidisers and nitrite oxidisers was puzzling as the energy generated by complete nitrification from NH_3 to NO_3 in an organism was greater than either of these processes ($\Delta G°'$ – 349 kJ mol^{-1} NH_3) (Daims *et al*., 2015). It would therefore be advantageous for microbes to be able to perform complete ammonia oxidation to NO_3 (comommox) over microbes that are only able to perform either ammonia oxidation or nitrite oxidation. After much postulation that comommox existed, in 2015, the initial discovery was made of two *Nitrospira* species of bacteria that possessed all the necessary enzymes to perform both ammonia oxidation and nitrite oxidation (Equation 5) (van Kessel *et al*., 2015). Comommox bacteria are now known to be ubiquitous in the environment and found in various ecosystems. However, due to their recent discovery, little is

known about their physiology and application to the nitrogen cycle. The most well-studied culturable comommox species to date is *Nitrospira inopinata.* This bacterium possesses the key enzyme to nitrite oxidation, nitric oxide reductase (Nor); as well as homologues enzymes to AMO and HAO, key to ammonia oxidation (Daims *et al*., 2015). It has been discovered that *N. inopinata* has a high growth yield when exposed to low NH_3 concentrations, indicating that comommox would be highly advantageous in nutrient-limited environments (Sakoula *et al*., 2021).

Equation 5:

$$NH_3 + 2O_2 \rightarrow NO_{3^-} + H_2O + 2H^+$$

1.1.4 Dissimilatory nitrate reduction to ammonia

Dissimilatory nitrate reduction to ammonia (DNRA) is a 2-step process whereby NO_3 is first reduced to NO_2 and then further to NH_3 (Pandey *et al*., 2020). During ammonification, NO_3 and NO_2 are terminal electron acceptors in place of O_2 (Mohan *et al*., 2004). *Escherichia coli* (*E.* coli) became a model organism for understanding the DNRA process and has led to the discoveries of enzymes that are required in this process (Stremińska *et al*., 2012).

The periplasmic NO_3 reductase, Nap*,* is essential to the reduction of NO_3 to NO_2 (Equation 6). In *E. coli* the *nap* gene operon consists of *napEDABC*. *napA*, a molybdoprotein containing an MGD cofactor and an [4Fe-4S] cluster, is the catalytic subunit of the *nap* operon. *napBCD* and two c-type cytochromes have also been found to be essential to NO_3 reduction in the periplasm (Potter & Cole, 1999). Nap activity in cells is induced by the presence of NO_3 and has found to be unaffected by NH_3 and O_2. The second reduction stage of NO_2 to NH_3 (Equation 7), is mediated by a cytochrome c nitrite reductase, (ccNir), found on the periplasmic membrane, which allows NO_2 to be the terminal electron acceptor in place of O_2. ccNir is a homodimer containing five c-type heme cofactors per monomer with a catalytic subunit of *nrfA* (Einsle *et al*., 1999).

Equation 6:

$$NO_{3^-} + 2H^+ + 2e^- \rightarrow NO_{2^-} + H_2O$$

Equation 7:

$$NO_{2^-} + 8H^+ + 6e^- \rightarrow NH_{4^+} + 2H_2O$$

In the absence of ammonium in the soil, assimilatory nitrate reduction to ammonia (ANRA) may take place instead of DNRA. This process is similar to DNRA, however, it employs

unrelated NO_3 and NO_2 reductases. Instead of using the periplasmic Nap to reduce NO_3 to NO_2, ANRA employs a membrane bound NO_3 reductase, Nas. In *Paracoccus denitrificans* (*P. denitrificans*), this protein is controlled by the *nas* gene cluster, *nasTSABGHC* where *nasC* encodes NasC, the catalytic subunit, ensuring NO_3 is reduced to NO_2 in a two-electron process. The second stage of ANRA is the reduction of NO_2 to NH_3 which requires *nasB* to reduce NO_2 to NH_3 in a six-electron process (Felgate *et al*., 2012).

Nap was first discovered in *P. denitrificans* in 1993 and was initially thought to be the NO_3 reducing enzyme, respiratory NO_3 reductase (Nar). However, its cellular location along the periplasmic side of the cell membrane of *P. denitrificans,* as well as its inability to reduce chloride, differentiated Nap. Since the discovery of Nap, a variety of prokaryotes involved in the reduction of NO_3 to NO_2 in both the DNRA and denitrification pathways have been found to possess this enzyme (Sparacino-Watkins *et al*., 2014).

DNRA in prokaryotes has been identified in both terrestrial and marine environments, especially in areas of low O_2 levels such as deep anoxic environments found in water columns (Kamp *et al*., 2011). Diatoms are photosynthetic microalgae found in many marine and soil environments across the world and are responsible for around 50% of global photosynthesis in the oceans (Kamp *et al*., 2015) It has recently been discovered that diatoms use DNRA for short-term survival, explaining how they are able to survive in dark, anoxic aquatic zones where they are unable to carry out photosynthesis or respire aerobically (Kamp *et al*., 2013). This shows the use of NO_3 and NO_2 as terminal electron acceptors by microbes is vital for their survival and a key process in the nitrogen cycle (Kamp *et al*., 2015). DNRA has been described as a similar process to denitrification as both denitrifiers and NO_3 ammonifiers utilise NO_3 as electron acceptors and therefore are competing for NO_3. Ammonification differs to denitrification however, as it converts NO_3 to NH_3 which can be utilised in nitrification; whereas the end product of denitrification is N_2 which is not directly used in other biochemical processes in the nitrogen cycle.

1.1.5 Anammox

As has been previously discussed, nitrification involving the process of ammonia oxidation was initially thought to only be able to be performed in aerobic conditions. However, it was discovered that ammonia oxidation could also be carried out without the presence of O_2 in a process called anaerobic ammonia oxidation (anammox) (Strous *et al*., 1999).

Anammox is a process where microbes are able to convert NH_3 and NO_2 ions directly to N_2 and H_2O (Equation 8) (Kartal *et al*., 2012). Anammox is carried out by Plantomycetes, a phylum of bacteria which uses NO_2 as an electron acceptor to produce N_2. Alongside denitrification, anammox is one of the few biological processes capable of producing N_2. In

1995, organisms capable of anammox were discovered and since then it has been found that anammox bacteria are found in almost all anoxic environments (van de Graaf *et al*., 1995). Anammox bacteria all contain an anammoxosome, a key component containing necessary enzymes for the reduction of NH_3 to N_2 (Cabello *et al*., 2009.). Research has found that NO_2 is initially reduced to NO which is then used as an oxidising power by the multiheme protein hydrazine hydrolase (hzh), to convert NH_3 into the poisonous compound hydrazine (N_2H_4). Oxidation of hydrazine catalysed by the enzyme hydrazine oxidoreductase (hzo) then results in N_2 (Cabello *et al*., 2009). It had previously been thought that due to NH_3's inert state in anoxic conditions, that anammox would not exist. However, the ability of anammox bacteria to oxidise NH_3 in anoxic conditions has led to anammox becoming the latest major discovery of the nitrogen cycle. Current estimates suggest that around 50% of fixed nitrogen in the ocean is released by bacterium capable of anammox (Kartal *et al*., 2012). As well as being a fundamental N_2 source, they have been used in wastewater treatment plants where they are key to the removal of NH_3. Currently there are 5 known genera of anammox bacteria, all belonging to the phylum *Plantomycetota*. These are: *Brocadia, Kueneunia, Scalindua, Anammoxoglobus and Jettinia*. These five genera share similar structures and metabolism, indicating they all evolved the ability to oxidise NH_3 from a single evolutionary event (Huub *et al*., 2007).

Equation 8:

$$NH_3^- + NO_2^- \rightarrow N_2 + 2H_2O$$

1.1.6 Assimilation

Microbes and other organisms such as plants are able to assimilate NO_3 and NH_3 to be incorporated into plant proteins and nucleic acids (Moreau *et al*., 2019). Assimilation is a major source of inorganic nitrogen for plants whereby NH_3 ions are actively transported into plants via NH_3 transporters. This is before one of two major assimilatory pathways occur, which convert the absorbed NH_3 into essential amino acids that can be used in a number of different physiological pathways in plants. The two major pathways are: the glutamate dehydrogenase pathway and the glutamine synthase – glutamine synthase (GS/GOCAT) pathway (Harper *et al*., 2010).

Firstly, looking at NH_3, in environmental conditions where there is a relatively high abundance of NH_3 (>0.1 mM), the enzyme glutamate dehydrogenase (GDH) is able to combine α-Ketoglutarate with NH_3 acting with Nicotinamide adenine dinucleotide phosphate (NADPH) as a coenzyme, to form glutamate. This can be seen in Figure 2. Glutamate is vital to a number of processes in plant growth and development such as root architecture, pollen germination,

pollen tube growth and seed germination (Qiu *et al*., 2020). This pathway takes place in environments of high NH_3 as GDH has a relatively low affinity for NH_3 (Plaitakis *et al*., 2017).

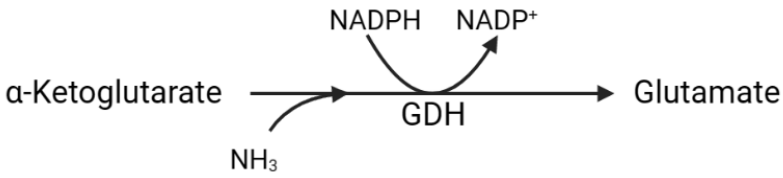


Figure 2: The glutamate dehydrogenase pathway. NH_3 and α-Ketoglutarate are combined to form glutamate, generating energy from the conversion of NADPH to $NADP^+$

However, in most soils, NH_3 concentration is relatively low, initiating the second major pathway, the GS/GOGAT pathway. The first stage of this pathway is the combination of glutamate and NH_3 to form glutamine, catalysed by the enzyme glutamine synthase (GS) and acquiring energy through hydrolysis of ATP.(Cruzat *et al*., 2018). This process is very energetically demanding and it is thought that around 15% of the cell's ATP is required to carry out this process, meaning production of glutamine is highly regulated at all cellular levels (Harper *et al*., 2010). Glutamine is an L-α- amino acid and an essential component to all organisms for growth and repair of cells and tissues. The second step in this process transfers NH_3 from glutamine to α-Ketoglutarate, forming two molecules of glutamate. This pathway can be seen in Figure 3. This is catalysed by the enzyme glutamate synthase. This pathway is favourable in soil conditions of low NH_3, as GS has a high affinity to NH_3.

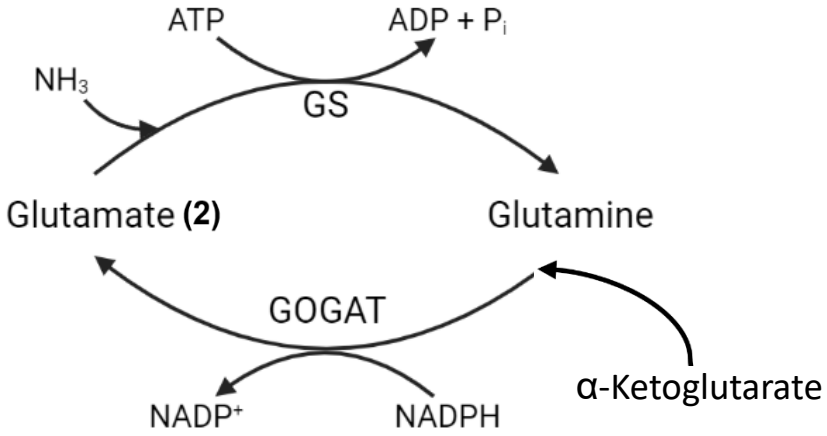


Figure 3: The glutamate synthase pathway. Glutamate and NH_3 are first combined to form glutamine, generating energy from the hydrolysis of ATP to ADP; before glutamine and NH_3 combine to form glutamate, generating energy from the breakdown of NADPH to $NADP^+$

NO_3 found in the soil must first be converted to NH_3 before it can be assimilated into plants. Therefore, NO_3 is converted to NO_2 in the cytosol by the assimilatory nitrate reductase, Nas, using NADPH as a coenzyme. NO_2 is then converted to NH_3 in the chloroplast by the assimilatory nitrite reductase, Nir. Once converted to NH_3, this is then assimilated into plants by the GS/GOGAT pathway.

1.1.7 Denitrification

Denitrification is the sequential reduction of NO_3 to N_2 signifying the end of the nitrogen cycle. In oxygen-limited environments, bacteria must switch from aerobic respiration to anaerobic respiration (Mohan *et al*., 2004). They can do this through denitrification whereby NO_3 is the electron acceptor instead of O_2. An incomplete denitrification pathway can lead to the release of the harmful greenhouse gas N_2O instead of N_2 which can have detrimental effects on the environment. The denitrification pathway will be explored in great detail throughout this thesis and is explained further in section 1.3.

1.2 Nitrous Oxide: The most significant greenhouse gas of the 21st Century

1.2.1 A potent greenhouse gas

Since the industrial revolution, modern human activities such as agricultural practices and fuel combustion have caused a significant increase in greenhouse gas (GHG) emissions, especially in the last 40 years (Lamb *et al*., 2021). CO_2 and Methane (CH_4) are the main contributors to global greenhouse gas emissions, accounting for approximately 76% and 16% of global emissions respectively. A less well-documented greenhouse gas nitrous oxide (N_2O), more commonly known as laughing gas accounts for 6% of global emissions (Gillman, 2018; EPA, 2022). In recent years, global efforts have been put in place to manage greenhouse gas emissions. However, currently not all goals are on target to be

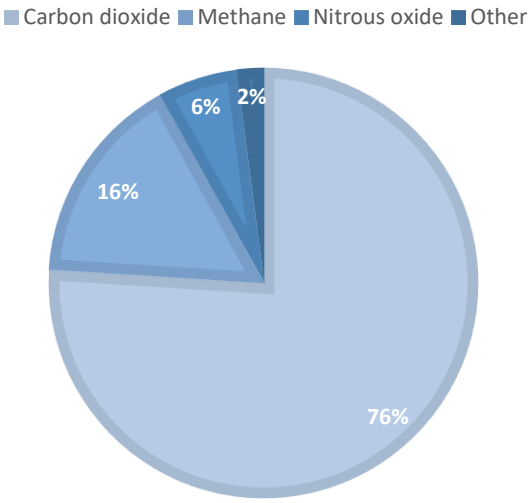


Figure 4: The primary global greenhouse gasses. The percentage of greenhouse gas emissions that each gas is responsible for is also given (EPA, 2022)

reached. The Paris Agreement in 2015 set a global aim to see a temperature increase of no more than 1.5°C above pre-industrial levels by the year 2030, at the time requiring a 45% reduction in greenhouse gas emissions (UNFCCC, 2015). However, a recent report published by The United Nations Environment Programme – 'The Closing Window' – suggested that current emission rates will see a 2.8°C rise in global temperatures by 2030 (UNEP, 2022). An increase as high as this will be devastating to the environment, causing issues such as: severe weather events including flooding and droughts, rising sea levels, mass extinction of species and starvation caused by food shortages (Tian *et al*., 2020).

This thesis will focus on N_2O as a contributor to climate change and will aim to understand its harmful global effects and mitigation strategies in greater detail. N_2O is a long-lived, ozone-depleting greenhouse gas of which emissions are increasing at an alarming rate. Although N_2O is emitted in far lesser quantities than the primary greenhouse gas CO_2, its potential global warming effects are ~300 times as potent (Solomon *et al,* 2007). Global N_2O emissions in 2016 were 10% greater than in the 1980s, the fastest rate of increase seen by any of the gasses monitored by the Intergovernmental Panel on Climate Change (IPCC) (Martin *et al*., 2021). Between 1750 and 2021, atmospheric N_2O concentrations increased by over 20% from 270 parts per billion (ppb) to 334.5 ppb. With N_2O global emission rates still increasing by ~0.2-0.3% each year, and with an atmospheric lifespan of around 114 years, it has been described as the most significant ozone-depleting gas of the century (Zhang *et al*., 2019; Uraguchi *et al*., 2009).

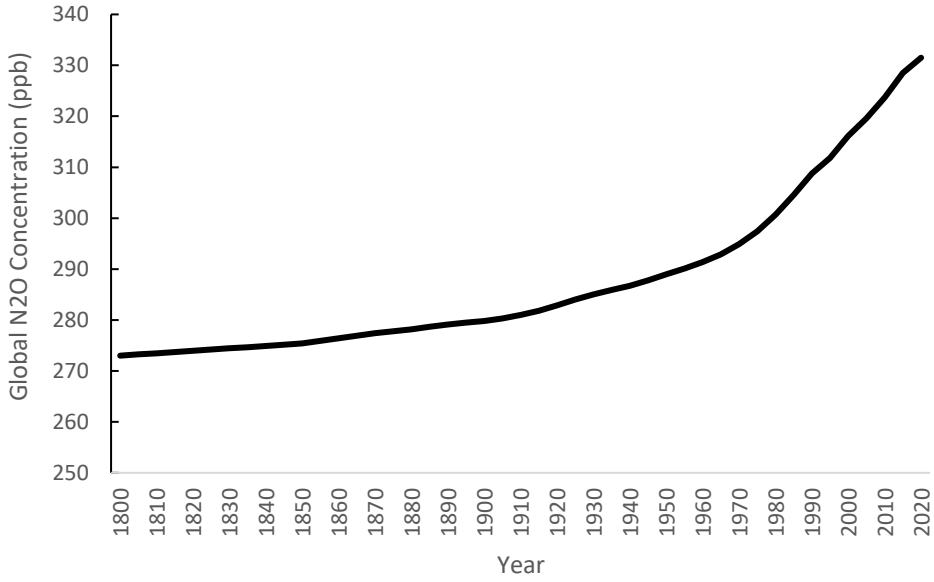


Figure 5: Atmospheric N_2O concentrations. In ppb between 1800 and 2020 (UNEP, 2013)

N_2O can be released from the nitrogen cycle through incomplete nitrification and denitrification pathways. Anthropogenic influences, such as in agricultural practices have largely contributed to disruptions to the denitrification pathway and increases in N_2O emissions. With the global population having just reached the milestone of 8 billion people, effective yet detrimental agricultural practices show no signs of slowing in order to feed the ever-growing world population. Therefore, it is becoming increasingly important to uncover novel mitigation strategies to help combat N_2O emissions. N_2O emissions have caused a serious threat to the environment, targeting the Earth's ozone layer leading to increased ozone depletion. Recent major climate summits have expressed aims of fighting climate change by reducing anthropogenic greenhouse gases including N_2O (UNFCCC, 2015; UNFCCC, 1997; IPCC SROCC, 2021). The recent Cop27 event in Egypt again highlighted the issues of greenhouse gases including N_2O with the World Meteorological Organisation (WMO) delivering a report stating that N_2O is currently at an all-time high, having risen by 1.3 ppb between 2020 and 2021 (WMO, 2022). Increasing levels of research are being conducted into the areas of climate change and N_2O. Due to its longevity in the atmosphere, many of the harmful effects caused by N_2O are now unavoidable and irreversible. This section will recognise the main sources of N_2O and reasons as to why N_2O emissions have increased exponentially in recent years. The future projections of N_2O will also be examined to understand what the serious implications may be if this harmful greenhouse gas is not controlled.

1.2.2 N_2O ozone depletion

The ozone layer is formed by the accumulation of ozone (O_3) particles in the Earth's stratosphere (Anwar *et al*., 2016). Solar formation of O_3 occurs when UV radiation causes O_2 to split into two separate O atoms. Each O atom then collides with separate O_2 molecules to form O_3 (van der Leun, 2004). The ozone layer acts as a protective layer to life on Earth by absorbing harmful UV radiation from the sun.

The first literature that focussed on ozone depleting substances (ODSs) was in the 1970s and focussed on the harmful NO_x emissions that could be emitted by supersonic aircraft flying in the stratosphere (Johnston, 1971). However, as attention to this issue subsided, more focus was drawn to other ODSs such as the increasing presence of Chlorofluorocarbons (CFCs) and other halogenated chemicals during the late 20th Century. Chlorofluorocarbons were widely used as propellants, refrigerants and solvents (Manzer, 1990). However, the sudden increase of ODSs such as these in the late 20th Century called for an urgent climate summit, The Vienna Convention for the Protection of the Ozone Layer. This convention led to a general agreement that the use of ODSs including CFCs must be heavily regulated to reduce harmful effects on the ozone layer (Weiss, 1985). From this, the Montreal Protocol was created which enforced strict regulations on the use of ODSs including CFCs, halons and methyl bromide

(Abadin Abid *et al*., 1987). The Montreal Protocol has been highly successful in reducing the usage and harmful effects of these ODSs. However, the recent increase of N_2O in the stratosphere and its effect on ozone depletion has not received as much attention. N_2O reacts with light in the stratosphere, reducing it to N_2 and O_2. N_2O also reacts with highly energetic O_2 atoms in the stratosphere which produces NO_x, known to cause ozone layer depletion. N_2O was not discussed at the Vienna Convention and is therefore not under regulation by the Montreal Protocol. Instead, emissions have been increasing exponentially overtime with little regulation.

Ozone Depletion Potential (ODP) is a metric used to measure how harmful substances are in depleting the ozone layer. In 2009, N_2O had an ODP of around 0.017 which was similar to harmful substances controlled by the Montreal Protocol such as HCFC-123 (0.02) and HCFC-124 (0.022). Whilst substances regulated by the Montreal Protocol have shown significant reductions in emissions, N_2O is projected to increase in coming years meaning it can now be described as the primary contributor to global ozone depletion.

1.2.3 Sources of N_2O

Since the beginning of life on Earth, N_2O has been produced naturally with its atmospheric concentration being low and relatively stable until around 1850. However, modern human influences such as industrialisation and the revolutionary Haber-Bosch process have caused a rapid increase in N_2O emissions since the 1850s and these emissions have been increasing exponentially to the present day (Chen *et al,* 2019). In pre-industrial times, natural N_2O emissions were approximately equal to the removal of N_2O by N_2O sinks – primarily the breakdown of N_2O in the stratosphere by light and O_2. However, nowadays that equilibrium no longer exists and N_2O emissions greatly outweigh removal rates.

The IPCC's 4th assessment report calculated that the current quantity of naturally sourced N_2O being released into the atmosphere is at around 11.0 Tg N_2O-N yr^{-1}. This includes N_2O stemming from terrestrial (6.6 Tg N_2O-N yr^{-1}), marine (3.8 Tg N_2O-N yr^{-1}) and atmospheric (0.6 Tg N_2O-N yr^{-1}) sources. Gross anthropogenic emissions of N_2O can be estimated at 6.2 Tg N_2O-N yr^{-1} and net N_2O emissions at 5.3 Tg N_2O-N yr^{-1} (UNEP, 2013).

Agricultural practices are by far the anthropogenic influence that has caused the most N_2O emissions, accounting for an estimated 66% of gross emissions (4.1 Tg N_2O-N yr^{-1}). As previously discussed, the introduction of the Haber-Bosch process and the subsequent overuse of nitrogen additives to soils is a major contributor to this and has led to major disruption on both an ecosystem level and atmospheric level. As previously mentioned in section 1, incomplete nitrification and denitrification can lead to the release of N_2O into the atmosphere. Increased addition of man-made N_r to soils has ultimately accelerated the

nitrogen cycle leading to imbalances in physiological pathways and an increase in the release of N_2O from soil denitrifiers. However, N_2O emissions may not just originate from the soil as a result of harmful agricultural practices. Excess N_r may be transported to water bodies through leaching which may lead to further N_2O emissions. Studies have found a clear correlation between increased levels of agricultural practices in the northern hemisphere compared with the southern hemisphere and a higher atmospheric N_2O abundance in the northern hemisphere (UNEP, 2013).

Industry and fossil fuel combustion accounts for around 15% of the gross N_2O emissions released (0.9 Tg N_2O-N yr^{-1}). Nitric acid, which is used in the creation of explosives and nitrogen fertilisers; and adipic acid, which is used in synthetic fibre production are the key industrial sources of N_2O emissions. Biomass burning is responsible for around 11% (0.7 Tg N_2O-N yr^{-1}) of gross anthropogenic N_2O emissions, taking into account of crop burning and wildfires as well as household biomass burning such as heating. This figure takes into account both natural and human-caused biomass burning as it can be difficult to distinguish between the two in some cases. Wastewater and aquaculture accounts for N_2O emissions due to N_2O escaping from wastewater including sewage as well as through wastewater treatment. Wastewater and aquaculture equate to around 4% (0.21 Tg N_2O-N yr^{-1}) of gross anthropogenic N_2O emissions (UNEP, 2013).

1.2.4 Possible solutions to the N_2O challenge and future projections

Although N_2O was not discussed at the Vienna convention and was not a regulated ODS of the Montreal Protocol, recent climate summits have recognised the need to address N_2O and have discussed protocols to decrease emissions of this gas in the future. However, currently N_2O emission rates are predicted to increase exponentially year on year, as mitigation strategies are not strong enough to have a significant impact.

Agricultural practices is the most obvious anthropogenic N_2O source to target as it has, by far, caused the highest emission rates. Although anthropogenic N_2O emissions stemming from agriculture seem inevitable in the modern world with increasing numbers of mouths to feed, there are steps that could be taken to significantly reduce emissions. Firstly, changes in diets could lead to reduced N_2O emissions. N_2O emissions stemming from animal-protein is 10-fold greater than from plant protein (Mark *et al*., 2011). It has also been found that the average human consumes 70% more protein than is needed in accordance with the World Health Organisation (WHO, 2007). Therefore, reducing intake of animal protein would reduce demand for these products and therefore reduce N_2O emission rates. Reducing food wastage could also lead to reduced N_2O emissions. It is thought that British civilians throw away around 33% of purchased food (UNEP, 2012). Similarly, to changing diets, reducing food waste by

only purchasing what is needed would lead to less demand and therefore reduced N_2O emissions. Another way to reduce N_2O through agriculture would be to improve the nitrogen use efficiency (NUE) of crops. Increased NUE leads to reduced N_2O emissions as well as improvements in crop yield. Studies have found that NUE in crops past an optimal level see sharp rises in N_2O emissions as the nitrogen input level into the soil has been reached and extra nitrogen additives are not being utilised by crops (Venterea *et al*., 2010). Management strategies such as the '4R nutrient Stewardship' have encouraged the correct level of nutrient application to soils (Mark *et al*., 2011). An increase in management strategies such as this would see a significant decrease in N_2O emissions from soils.

Future projections of N_2O are currently showing no signs of slowing. Management strategies into agriculture as well as into other key areas of N_2O emissions including fossil fuel combustion and biomass burning, will all need to be implemented if we are to see improvements in climate issues surrounding N_2O (Lamb *et al*., 2021).

1.3 Denitrification – A major N_2O sink

1.3.1 Introduction

As was briefly mentioned in section 1.1.7, the denitrification pathway is the sequential reduction of NO_3 to N_2 and is a significant contributor to global N_2O emissions due to truncation of the pathway at the final reduction stage. This anaerobic process occurs in O_2-limited environments such as in waterlogged soils after heavy rainfall where the availability of O_2 is greatly reduced (Robertson & Groffman, 2006). The reduction of NO_3 to the harmless gas N_2 is via a four-step reduction process (Figure 4). This reduction involves the intermediates NO_2, nitric oxide (NO) and N_2O (Mohan *et al*., 2004). Firstly, NO_3 is reduced to NO_2 by nitrate reductase (Nar) (Equation 9). NO_2 must then be reduced to NO by nitrite reductase (Nir) (Equation 10). Next, NO is reduced to N_2O catalysed by the reductase nitric oxide reductase (Nor) (Equation 11). The final step in the denitrification pathway is the reduction of N_2O to N_2 by way of nitrous oxide reductase (Nos) (Equation 12). If any stage in the denitrification process is not completed, the pathway will be truncated and N_2 will not be released. Instead, one of the previous intermediates in the pathway will be released instead.

Equation 9:

$$2NO_{3^-} + 4H^+ + 4e^- \rightarrow 2NO_{2^-} + 2H_2O$$

Equation 10:

$$2NO_{2^-} + 4H^+ + 2e^- \rightarrow 2NO + 2H_2O$$

Equation 11:

$$2NO + 2H^+ + 2e^- \rightarrow N_2O + H_2O$$

Equation 12:

$$N_2O + 2H^+ + 2e^- \rightarrow N_2 + H_2O$$

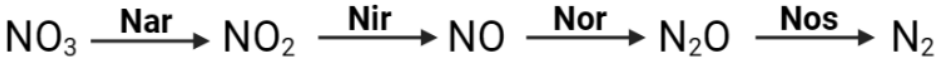


Figure 6: The complete denitrification pathway showing the sequential reduction of NO_3 to N_2 by the reductases Nar, Nir, Nor and Nos.

Denitrification is carried out by a diverse range of prokaryotes including bacteria, archaea and eukarya (Girkin & Cooper, 2022). Over 50 genera and over 125 different species having been identified including organotrophs, chemotrophs, thermophiles and pathogens (Robertson & Groffman, 2006). Extensive research on such an abundance of diverse denitrifiers has led to extensive knowledge about the characteristics of denitrifiers and their mechanisms of action. However, not all denitrifiers are culturable in a laboratory. The most culturable denitrifiers are facultative anaerobes belonging to 3-6 genera, mainly *Pseudomonas* and Alcaligenes. It is thought that denitrifiers make up around 0.1-5% of culturable microbes found in soil and make up around 20% of all microbes found in the soil (Robertson & Groffman, 2006). Denitrifying organisms can be partial denitrifiers which are unable to complete the entire pathway, or complete denitrifiers which are able to carry out the full denitrification process (Hallin *et al*., 2007).

Environmental factors including temperature, pH, oxygen availability and NO_3 concentrations in the soil are all known to have significant effects on denitrification rates (O'Neill *et al*., 2020). At a gene level, the reductases involved in denitrification require different metal cofactors to perform each stage of the pathway. For example, Nos requires copper (Cu) to reduce N_2O to N_2; meaning in soils of low Cu concentrations, the final stage of the denitrification pathway may be incomplete and N_2O is released instead of N_2 (Sullivan *et al*., 2013). Reductases involved in denitrification are activated or repressed by transcriptional regulators (TRs) belonging to the FNR (fumarate and nitrate reduction)/CRP (cyclic-AMP receptor protein) superfamily of TRs. FNR TRs are particularly influenced by environmental factors such as O_2 availability and NO concentration in soils.

As previously discussed in section 1.2, a significant environmental issue surrounding denitrification occurs when the pathway is truncated at the last stage of the pathway and the harmful greenhouse gas N_2O is released into the atmosphere instead of N_2. The release of

N_2O has serious implications for global climate issues and has been described as the most significant gas of the 21st century (Uraguchi *et al*., 2009).

In this section, the denitrification pathway will be examined in greater detail. Firstly, the key reductases involved in the denitrification pathway will be explored focussing on their structure and catalytic functions, then, understanding of environmental and transcriptional regulation and of these reductases will also discussed paying particular attention to the model bacterial denitrifier, *P. denitrificans.*

1.3.2 Nitrate Reductase

Prokaryotic nitrate reductases are molybdenum dependent enzymes that can be split into three groups depending on their operon organisation, active site structure and cellular location within cells (Pinchbeck *et al.,* 2019). Two of these three nitrate reductases are dissimilatory, (Nar and Nap) whereas the other nitrate reductase is assimilatory (Nas). Each nitrate reductase is distinct in that it reduces NO_3 to NO_2 for specific purposes within a cell.

Although Nar and Nap both reduce NO_3 by way of dissimilatory nitrate reduction they are involved in separate pathways within the nitrogen cycle. Nar is involved in the reduction of NO_3 to NO_2 in the denitrification pathway whereas Nap is involved in the reduction of nitrate to nitrite involved in the DNRA process. Assimilatory nitrate reduction on the other hand, incorporates nitrogen found in NO_3 into a plant's biomass through the process of assimilation (Sparacino-Watkins *et al*., 2014).

The cellular locations of these nitrate reductases have greatly defined them. Nar and Nap are membrane-associated which is appropriate in terms of their roles involved in processes such as energy acquisition and redox regulation. Nar orientates towards the cytoplasm of a cell and couples proton translocation and nitrate respiration through the creation of an electrochemical gradient which can be used to generate ATP synthesis. Nap on the other hand is associated with the periplasm. The location of Nas is within the cytoplasm (Sparacino-Watkins *et al*., 2014).

Nar has been isolated from a number of well-characterised denitrifiers including those belonging to the genera *Pseudomonas, Paracoccus* and *Thiosphaera* (Baumann *et al*., 1996; Bedzyk *et al*., 1999; Bell *et al*., 1990). These multimeric enzymes are encoded by a number of genes with the genes *narGHI* encoding the metalloenzyme NarGHI complex that reduces NO_3 to NO_2 (Pinchbeck *et al*., 2019). The catalytic subunit, NarG (α) (140 kDa), contains a molybdopterin guanine dinucleotide (MGD) cofactor and an iron-sulfur centre [4Fe-4S]. The soluble subunit, NarH (β) (60 kDa), contains one [3Fe-4S] and three [4Fe-4S] centres. The membrane bound subunit, Narl (γ) (25 kDa), contains two *b*-type hemes (González *et al*.,

2006). The NarGHI complex reduces NO_3 in a 2-electron transfer whereby electrons are drawn from ubiquinol, a form of coenzyme Q found in cells that is rich in electrons (Craske *et al*, 1986). The active site of NarG faces the cytoplasm, meaning NO_3 must be transported into the cell across the cytoplasm to be reduced before being transported back across the cytoplasm as NO_2. This translocation of NO_3 is usually carried out by transport proteins belonging to the NarK family. NarJ is tasked with assembly of NarGH prior to the attachment of NirI (Blasco *et al*., 1998).

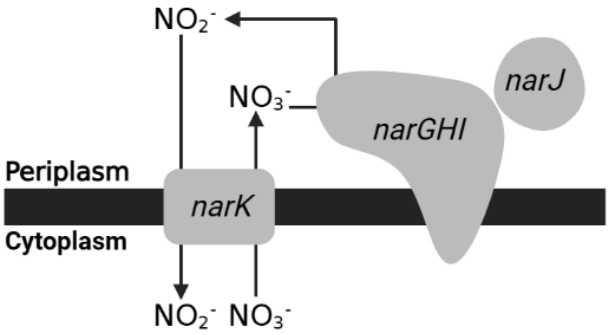


Figure 7: Organisation of Nar within cells showing cellular location of the NarGHI complex and NarKJ in relation to the periplasm and cytoplasm of a cell (Blasco *et al*., 1998).

The catalytic subunit of Nap is NapA and like NarG, contains the MGD cofactor and a [4Fe-4S] cluster. NapB contains a cytochrome *c* and forms a NapAB complex with NapA. NapC is responsible for the electron transfer to NapB from the ubiquinone pool. NapF has found to be associated with assembling of NapA and NapD has been found to be involved with NapA maturation (Cabello *et al*., 2009).Once again, the catalytic subunit of Nas, NasC, contains anMGD cofactor. NasC is where the reduction of nitrate to nitrite takes place through a two-electron process. (Moreno-Vivia *et al*., 1999).

1.3.3 Nitrite reductase

Nir is a periplasmic enzyme responsible for the reduction of NO_2 to NO in a one electron transfer reaction. Studies have shown that there are 2 distinct classes of Nir: those that contain heme as a cofactor (cytochrome cd_1 nitrite reductase) encoded by the *nirS* gene; and those that contain Cu as a cofactor (copper-containing Nir) encoded by the *nirK* gene (Rinaldo & Cutruzzolà, 2007). Although cd_1Nir and CuNir perform the same reduction in the denitrification pathway, they rarely coexist in the same bacterial species and were thought not to at all, until *Bradyrhizobuim nitroreducens* was discovered to possess both types of Nir in 2018 (Jang *et al*., 2018).

cd_1Nir has been found in a number of denitrifiers such as *Pseudomonas aeruginosa* and *Pseudomonas nautica* and *P. denitrificans* (Rinaldo & Cutruzzolà, 2007). cd_1Nir is a

homodimer consisting of two 60 kDa subunits, each with one α-helical domain containing c-type heme where the electron acceptor pole is situated; and one β-propeller d_1-type heme where the active site is situated (Farver *et al*., 2003). Non-covalent interactions between the two monomers are very strong, and the domains within the monomers share segments of their N-terminal domains with each other. Catalysis is initiated when the substrate binds to the d_1-type heme, producing a nitrosyl intermediate and then finally NO (Cabello *et al.* 2009).

CuNir is present in gram-negative and -positive bacteria and archaea including *Alcaligenes faescalis* and *Rhodobacter sphaeroides* (Murphy *et al*., 1995; Olesen *et al*., 1998). The structure of CuNir differs to that of cd_1Nir in that it is a homotrimer made up of 3 monomers (~40 Kda), consisting of 3 identical subunits containing both type 1 and type 2 Cu centres. The type 1 Cu centre acts as a redox centre and from here electrons are transferred to the type 2 Cu centre where the substrate binds. The catalysis reaction is similar to that of cd_1Nir whereby nitrosyl is produced as an intermediate (Cabello *et al.* 2009).

1.3.4 Nitric oxide reductase

Nor is a transmembrane protein belonging to the heme-copper oxidase (HCO) super-family of reductases (Saraste & Castresana, 1994) and is responsible for the reduction of NO to N_2O. It is important that this reduction stage is rapid due to the high cytotoxicity level of NO. NO-induced cell death can be caused by disrupted energy metabolism, DNA damage, oxidative stress and dysregulation of cytosolic calcium. Disruptions such as these can lead to apoptotic or necrotic cell death, depending on the severity effects caused by NO (Murphy., 1998). Nor is of particular interest in relation to the environment and as it reduces NO to the harmful greenhouse gas, N_2O. There are three types of Nor: cNor, qNor and qCu_ANor.

Cytochrome *c* dependent (cNor) is key to the penultimate reduction stage in denitrification and is found only in denitrifying species such as the well-studied denitrifiers: *P. denitrificans, Pseudomonas stutzeri* and *P. aeruginosa* (Hutchings & Spiro, 2000; Lichtenberg *et al*., 2021; Zumft *et al*., 1994). cNor, in denitrifying bacteria, consists of NorB and NorC subunits (Zumft, 2005). NorC is the smaller of the two subunits (17kDa), consisting of a heme *c* with His and Met residues acting as axial ligands. NorC is responsible for receiving electrons from electron donors. These donors are either c-type cytochromes, azurin or pseudoazurin which are allocated in the periplasm (Spiro, 2012). NorB (53 kDa) is a transmembrane protein where the catalysis of the reduction of NO to N_2O takes place. NorB contains two *b*-type hemes and one non-heme iron. One of the two *b*-type hemes is responsible for transferring electrons from heme *c* in NorC to the catalytic centre of the protein in NorB consisting of another *b*-type heme (b_3) and non-heme iron (Fe_B) (Shiro, 2012). Two NO molecules must be transferred to the catalytic centre of the protein where electron transfer from b_3 and Fe_B leads to the formation of

the intermediate, hyponitrite. Hyponitrite consists of two N atoms and two O_2 atoms (O-N **=** N-O) (Wright & Hayton, 2015). The bond between N and O_2 of hyponitrite is then broken, producing N_2O and H_2O (Shiro, 2012).

Along with *norCB,* a number of other genes are commonly transcribed in the Nor gene operon including *norQDEF*. In all denitrifiers, *norQ* and *norD* are linked to *norCB* whereas *norE* and *norF* may be found at distant locations or not found at all in some genomes (Zumft, 2005). The functions of *norQDEF* are not yet well understood.

qNor has been isolated from denitrifiers such as *Ralstonia eutropha* and *Pyrobaculum aerophilum* (Cramm & Strube, 2008). It is also found in non-denitrifying, pathogenic species of bacteria where it plays a key immune response role in NO detoxification. qNor are single subunits (80 kDa) containing *b*-heme but lacking heme c. Unlike cNor, qNor use ubiquinol or menaquinol as electron donors (Cabello *et al*, 2009). The final Nor, qCu_ANor has been isolated in the bacterium denitrifier *Bacillus azotoformans* and contains on subunit, similar to NorB. It receives electrons from menaquinol and cytochrome c551 (Suharti *et al*., 2004).

1.3.5 Nitrous oxide reductase

The periplasmic enzyme, Nos, catalyses the reduction of N_2O to N_2 in the last stage of the denitrification pathway and has been extensively studied due to it being the only natural biological sink of the harmful N_2O. If Nos does not function correctly, the denitrification pathway is truncated and N_2O is released into the atmosphere instead of N_2 (Richardson *et al,* 2009). Research into Nos function is therefore highly important in the fight against N_2O emissions.

Nos is a periplasmic, homodimeric protein with two multinuclear Cu centres (120 – 160 kDa) (Carreira *et al*., 2017). Each monomer contains a binuclear Cu_A centre in the C-terminal domain, formed by two Cu atoms and is the electron entry site into Nos. Cu_A also contributes to Nos stability. At the N-terminal domain is found a sulfide-bridged tetranuclear Cu_Z centre, formed of four Cu atoms where catalysis occurs (Solomon *et al*., 1996). Electrons are most commonly transferred to the Cu_A centre of Nos via small electron carriers such as cytochrome c550 or pseudoazurin in the periplasmic space, originating from the cytochrome *bc*1 complex. This can vary between denitrifying genera. For example, whereas Nos in *R. capsulatas* and *R. sphaeroides* receive electrons via a c-type cytochrome (Richardson *et al*., 1991), *P. pantotrophus* receives electrons via pseudoazurin (Berks *et al*., 1993). The Cu_Z is unique as it has, up until now, only been identified in Nos.

As the structure of Nos constitutes two multinuclear Cu centres, it is of no surprise that the presence of Cu can affect the efficacy of Nos in the final reduction stage of the denitrification pathway. It was first found by Matsubara *et al*(1982) whilst studying *Pseudomonas perfectomarinus*, that the end product of the denitrification pathway was N_2O in a Cu low

environment (Matsubara *et al*., 1982). It was later discovered by Felgate *et al*(2012) that in Cu-depleted conditions, that *P. denitrificans* released ~40% of NO_3 as N_2O during denitrification studies. In fact, by the end of this investigation, the N_2O released by *P. denitrificans* in Cu-limited conditions was more than 1000-times greater than in Cu-sufficient cultures (Felgate *et al*., 2012). Research into this continued with Sullivan *et al*(2013) producing the first study into the genetic control of *nos* genes by extracellular Cu levels (Sullivan *et al*., 2013). In this study, genes encoding Nar, Nir, Nor and Nos were all subject to growth in denitrifying conditions of high and low Cu. Genes encoding Nar, Nir and Nor showed no changes in expression between the two growing conditions. However, genes found in the *nos* operon *nosRZDFYLX* were all significantly downregulated in Cu-L conditions indicating that these genes are strongly regulated by the presence of Cu (Sullivan *et al*., 2013). NosZ is the catalytic subunit of Nos and is responsible for binding 12 Cu ions per functional homodimer in Cu_A and Cu_Z. Therefore, it is no surprise that in Cu-L conditions, this protein is strongly downregulated. Whilst a definitive role for *nosDFYLX* has not yet been confirmed, it is thought that they may be involved in NosZ assembly and activation (Ulrike Honisch & Zumft, 2003). Interestingly, *nosC* was up regulated in Cu-L conditions. NosC is a hypothetical protein with an unknown function. However, due to its increase in expression in Cu-L, this indicates a potential role in Cu regulation in Nos. Another gene in the Nos operon, *nosR* is found adjacent to *nosZ* in the majority of denitrifying bacterial genomes and is therefore thought to play a role in N_2O reduction. Sullivan *et al* wanted to test whether *nosC* and *nosR* were important in Cu regulation and therefore created mutant *P. denitrificans* strains where each gene was independently deleted. In the mutant *nosC* strain N_2O reduction was significantly reduced in Cu-H and Cu-L cultures. This study also wanted to understand whether *nosC* and *nosR* were involved in the regulation of *nosZ.* Therefore, the transcription of *nosZ* in mutant *nosC* and *nosR* strains were analysed. Interestingly, the transcript levels of *nosZ* in both mutant strains did not change between Cu-H and Cu-L conditions. This is significant as in wildtype strains, it would be expected that *nosZ* would show a significant change in expression between Cu-H and Cu-L conditions. Therefore, the fact that *nosZ* expression does not change when *nosC* and *nosR* were mutated suggests that *nosC* and *nosR* play key roles in the regulation of *nosZ* in response to Cu. However, the exact mechanisms are not yet fully understood (Sullivan *et al*., 2013).

1.3.6 *Paracoccus denitrificans*

Denitrifying bacteria have an important role in regulating N_r in conditions of low O_2 concentrations in a wide range of environments. Denitrification is crucial to the survival of these bacteria where they are able to switch between respiring O_2 and NO_3. *P. denitrificans* is a model bacterial denitrifier that has been studied extensively as a laboratory model in

denitrification studies. *Paracoccus* was first isolated in 1910 by Dutch microbiologist, Martinus Beijerinck, and its genome was fully sequenced in 2004 (Beijerinck & Minkman, 1910). *P. denitrificans* is an organo-lithotrophic, gram negative alpha proteobacteria which is ubiquitous in nature, represented in both aerobic and anoxic environments such as soil, wastewater treatment plants and marine sediments (Baumann *et al*., 1996). It is genetically tractable and easy to control expression of its genes. As well as this, *P. denitrificans* is a complete denitrifier, able to complete each stage of the denitrification pathway (Spiro, 2016). The exact enzymatic mechanisms of the denitrification pathway in *P. denitrificans* have become well characterised and have greatly improved understanding in this field. *P. denitrificans* has been cultured under a number of different environmental conditions and has been used to uncover the strong dependence of *nosZ* on Cu availability in soils (Sullivan *et al*., 2013). *P. denitrificans* was the model denitrifier focussed on during this study.

1.4 Transcriptional Regulation of Denitrification and environmental influences

1.4.1 Transcriptional Regulation in bacteria

During transcription, DNA is transcribed into readable genetic code (mRNA) by RNA polymerase, which is then translated by ribosomes into amino acids and proteins. In bacteria, it is widely accepted that the expression of genes is controlled by TRs of which can be specific to a single gene or can regulate a network of genes. Through binding with the promoter region of a gene, TRs either upregulate or downregulate the gene's expression. TRs are known to be greatly controlled by environmental signals which allows organisms to correctly respond to these signals and mount a response through gene repression or activation. Transcriptional regulation is vital to all living organisms for survival. Sigma factors (σ) are important in transcription within bacteria as they help to enhance binding between RNA polymerase and the DNA template strand, creating an RNA holoenzyme. In bacteria, σ^{70} is responsible for the transcription of most essential growth-related genes.

1.4.2 Transcriptional regulation of denitrification

As has previously been discussed, the denitrification pathway consists of the key reductases Nar, Nir, Nor and Nos, which are all essential to the completion of the denitrification pathway. Due to their key role in the denitrification pathway, the gene operons of these reductases must be closely monitored and are constantly being regulated by TRs.

In *P. denitrificans,* this regulation is carried out by TRs belonging to the FNR (fumarate and nitrate reduction) / CRP (cyclic-AMP receptor protein) super-family of TRs. The first FNR protein was discovered in *E. coli* and played a role in the transition between aerobic and anaerobic respiration (Lambden A N & Guest, 1976). The structure of FNR proteins consists of two domains: an N-terminal domain with a $[4Fe\text{-}4S]^{2+}$ or $[2Fe\text{-}2S]^{2+}$ cluster that is bound by four cysteine residues; and a C-terminal domain with a DNA-binding helix-turn-helix domain. In the presence of O_2, the $[4Fe\text{-}4S]^{2+}$ in the N-terminal domain is oxidated and the protein becomes unable to bind to its target gene (Van Spanning *et al*., 1997). Therefore, FNR-family TRs often have regulatory roles in environments where O_2 levels have a significant impact on the survival of organisms. (Hoe *et al*., 2013). Most FNR-family TRs share these similar features: 1. An N-terminal domain containing 3 cysteine residues, combining with a fourth cysteine residue in the central domain to form a [4Fe-4S] cluster. 2. Residues found in the central domain that may expose a loop that RNA helicase recognises and is able to bind to. 3. An alpha-helical domain involved in dimerization. 4. A helix-turn-helix (HTH) binding motif in the C-terminal end recognising the consensus sequence TTGAT-N4-ATCAA. This is also known as the FNR box (Hutchings *et al*., 2002). FNR proteins are therefore sequence specific which is common amongst regulators involved in important metabolic pathways such as denitrification.

The sigma factor σ^{54} has been found to be involved in the regulation of a number of different genes in denitrifying bacteria. For example, *Ralstonia eutropha* requires σ^{54} for growth and studies into the roles of this sigma factor have found it to be important in the regulation NorR to control expression of the *nor* operon (Pohlmann *et al*., 2000). *P. denitrificans* has been found to encode a σ^{54} homologue which may also play a denitrifying role similar to that seen in *R. eutropha.*

1.4.3 FnrP, NNR and NarR

P. denitrificans employs three FNR-family TRs to control the expression of Nar, Nir, Nor and Nos. These are: fumarate and nitrate reduction protein (FnrP); nitrite reductase and nitric oxide reductase regulator (NNR); and nitrate reductase regulator (NarR) (Van Spanning *et al*., 1997). All three of these proteins are key to the completion of the denitrification pathway. They are all highly sensitive to environmental conditions.

FnrP is involved in activation of the gene operons of Nar and Nos. An FnrP mutant strain of *P. denitrificans* was found to have a 5-fold reduction in growth rate in anaerobic denitrifying conditions when compared with the wildtype (Van Spanning *et al*., 1997). The Nar activity in this strain was reduced by 3-fold showing the importance of FnrP to the first reduction stage of the denitrification pathway (Van Spanning *et al,* 1997). A recent study demonstrating the

transcriptional profile of *P. denitrificans* in an FnrP mutant strain found that FnrP was also responsible for regulation of Nos, the key reductase in the final stage of the denitrification pathway (Bouchal *et al*., 2010). In *P. denitrificans,* FnrP is formed of a $[4Fe\text{-}4S]^{2+}$ cluster that is coordinated by four conserved cysteine clusters (Cys14, 17, 25 and 113). However, this cluster is greatly affected by the presence of O_2 where it goes through O_2-driven cluster conversion from [4Fe-4S] to [2Fe-2S]. Recent work has found that the presence of NO can also cause dissociation of the [4Fe-4S] cluster into monomers (Crack *et al*., 2016).

NNR is homologous to the FnrP protein, however, it lacks the N-terminal cysteines found in FnrP. NNR is the second FNR protein that *P. denitrificans* employs in the denitrification pathway. Like FnrP, an NNR mutant strain was found to show a reduction in growth rate in anaerobic denitrifying conditions compared to wildtype *P. denitrificans*; in this case of 2-fold. This mutant strain showed an accumulation of nitrite which was discovered to be due to the fact that Nir activity in this strain had been completely stopped (Van Spanning *et al*., 1995). This shows that NNR is important to the regulation of Nir in the second reduction stage of the denitrification pathway. NNR is also responsible for the expression of genes encoding Nor in the third reduction stage of denitrification. Interestingly, recent investigations into NNR have also found it to be responsible for expression of genes involved in Nos (Bergaust *et al*., 2012). Similarly to FnrP, NNR function is rapidly inactivated in the presence of O_2. This was confirmed by a low number of NirS mRNA transcripts when an anaerobic culture of *P. denitrificans* was exposed to aerobic conditions (Baumann *et al,* 1996). However, unlike FnrP, transcription by NNR is activated in the presence of NO. NNR is able to work as a dual-sensor between O_2 and NO (Lee *et al*., 2006).

The least studied and third FNR protein encoded by *P. denitrificans* is NarR. NarR responds to both nitrate and nitrite and is required for maximal expression of Nar. The C terminal of NarR possesses a helix-turn-helix domain which is homologous to NNR, meaning NarR is able to bind to FNR-like cognate sites. The gene *narR* is found just upstream of *narK* whose expression it controls (Bergaust *et al*., 2012).

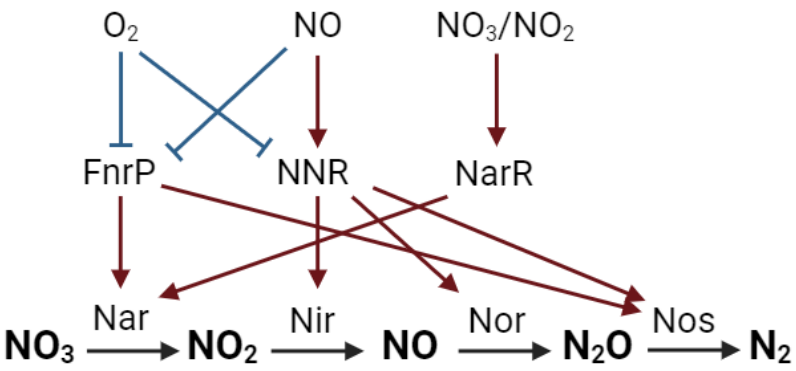


Figure 8: The denitrification pathway with the key reductases indicated; the FNR transcriptional regulators that regulate each reduction stage; and the environmental factors that affect the expression of these FNR transcriptional regulators. Red arrows indicate an activation whereas blue lines indicate inhibition.

1.4.4 FNR homologues

FnrP, NNR and NarR proteins are of great importance to the denitrification pathway in *P. denitrificans.* However, there are other homologous FNR proteins that exist in other denitrifying species and carry out similar denitrifying roles.

Denitrifiers belonging to the *Pseudomonas* genera employ members of the FNP/CRP superfamily of TRs similar to *P. denitrificans*. ANR (anaerobic regulator of arginine deiminase and nitrate reductase) is a TR found in *P. aeruginosa* and plays a similar role to FnrP found in *P. denitrificans* in that it encodes Nar in O_2-limited environments (Schreiber *et al*., 2007). The structure of ANR is similar to FnrP in that it is made up of a [4Fe-4S] cluster that is dissociated to [2Fe-2S] in the presence of O_2 and NO (yoon *et al*, 2007). ANR also promotes transcription of dissimilatory nitrate respiration regulator (DNR) which is responsible for the transcription of *nir and nor* genes in response to NO, much like NNR (Aral *et al*., 1997). A separate *Pseudomonas* species, *P. stutzeri* encodes the FNR homologues dnrD, dnrE and dnrS. However, these homologues do not contain the [4Fe-4S] cluster seen in most FNR regulators involved in anaerobic environments. DnrD has been found to be key in denitrification regulation activating gene expression of *nir* and *nor* operons (Schreiber *et al*., 2007).

The marine denitrifier, *Dinorosebacter shibae* encodes seven FNR/CRP homologues of which four (FnrL, DnrD, DnrE and DnrF) are known to play a role in denitrification (Wagner-Döbler *et al*., 2010). Out of these, FnrL contains cysteine residues that may be involved in iron-sulfur cluster formation and therefore could play a key role in sensing O_2 in the regulation of denitrifying genes in *D. shibae* (Ebert *et al*., 2017).

An NNR homologue is encoded by *Rhodobacter sphaeroides* named NnrR. It has been found that an NnrR mutant strain in *R. sphaeroides* leads to a reduction in NO_2 and NO due to NnrR being a transcriptional activator of Nir and Nor. NnrR also encodes *nnrS,* an additional TR of which its expression controlled by NnrR (Bartnikas *et al*., 2002).

Bradyrhizobium diazoefficiens is best known for N_2-fixation and is a soybean symbiont. However, it is also known to be able to complete denitrification. *B. diazoefficiens* encodes NnrR, which is homologpus to NNR of *P. denitrificans* and NnrR of *R. sphaeroides.* In *B. diazoefficiens,* NnrR regulates FixLJ-$FixK_2$ that in turn regulate key genes involved in denitrification. FixL, a histidine kinase acts as an O_2 sensor in which low O_2 levels cause autophosphorylation of FixL whereby a phosphate group is transferred to FixJ. FixJ then activates $FixK_2$ which is then responsible for the expression of *napEDABC, nirK, norCBQD* and *nosRZDYFLX* (Bueno *et al*., 2017). NnrR therefore improves control of the FixLJ-$FixK_2$ cascade and in turn Nar, Nir, Nor and Nos. As well as this, proteins RegS and RegR in *B. diazoefficiens* are known to play a role in *nor* and *nos* gene operon expression in response to NO_3 and low levels of O_2. *P. denitrificans* shares the homologous proteins RegAB. However, the role of these proteins in denitrification is still yet to be determined (Torres *et al*., 2014).

1.4.5 Environmental factors affecting denitrification

It has already been discussed that FnrP, NNR and NarR are all regulated by the presence of O_2 denitrifying intermediates such as NO. The regulation of TRs by environmental signals is vital to ensuring biological pathways operate efficiently and to achieve maximum energy yields to facilitate an organisms survival. Research into the environmental regulation of denitrification has increased over recent years as scientists have aimed to understand the denitrification pathway in greater detail. As reducing N_2O emissions has become increasingly important, this has led to a greater importance of understanding the environmental signals that cause N_2O to be released. Whilst some environmental signals such as O_2 can directly affect TRs involved in denitrification, other environmental signals directly affect the pathway and target the key reductases involved.

As has been mentioned in detail previously (1.3.5), Nos, the final reductase in the denitrification pathway is heavily reliant on the presence of Cu to carry out the reduction of N_2O as it strongly regulates the activity of NosZ (Sullivan *et al*., 2013). The demand for Cu by NosZ is high as its activity relies on the presence of Cu_Z and Cu_A to bind to N_2O. Whilst some bacterial enzymes that normally require the presence of Cu are able to find alternatives to carry out their functions, NosZ activity has been shown to completely fail in the absence of Cu leading to the truncation of the denitrification pathway and the release of N_2O instead of N_2. Interestingly, this same study found that *nosC,* was up-regulated in response to Cu limitation,

differing from the other genes of the *nos* operon. *nosC* and *nosR,* which is found adjacent to *nosZ* in most Nos operons, were both found to play a part in copper regulation in *nosZ* yet their specific roles are yet to be uncovered (Sullivan *et al,* 2013).

Zinc is a key element in the survival of many organisms and plays a key catalytic role for many enzymes and in the organisation of protein structures (Coleman, 1992). However, zinc in excessive levels can be toxic, for example it can displace other key metal elements such as manganese and iron (Waldron & Robinson, 2009). Therefore, the control of zinc uptake and zinc homeostasis must be strongly controlled by organisms. A study into the effects of zinc on genes in *P. denitrificans* identified 147 genes were differentially expressed across growth conditions of varying levels of Zinc. The majority of these genes were upregulated in growth conditions of low Zinc. Along with several transcription factors and transition metal transporters, genes encoding Nor (*norCB*) and genes encoding Nir (*nirS*) were up-regulated by way of zinc-depletion. Interestingly, *norC* showed up-regulation of nearly 10-fold. The study also found that *norCB* were regulated by the zinc uptake regulator (Zur) (Neupane *et al*., 2017) This could mean that a lack of zinc may cause a decrease in the reduction of nitrite and nitric oxide in denitrification and in turn a reduction in later intermediates including N_2O and N_2.

The pH of soil has previously been described as a 'master variable' due to its significant effects on a number of physiological pathways. This is thought to be due to the enzymes in these physiological pathways becoming denatured under acidic conditions and therefore, not being able to carry out their physiological roles. It has been found to play a role in the denitrification pathway with a study finding that the ratio of N_2O:N_2 is increased when pH of soils is reduced (Šimek & Cooper, 2002). It has been found that emissions of N_2O, N_2 and NO are reduced in acidic soils compared to neutral or alkaline soils. However, various factors may affect the optimum pH for denitrification to take place such as: the number of denitrifying bacteria per unit of soil, species composition within the soil and the activity of denitrifying enzymes within the soil. It is therefore very difficult to attribute an optimum pH for denitrification to take place without knowing other variables involved.

Temperature has also been found to have a role in denitrification rate. Temperature is particularly influential in affecting the growth and reproduction of denitrifying microorganisms and therefore the denitrification rate (Qu *et al*., 2022). Temperature also affects enzyme activity and denaturing of enzymes may take place when optimal temperatures are exceeded. It is thought that denitrification can take place between 2 °C and 50 °C. The reason for such a range in temperature is due to microorganisms having varied optimal temperatures and having the ability to adapt to changes in temperature. However, in the majority of microorganisms, denitrification rate works most optimally between 25 °C and 35 °C (Feng *et*

al., 2021). A study by Qu *et al*(2022) investigated the removal of NO_3 from groundwater at four different temperatures: 15 °C, 25 °C, 40 °C and 45 °C, as well as how the microbial community changed. The study found that the NO_3 removal rate was highest at 40 °C with a removal rate of 99.26 %. This was greater that the conditions of 15 °C, 25 °C and 45 °C by 55.62 %, 12.32 % and 6.52 % respectively. Interestingly, the diversity of microbial organisms found in the 15 °C conditions was the highest. However, the diversity of microbial organisms in the other three conditions increased over time indicating an ability of adaption the temperature by soil communities.

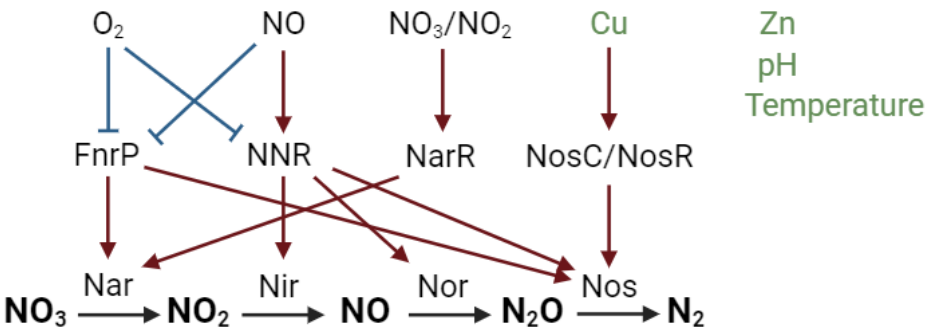


Figure 9: The denitrification pathway with the key reductases indicated; the FNR-family TRs that regulate each reduction stage; and the environmental factors that affect the expression of these FNR-family TRs, including other environmental factors that do not directly affect FNR proteins in green. Red arrows indicate an activation whereas blue lines indicate inhibition.

1.5Small RNAs

1.5.1 What are small RNAs?

Microorganisms undergo a lot of external stress and changes in their environment; therefore, they must quickly adapt fast to in order to survive. Aside from gene regulation by way of TRs, small RNAs (sRNAs) are a relatively new area of study which have also been found to control gene expression in changing environments. sRNAs are approximately 40-500 nucleotides in length and have been found to play roles in numerous important physiological processes (Storz *et al*., 2011). The first characterised sRNA, MicF, was discovered in 1984 in *Escherichia coli* (*E. coli*). MicF is a 174-nucleotide sRNA with a function of inhibiting synthesis of OmpF, an outer membrane protein, at the level of translation (Mizuno & Chou, 1984). In 2001, further research took place into the intergenic regions (IGR) of *E. coli*. Genome-wide analysis, using a number of advanced techniques, was able to uncover hundreds of sRNAs. Some of the sRNAs were found to play key roles in regulatory pathways within *E. coli* such as pathogenesis and stress responses (Storz *et al*., 2011).

The majority of sRNAs act through base-pairing with a small segment of DNA called a seed region, leading to changes to the stability or translation in the target (Hör *et al*., 2020). Base pairing of an sRNA to its target gene facilitates either repression or activation of the cognate target gene. In order for effective base pairing to take place, a certain degree of complementarity between an sRNA and its cognate target gene must exist (Wagner & Romby, 2015). sRNAs either originate from a gene of interest or are processed from the 5' or 3' untranslated regions (UTRs). Some are then further processed into sRNA fragments by RNase E which can lead to an increase in gene regulation. For example, this can be seen in RoxS in *Bacillus subtilis* (Durand *et al*., 2015).

sRNAs can either be *cis*-encoded or *trans*-encoded. *Cis*-encoded sRNAs are transcribed from the same DNA sequence from which their target RNA is transcribed. Therefore, *cis*-encoded sRNAs have high complementarity to their targets (Brantl, 2007). An example of *cis*-encoded sRNAs can be found in *E. coli* where five Sib antitoxin RNAs are found to play a pivotal role in repressing mRNA targets which are known to encode Ibs toxins (Han *et al,* 2010). By contrast, *trans*-encoded sRNAs are encoded from regions that are unrelated to their target genes, meaning they share less complementarity with these target genes compared to *cis*-encoded sRNAs. However, they are able to bind with a larger number of mRNA targets and have greater control over many physiological responses.

sRNA interactions with an mRNA target initially begin with a 'kissing' interaction in which nucleotides in the seed region of the sRNA are exposed to the mRNA target gene. This leads

to additional nucleotide sRNA-mRNA interactions, normally caused by rearrangement of the sRNA structure (Updegrove *et al*., 2015). Because of *trans*-encoded sRNAs sharing less complementarity with their target regions, they often require an sRNA chaperone to ensure stability of the sRNA and facilitate binding to its target site (Gottesman, 2005).

Research into the global roles of sRNAs has already uncovered sRNAs with the ability to respond to changes in their environment such as in stress response and pathogenesis. However new research has begun to uncover their roles in environmental pathways including the denitrification pathway (Moeller *et al*., 2021).

1.5.2 sRNA chaperones

Hfq, a homohexameric-shaped protein, is an Sm-like (Lsm) protein and a well-studied RNA chaperone, that is critical for sRNA-mRNA binding and stability in a wide array of sRNAs (Moeller *et al*., 2021). Hfq was first identified in *E. coli* for its role in acting as a **h**ost **f**actor for replication of the bacteriophage **Q**β (Franze De Fernandez & August, 1968). Since then, Hfq has also been found to play a key role in both gene activation by protection of sRNAs from degradosome, an enzyme known to repress the expression of an sRNA-mRNA complex (Georg *et al*., 2020). DsrA, an sRNA found in *E. coli,* is able to positively regulate its mRNA target, *rpoS.* The binding of Hfq to the binding motif of *rpoS* leads to confirmational change to the mRNA structure allowing DsrA to bind (McCullen *et al*., 2010). A novel sRNA chaperone, ProQ, has recently been discovered and has been found to play a role in target binding and sRNA stability, however, the exact mechanisms of how ProQ functions are not yet known (Olejniczak & Storz, 2017). In *S. typhimurium,* an sRNA named RiaZ is dependent on ProQ in the base pairing of RaiZ to the ribosome binding site (RBS) of *hupA,* a gene that encodes the protein HU-α. However, the presence of RaiZ at the RBS of *hupA* greatly inhibits the translation of this protein (Smirnov *et al*., 2017).

1.5.3 Gene repression

The majority of sRNA-mRNA interactions lead to the repression of an mRNA target gene. This can be due to a number of different mechanisms. These mechanisms are explained below with a visual representation found in Figure 10.

Firstly, sRNAs can bind with an mRNA RBS, subsequently preventing translation of that mRNA taking place. In order for translation to take place, a 30s ribosome subunit must be present. When an sRNA blocks the RBS of an mRNA target, this prevents the entry of a 30s ribosome, meaning translation is not possible (Jagodnik *et al*., 2017). sRNAs have been found that mask the AUG start codons of genes to block translation; RhyB in *E. coli* for example, interferes with the translation of non-essential Fe-binding proteins when Fe is limited (Massé & Gottesman, 2002). Other studies have found that sRNA masking of up to the 5th codon can

repress translation (Bouvier *et al*., 2008). For example, OxyS has been found to control the expression of over 40 genes through hybridisation of the RBS (Altuvia *et al*., 1998). One of these targets, an *rpoS*-encoded σ^s subunit RNA polymerase, is responsible for regulating genes induced by osmotic stress and starvation. OxyS represses the activity of *rpoS* and protects against these stress responses caused by hydrogen peroxide (Zhang *et al*., 2002). Hfq is involved in the repression of gene targets through interfering with ribosome binding and consequently stopping transcription from taking place. Spot42, an sRNA found in *E. coli,* binds to a seed region upstream of the sRNA *sdhC,* a subunit of succinate dehydrogenase*.* However, it does not directly interfere with inhibition of a 30s ribosome. Instead, Spot42, binds 42 nucleotides upstream of the *sdhC* start codon and recruits Hfq which directly interferes with binding of the 30s ribosome and therefore the initiation of translation.

A second way that sRNAs can cause repression is through the recruitment of Rnases that cause degradation of sRNAs and mRNAs. When an sRNA binds to its target and initiates either activation or repression of a gene, the recruitment of a ribonuclease must take place in order to remove the sRNA from its target for rapid turnover of the RNAs (Durand *et al*., 2015). In bacteria, the predominant ribonuclease is Rnase E which has been found to play a key role in repression of mRNA targets (Chao *et al*., 2017). Hfq has also been found to play a role in mRNA decay by binding to a C-terminal domain in Rnase E forming a ribonucleoprotein complex with the sRNA leading to mRNA decay. This ribonucleoprotein complex has been found to increase Rnase E concentration in the area around the target, leading to further degradation of mRNA targets. mRNAs involved in encoding Fe-storage and binding proteins during Fe depletion are known to be regulated by Rnase E in stress conditions. RhyB is responsible for this degradation (Morita *et al*., 2005). Although Rnase E is the most common ribonuclease in bacteria, a number of others have been found to play a role in mRNA degradation, such as Rnase Z and Rnase III (Chen *et al*., 2016; Lalaouna *et al*., 2013).

sRNAs are also able to repress gene expression through premature termination of transcription. This has been found in the virulence gene, *icsA* in *Shigella flexneri* by way of the sRNA RnaG. Due to the presence of the promoter for RnaG and the gene encoding *icsA* being less than 120bp apart, the movement of RNA polymerase is blocked by the sRNA leading to the attenuation of *icsA* transcription (Giangrossi *et al*., 2010).

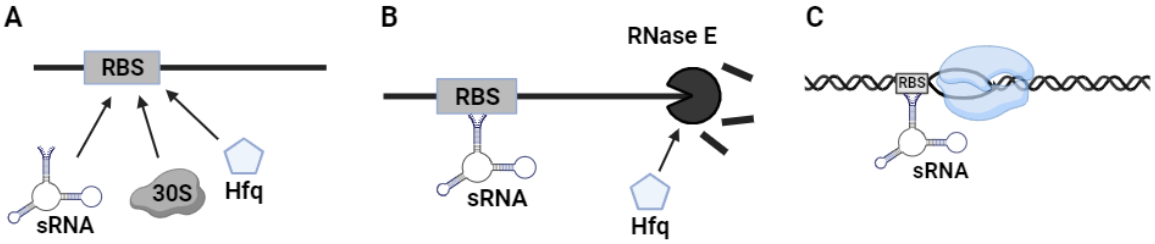


Figure 10: Gene repression by sRNAs. (A) sRNA binding with the RBS to inhibit the 30S ribosomal subunit from binding and therefore inhibiting translation; Hfq is also able to interfere with ribosomal binding. **(B)** sRNA binding to the RBS and recruiting RNase E to degrade the mRNA structure; Hfq can form a ribonucleoprotein with RNase E leading to degradation of the mRNA structure. **(C)** sRNA binding can cause premature termination of transcription by blocking DNA polymerase.

1.5.4 Protein sequestration

Aside from gene repression, some sRNAs have been found to directly bind to proteins to inhibit them from carrying out their function. This way, sRNAs are able to modulate the expression of the genes these proteins are responsible for controlling without directly interacting with the genes. CsrA for example, is a post-transcriptional regulator that controls a number of genes involved in bacterial stress response. The sRNA CrsB, inactivates CsrA activity and therefore indirectly represses the expression of the genes CrsA controls (Babitzke & Romeo, 2007).

1.5.5 Gene Activation

sRNAs are also capable of activating gene expression through a number of mechanisms. These are explained below with a visual representation found in Figure 11.

mRNAs are often unstable and susceptible to degradation by ribonucleases such as Rnase E. However, base pairing of an sRNA to an mRNA can stabilise its structure, making it less likely to be degraded by a ribonuclease. Therefore, leading to greater expression of a target gene (Opdyke *et al*., 2011). An example of this process taking place can be seen with the sRNA GadY. In *E. coli, gadX* is required to protect the bacteria from acid stress. However, it is sensitive to the ribonuclease RNase E, which inhibits its expression (Tramonti *et al*., 2002). GadY is a *cis*-encoded sRNA, encoded on the opposite strand to the gene *gadX,* and therefore its seed region shares complementarity to that of *gadX* (Weber *et al*., 2005). Instead of blocking the Rnase E binding site on the *gadX* mRNA, GadY recruits endonucleases including Rnase III and other enzymes to cleave the mRNA region that is sensitive to degradation by Rnase E (Opdyke *et al*., 2011).

mRNA gene expression is often inhibited by an intrinsic secondary structure which blocks the RBS. Certain sRNAs, known as ‘anti-antisense’ sRNAs, are able to remove this secondary structure by binding to the mRNA target leading to unfolding of this secondary structure. In *E. coli, rpoS,* a stationary phase sigma factor of RNA polymerase, requires sRNA binding to liberate its RBS. The RBS is located in the 5’UTR of *rpoS* which is sequestered and not

accessible for translation (Sedlyarova *et al*., 2016). Three sRNAs, DsrA, RprA and ArcZ, are needed to bind to a section of the *rpoS* 5'UTR exposing the RBS so translation can take place. It has been found that Hfq is able to bind to *rpoS* and restructure it to facilitate binding of these three sRNAs. The binding of these sRNAs also protects *rpoS* from cleavage by Rnase E (Bossi & Figueroa-Bossi, 2016).

rpoS has also been found to be regulated in a separate gene activating sRNA process known as transcription antitermination. Rho is a hexameric helicase protein which acts as a transcription termination factor that has been found to stop transcription in hundreds of bacterial genes. It works by binding to an RNA leading to an increase in ATPase activity and the termination of transcription. Rho normally acts to terminate transcription in *rpoS.* However, the binding of sRNAs DsrA, RprA and ArcZ interferes with Rho binding, inhibiting transcription (Sedlyarova *et al*., 2016).

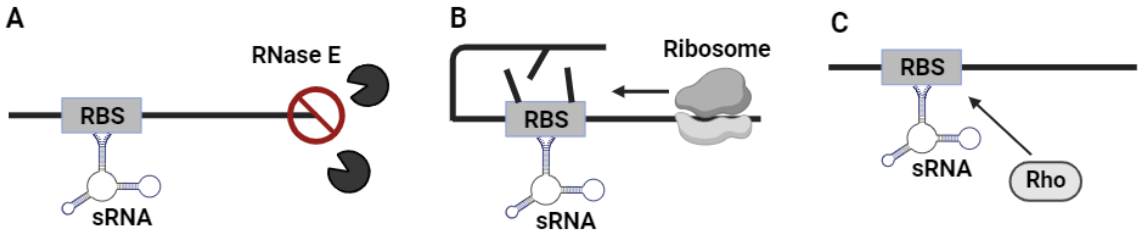


Figure 11: Gene activation by sRNAs. (A) sRNA binding to the RBS can stabilise the mRNA structure meaning RNase E is unable to bind and therefore unable to cause degradation of the mRNA. **(B)** Binding of the sRNA can alter the mRNA structure leading to the RBS being unmasked and allowing transcription to take place. **(C)** sRNA binding interferes with Rho binding, halting premature termination of transcription.

1.5.5 Physiological roles of sRNAs

Due to sRNAs being significantly smaller than mRNAs and other proteins, they have an energetic advantage in that they are able to carry out physiological responses yet do not require translation into a protein. (Beisel & Storz, 2010).

sRNAs have been found to play a role in pathogenesis within cells. This may be expected as a bacterial infection involves many unexpected changes from a homeostatic environment that must be efficiently responded to. Members of the CsrB family of sRNAs, for example, have been shown to respond to infection in *Salmonella, Erwinia, Yersinia, Vibrio* and *Pseudomonas* (Waters & Storz, 2009). CsrB sRNAs bind to CsrA proteins, which are global regulators of virulence genes, initiating an immune response. The sRNA, RhyB, found in *Shigella* has been found to repress two sRNAs: RNAIII in *Staphylococcus* and Qrr in *Vibrio*, both of which are transcriptional activators of virulence genes (Heroven *et al*., 2008). Some sRNAs encoded into the pathogenicity islands belonging to *Salmonella* and *Staphylococcus* have been found to

show differential expression under pathogenic conditions indicating a response to pathogenic conditions (Pfeiffer *et al*., 2007; Pichon & Felden, 2005).

sRNAs have been found to be involved in mediating responses to changing environmental conditions and have roles in controlling metabolic pathways and initiating stress responses. For example, the CsrB and 6S families of sRNAs respond to a decrease in nutrients availability by repressing certain global regulators allowing the genes they control to be expressed. Many *trans*-encoded sRNAs have been found to survive in changing environmental conditions by repressing the translation of proteins or regulators that are not needed by an organism at that time (Waters & Storz, 2009). sRNAs have also been found to be involved in stress responses such as changes to oxygen availability, changing between aerobic and anaerobic metabolism and osmotic stress.

Although the study of sRNAs has primarily focussed on physiological roles such as pathogenesis and in stress responses, recent studies have found that sRNAs also play key roles in a number of environmental pathways (Moeller *et al*., 2021). Interestingly and relevant to this study, sRNAs have been found to be involved in the nitrogen cycle. The sRNA NfiS, for example, was identified in *P. stutzeri* A1501 and has a role in binding to the *nifK* gene mRNA which encodes a subunit of the key nitrogen fixation enzyme Nif (Zhan *et al*., 2016). In *P. aeruginosa,* the sRNA NalA is involved in nitrate assimilation needed for plant growth. A deletion mutant not containing NalA was unable to grow in the presence of NO_3 as the only nitrogen source. It was, however, able to grow in the presence of ammonium. This showed that NalA is essential to nitrate assimilation (Romeo *et al*., 2012). The fact that sRNAs such as these examples have been found to have roles in the nitrogen cycle makes it more likely that sRNAs play a role in the denitrification pathway.

1.5.6 sRNAs involved in Denitrification

The role of sRNAs in the denitrification pathway is a relatively recent area of research. In 2016, 167 sRNAs were discovered across the *P. denitrificans* genome and, of these, 35% were found to be differentially expressed between aerobic, N_2-producing conditions and anaerobic N_2O-producing conditions. This suggests that these sRNAs may play a role in the reduction stage from N_2O to N_2. Some of these sRNAs showed sequence homology with other species of denitrifying bacteria indicating a potentially conserved role in denitrification (Gaimster *et al*., 2016). A separate study by Gaimster *et al* (2019) found that one of these sRNAs, sRNA29, which was later named DenR*,* played a key role in the denitrification pathway by regulating the expression of a previously unknown GntR-type regulator, NirR. NirR was then discovered to be able to repress the expression of NirS, resulting in reduced N_2O emissions. Overexpression of DenR also resulted in a change in expression levels of 53 other genes in

P. denitrificans that were found to be involved in transport, metabolism or had an unknown cellular function. DenR has been found to be conserved in other denitrifying species of bacteria including *Paracoccus aminophilus, Ruegeria pomeroyi* and members of the *Rhodobacteraceae* genus (Gaimster *et al*., 2019).

Of the other 166 sRNAs in *P. denitrificans,* it is thought that it is likely more of these will also play important roles in the denitrification pathway. This is of great significance as, if found, sRNAs involved in regulation of the reduction of N_2O could open the door to novel mitigation strategies against N_2O emissions. Currently, there has been little research on sRNAs involved in denitrification and therefore many of these sRNAs and their functions are yet to be discovered.

1.6 Aims

It has previously been shown that the FNR-family of transcriptional regulators FnrP, NNR and NarR play a vital part in regulation of denitrification. The relatively recent discovery of the global physiological roles of sRNAs has found them to be important in many processes including denitrification. The previous discovery of sRNA-29 in the model denitrifier *P. denitrificans* and its roles in regulation of denitrifying genes in *P. denitrificans* suggests that it is possible that other sRNAs found on the *P. denitrificans* genome could also play a key role in the denitrification pathway. We hypothesised that FNR-regulated sRNAs are likely to play an important role in the denitrification pathway and potentially the reduction of N_2O emissions and used putative FNR family regulation as a way to select sRNAs for further investigation.

The first aim of this study was to identify candidate sRNAs that possess an FNR binding motif within 200bp of their promoter region on the *P. denitrificans* genome.

The next aim of this study was to use computational characterisation software to determine which of the candidate sRNAs found to possess an FNR binding motif upstream, demonstrate characteristics that may indicate they play a role in denitrification. This takes into consideration the secondary structures, target sites and sRNA sequence homologues which all gives indication into the candidate sRNAs' physiological roles within *P. denitrificans.*

Once these computational characterisation steps have been completed and the identity of the candidate sRNAs is better understood, we aimed where time allowed, to take candidate sRNAs forward for experimental validation.

Chapter 2: Materials and Method

2.1 Computational characterisation techniques

2.1.1 Identification of FNR binding sites upstream of sRNAs

The Artemis genome browser (Sanger Institute) was utilised to visualise and interrogate the *P. dentrificans* genome. The navigator function tool was used to search for different variations of FNR family binding motif using data of previously discovered putative FnrP, NNR and NarR binding motifs across the *P. denitrificans* genome (Bouchal *et al,* 2010). Any sRNA with an FNR binding site within 200 bp upstream of the promoter region was analysed further.

2.1.2 Prediction of secondary structure

The RNA folding and hybridisation software, Mfold, was used to predict the secondary structures of candidate sRNAs. Candidate sRNA nucleotide sequences were input into the MFold database, producing an image of a predicted secondary structure as well as a minimum free energy value. Mfold also listed thermodynamic details including the number of hairpin loops found in the sRNA secondary structure (Zuker, 2003).

2.1.3 Prediction of putative sRNA targets

Candidate sRNA sequences were input into TargetRNA2. The parameters of the mRNA targets were set so that only targets from nucleotide -80 to nucleotide +20 relative to the transcription start site were found. TargetRNA2 produced the positioning of the sRNA target binding region on the sRNA and the positioning of the seed region on the mRNA target. TargetRNA2 also output the binding sequences for the sRNA-mRNA interaction. Potential gene targets found on chromosome 1 and 2 and the plasmid of *P. denitrificans* were listed. From this list, the thermodynamic energy (kcal/mol) of hybridisation, as well as the p-value, was listed (Kery *et al*., 2014).

2.1.4 Analysis of sRNA sequence conservation

To identify whether the sRNA sequences are conserved in other denitrifying bacteria, BLAST (Basic Local Alignment Search Tool) analysis was performed on candidate sRNAs. This tool finds local similarities between nucleotide sequences, calculating their statistical significance. sRNA sequences were run through the BLASTn database, which is used when searching for shorter queries between nucleotide sequences as well as cross-species comparisons. BLASTn showed whether there was potential conservation between sRNA sequences in *P. dentrificans* and bacteria. Only conserved bacteria with 80% query cover and 60% sequence identity were listed as potential sRNA homologues (Ye *et al*., 2006). To search whether conserved species of bacteria possessed genes encoding Nar, Nir, Nor, Nos or *nosZ,* KEGG (Kyoto Encyclopedia of Genes and Genomes) was used (Kanehisa *et al*., 2017).

2.1.5 Analysis of sRNA expression in varied environmental conditions

Data on sRNA expression in "high N_2O", "low N_2O" and "No N_2O" emitting conditions was collated from Gaimster *et al*(2016) and the expression data for each candidate sRNA was displayed in a bar chart. Each bar represented the fold change in sRNA expression relative to in "high N_2O" conditions.

2.2 Bacterial strains

The bacterial strains used in this study are listed in Table 1. Throughout this study, investigations into candidate sRNA growth and expression in different conditions was made possible by inserting the promoter region of each into the plasmid pLMB509 which has previously been reported to induce a 15-fold to 20-fold increase in its expression with the addition of 10mM taurine (Tett *et al*., 2012). These plasmids were inserted into *P. denitrificans* to create the strains PdsRNA18, PdsRNA36 and PdsRNA79. A *P. denitrificans* wildtype (WT) strain, named PdEmp was analysed alongside them throughout this study.

The strains used in this study were grown in the presence of antibiotics that they are resistant to acting as a selective growth factor. These antibiotics are listed in Table 1.

Table 1: Bacterial strains used in this study. Antibiotics that they are resistant to and their optimal growth temperature are also listed in the table below

Strain	**Antibiotic resistance**	**Optimal growth temperature**
PdEmp	Rifampicin/Gentamycin	30°C
PdsRNA18	Rifampicin/Gentamycin	30°C
PdsRNA36	Rifampicin/Gentamycin	30°C
PdsRNA79	Rifampicin/Gentamycin	30°C
Top10 *E. coli*	Tetracycline	37°C

2.3 Bacterial culture conditions

2.3.1 LB media preparation

Lysogeny broth (LB) was the medium used for bacterial growth. LB media contained 5 g L^{-1} of yeast extract, 10 g L^{-1} of tryptone and 10 g L^{-1} of sodium chloride (NaCl). LB media was autoclaved prior to use. The addition of 1.5% w/v agar before autoclaving created media that could be used to create LB agar plates.

2.3.2 Minimal Media preparation

A minimal salts medium containing essential components for bacterial growth of *P. denitrificans* was prepared at a pH of 7.5, containing core growth components: di-sodium orthophosphate (Na_2HPO_4), potassium di-hydrogen orthophosphate (KH_2PO_4), ammonium chloride (NH_4Cl), magnesium sulphate ($MgSO_4$), sodium nitrate ($NaNO_3$) and sodium succinate ($Na_2C_4H_6O_4$). The media was autoclaved prior to use. Once autoclaved, 2 ml L^{-1} of filter-sterilised trace element solution, based on a creation by Wolf Vishniac and Melvin Santer in 1957 (Vishniac & Santer, 1957), was added to the medium. This solution contained essential trace metals needed for growth. From the components listed in Table 2, NO_3 in $NaNO_3$ served as the respiratory electron acceptor; succinate was used as a carbon source and ammonium in NH_4Cl as a nitrogen source. The Cu content was altered depending on experimental requirements. For the purpose of this study, if the Vishniac and Santer solution contained Cu, it was known as Cu high (CuH); whereas if Cu was removed, it was known as Cu low (CuL). The Vishniac and Santer solution was prepared at 500-times stock solution.

Table 2: Chemical components required for denitrifying conditions. Used to create a minimal medium for growth of P. denitrificans. The Molecular weight of each component and the quantity needed to create minimal medium were also listed in the table.

Component	Molar weight (M_w)	Quantity (mM L^{-1})
Na_2HPO_4	141.96	29.0
KH_2PO_4	136.09	11.0
NH_4Cl	53.49	10.0
$MgSO_4$	246.48	0.4
$NaNO_3$	89.99	20.0
Succinate	270.14	30.0

Table 3: Essential components of CuH Vishniac and Santer solution, containing essential trace metals for bacterial growth. The Molecular weight of each component and the quantity needed to create the Vishniac and Santer solution were also listed in the table below.

Chemical	Molar Weight (M_w)	Quantity (mM L^{-1})
EDTA	292.24	130.00
$ZnSO_4$	287.55	7.64
$MnCl_2$	197.91	25.00
$FeSO_4$	278.01	18.50
$(NH_4)_6Mo_7O_24$	1235.90	0.89
$CuSO_4$	249.68	6.40
$CoCl_2$	237.93	6.72
$CaCl_2$	147.02	37.40

2.3.3 Short- and long-term bacterial storage

For short term bacterial storage, bacterial strains were spread aseptically across LB agar plates and stored at 4°C for up to two weeks. Long term storage of bacteria was achieved through storing bacterial strains at -80°C in 25% glycerol stocks.

2.3.4 Overnight cultures

Overnight (O/N) bacterial cultures were prepared from short term bacterial storage plates. A single bacterial colony was inoculated in 10 mL LB broth and supplemented with the correct antibiotic for selective growth of that bacteria. Cultures were grown at their optimal temperature with agitation at 200 rpm.

2.3.5 Antibiotics

Antibiotics and their working concentrations that are used throughout this study are listed in Table 4. Stock solutions of these antibiotics were dissolved using the correct solvent, filter sterilised and kept at 4°C for no longer than 8 weeks.

Table 4: Antibiotics used in this study, including their stock concentration, the solvent they were dissolved in and the working concentration used in experiments

Antibiotic	Stock concentration (mg ml^{-1})	Solvent	Working concentration (µl ml^{-1})
Gentamicin (Gm)	50	ddH_2O	25
Rifampicin (Rif)	25	ddH_2O	25
Tetracycline (Tet)	10	Ethanol	10

2.3.6 Anaerobic bacterial growth

Bacterial strains PdEmp, PdsRN18, PdsRNA36 and PdsRNA79 were grown in minimal media in 250 mL Duran bottles with gas-tight silicon caps. Prior to inoculation with 1 mL O/N culture, the media were sparged with N_2 for 15 minutes to create an anoxic environment. The corresponding antibiotics required for selective growth of the bacteria were also added to the Durans. Bacterial growth took place at 30 °C without agitation.

To create a CuH environment, 400 µL CuH V-S solution was added to the minimal media prior to O/N inoculation. To create a CuL environment, 400 µL CuL V-S solution was added instead. To test for overexpression 4 mL of taurine was added to induce expression of candidate sRNAs.

2.3.7 Optical density

Optical density (OD) readings to analyse bacterial growth rates were taken using a spectrometer, by shining light at a wavelength of 600nm through a bacterial growth sample in a 1 ml cuvette to determine bacterial population within the sample. Optical density readings were taken every 3 hours for anaerobic growth cultures.

2.3.8 Measurement of N_2O in culture

During anaerobic growth of bacterial cultures, gas samples were extracted from the headspace of the Duran culture bottles. 3 mL of gas was extracted every 3 hours using a 5 mL gas-tight syringe (Hamilton). Gas was transferred to 3 mL pre-evacuated screw cap exetainers (Labco). Gas from these exetainers was then analysed using a Clarus 500 Gas Chromatograph (Perkin Elmer). 50 µL was extracted from the exetainer and injected into an Elite-Q PLOT Phase Column using a gas-tight syringe (Hamilton). The headspace gas was detected using a $_{63}Ni$ Electron Capture Detector (ECD). The following instrument parameters were used for N_2O detection: carrier gas flow, 60 psi; auxiliary gas flow, 58 psi; injector temperature, 115 °C; column temperature, 90 °C; ECD temperature, 350 °C. This allowed for

a 5.2-minute retention time for N_2O. The total amount of N_2O was calculated using a Henry's Law constant for N_2O (at 30 °C) of $k_{cc\ H} = 0.5392$.

2.4 General laboratory techniques

2.4.1 Polymerase chain reaction

Polymerase chain reaction (PCR) was performed following the Phusion High-Fidelity PCR protocol (Thermo Fisher Scientific). PCR was used to amplify the promoter regions of candidate sRNAs using the components listed in Table 5. To prepare the bacterial template DNA required, a single colony of WT *P. denitrificans* was resuspended in 100 µl of ddH_2O and heated for 5 minutes at 100 °C ("boilate"). The bacteria was then centrifuged at 5000 rpm for 5 minutes and the supernatant was used as the bacterial template DNA. Forward and reverse primers were designed and ordered (Integrated DNA Technologies). The primers for each candidate sRNA are listed in Table 6.

Table 5: PCR components for reactions using the Phusion High-Fidelity PCR protocol (Thermo Fisher Scientific). The volumes of each PCR reagent are also listed in µl

PCR Reagent	Volume (µl)
Phusion HF/GC Buffer	10
10 µl dNTPs	1
10 µl F primer	2.5
10 µl R primer	2.5
Template DNA	2
Nuclease free water	31
Phusion DNA polymerase	1

Table 6: Candidate sRNA primer sequences used for PCR amplification of the sRNAs promoter regions

sRNA	Primer	Primer sequences
18	sRNA18PF_EcoR1	5' – AAAAGGAATTCGCGGGAACGCTGGCGACG – 3'
18	sRNA18PR_Pst1	5' – AAAACTGCAGGCGACCCAGTATGTCGGC – 3'
36	sRNA36PF_EcoR1	5' – AAAAGCAATTCGGGCTTTTTCATTTTGCG – 3'
36	sRNA36PR_Pst1	5' – AAAACTGCAGGAACGGAATGTCGTTAG – 3'
79	sRNA79PF_EcoR1	5' – AAAAGGAATTCAGCGCGGCCTTGAGCCGC – 3'
79	sRNA79PR_Pst1	5' – AAAACTGCAGGCGGGGCTTTAGACCGAC– 3'

The PCRs were performed using a Techne™ Prime Elite Thermal Cycler and followed the programme listed in Table 7.

Table 7: PCR cycle. The temperature needed for each PCR reaction stage and the amount of time of each stage are listed in the table below

Step	Temperature (°C)	Time (seconds)
Initial Denaturation	98	30
Denaturation, annealing, elongation (x30)	98 65 70	10 20 30
Final Elongation	72	10

2.4.1.1 PCR product purification

PCR product purification was achieved using a QIAquick PCR Purification Kit (QIAGEN) following manufacturer's instructions. Once purified, products were eluted in 30 µL dH_2O and stored at -20°C.

2.4.2 Agarose gel electrophoresis

Agarose gel electrophoresis was used to analyse PCR products (2.4.1), purified plasmids (2.4.3) and restrictions enzyme digests (2.4.4). Depending on the size of the DNA fragments being analysed, DNA was separated on a 1% or 2% (w/v) agarose gel containing 1x SYBR safe DNA gel stain (Invitrogen) in 1x Tris-Borate-EDTA (TBE). Before the gel electrophoresis, DNA was mixed with a Bioloine 5x loading buffer. DNA was run alongside a Bioline hyperladder used to determine the sizes of the DNA fragments. Gels were run for 50 minutes at 110 V and 400 mA. DNA was visualised using a Molecular Imager® Gel Doc™ (BioRad).

2.3.2.1 Gel extraction

DNA products were purified by gel extraction using a QIAquick gel extraction kit (Qiagen) following manufacturer's instructions. DNA was eluted with ddH_2O and stored at -20 °C.

2.4.3 Plasmid purification

The plasmid vector pMP220 was extracted from an O/N culture of Top10 *E. coli.* This was achieved using a QIAprep Spin Miniprep Kit (Qiagen) following manufacturer's instructions. Plasmid DNA was eluted in 30 µL ddH_2O and stored at -20 °C.

2.4.4 Restriction digests

It was necessary to create restriction digests for both candidate sRNA promoter regions and pMP220 so that they were able to effectively ligate (2.4.5). sRNA promoter regions had been amplified by way of PCR using primers that resulted in the presence of restriction sites at the 5' and 3' ends of the product (2.4.1), PCR products and pmP220 were digested with EcoR1

and Pst1. The digests were set for 4 hours at 37 °C. Restriction digest reaction compositions are shown in Tables 8 and 9. Products from the reactions were purified by gel extraction (2.4.2.1).

Table 8: Restriction digest reaction composition for digestion of sRNA promoter regions (insert DNA). The volume of each component is also listed in the table below.

Component	Volume (µL)
Sterile water	33
H buffer	5
EcoR1	1
Pst1	1
sRNA promoter region DNA	10

Table 9: Restriction digest reaction composition for digestion of pmP220 (vector DNA). The volume of each component is also listed in the table below.

Component	Volume (µL)
Sterile water	14
H buffer	2
EcoR1	1
Pst1	1
pmP220 DNA	2

2.4.5 Ligation

Ligations of digested insert DNA (Table 8) and vector DNA (Table 9) were performed using T4 DNA ligase (Promega) on ice in a 20 µL reaction with components listed in Table 10. The ligation reaction was left O/N at 4 °C.

Table 10: Composition of ligation reaction. The volume of each component is also listed in the table below.

Reaction component	Volume (µL)
Sterile water	6
10x Ligation buffer	2
Vector DNA	1
Insert DNA	10
Ligase	1

2.4.6 Bacterial transformation

Bacteria were transformed with ligation reactions (2.4.5)

2.4.6.1 Preparation of electrocompetent cells

Top10 *E. coli* cells were grown in 10 ml LB overnight cultures at 37 °C. These were recovered and inoculated (1% V/V) into two 50mL LB conical flasks. These two 50ml solutions were then incubated at 37°C at 200rpm until their OD_{600} was 0.4-0.6. The 50ml LB solutions were then transferred into two 50ml falcon tubes and the Top10 *E. coli* cells were harvested by centrifugation at 4000 x g at 4°C for 15 minutes. The supernatant was removed from each falcon tube and the cells were resuspended on ice using 15ml ice-cold 10% glycerol. The newly resuspended cells were then centrifuged once again at 4000xg at 4°C for 15 minutes. Cell harvesting and resuspension was repeated 3 times. During the final resuspension, 2ml glycerol was used instead of 15 ml, and the resuspended cells were then aliquoted as 100 µl volumes in 1.5 ml microcentrifuge tubes. These tubes were stored at -20°C.

2.4.6.2 Electroporation

Electrocompetent cells were kept on ice and mixed with 1-5 µL of each of the three ligation reactions. 100 µL aliquots of these solutions were added to electroporation cuvettes which had been chilled for 15 minutes at -20°C prior to use (BioRad). These cells were subjected to single electrical pulse at 2.5 Kv (EC2 setting on MicroPulser Electroporator (BioRad)). Following electroporation, 1 mL LB was immediately added to each electroporation cuvette and gently mixed with the cells. These cells were then transferred to 1.5 mL microcentrifuge tubes which were incubated at 37°C with agitation at 200rpm for 1.5 hours.

Following cell recovery, a 100 µL of each transformation was plated on an LB agar plate containing 10 µl/ml tetracycline. The remaining cells were spun in a microcentrifuge at 5000rpm for 30 seconds to form a pellet. The supernatant was removed and the pellet was resuspended in 100 µL LB. This was also plated as above. Plates were incubated at 37°C O/N.

2.4.7 β-galactosidase activity assay

2.4.7.1 Preparation of cells

Overnight bacterial cultures were diluted in fresh medium (1/100) and grown to a mid-log phase. To prepare the cells, cultures were incubated for 20 minutes on ice to stop growth and then washed. To wash these cultures, 2 mL of cells were pelleted at 4 °C by centrifuging them for 10 minutes at 6000 rpm in a Sorval SS34 rotor. The supernatant was then removed and the pellet was resuspended in 2 mL of chilled Z buffer. The OD_{600} of the resuspended cells was then measured against a blank (Z buffer). 0.5 mL cells were then diluted in 0.5 mL Z buffer. Cells were permeabilised by adding 100 µL chloroform and 50 µL 0.1% SDS (sodium

dodecyl sulphate sodium laurel sulphate). The cells were then vortexed and equilibrated in a 28 °C water bath for 5 minutes.

2.4.7.2 Assay

0.2 mL ONPG (o-nitrophenyl-B-D-galactosidase) (4 mg/mL) was added to the cells before being vortexed. The cells were then incubated at 28 °C. Once a sufficient yellow coloration has been developed, 0.5 mL Na_2CO_3 was added and the solution was once again vortexed. 1 mL of the solution was transferred into an Eppendorf tube and was centrifuged at maximum speed for 5 minutes to remove any debris and chloroform from the cells. The optical density of the solution was then recorded at 420 nm and 550 nm and from this the level of activity can be measured in Miller Units.

Chapter 3: Identification and computational characterisation of FNR regulation of sRNAs in *P. denitrificans*

3.1 The transcriptional profiles of FnrP, NNR and NarR

The next few sections take into account data from previous studies to help understand the transcriptional profiles of FnrP, NNR and NarR. This then aided further computational and experimental steps in later sections. A range of published data from a number of highly cited scientific studies was used. In each case, it is mentioned in the text when these studies have been used to help this study.

3.1.1 Gene expression in Δ*fnrP*, Δ*nnR* and Δ*narR*

As has been previously addressed, FnrP, NNR and NarR TRs are key to the completion of the denitrification pathway. A study in 2017 by Giannopoulos *et al* aimed to understand the transcriptional profile of these TRs in *P. denitrificans* (Giannopoulos *et al*., 2017). In order to achieve this, mutant strains of *P. denitrificans* without FnrP, NNR and NarR were constructed. These were Δ*fnrP*, Δ*nnR* and Δ*narR*. The mutant strains underwent whole genome analysis in anaerobic continuous cultures and gene expression across the *P. denitrificans* genome was analysed using type II microarray technology. The resulting gene expression data from each strain was compared with WT *P. denitrificans* undergoing the same anaerobic growth treatment. The relative expression between each mutant strain and the WT were calculated giving an indication of the genes regulated by FnrP, NNR and NarR. Genes were either repressed or activated in the presence of mutant strains and the level of repression or activation varied greatly between genes. In the Δ*fnrP*, strain, 547 genes were up-regulated and 350 genes were down-regulated by ≥2fold. In the Δ*nnR* strain, 519 genes were up-regulated and 350 genes were down-regulated by ≥2fold. In the NarR strain, 1511 genes were up-regulated and 138 genes were down-regulated by ≥2fold. Understanding of changing gene expression in different mutant strains compared to the WT helps to better understand the transcriptional profiles of FnrP, NNR and NarR and helps to confirm their roles in the denitrification pathway. Genes of interest involved in the denitrification pathway that showed a change of expression between mutant and WT strains are shown in Table 11. Values showing a ≥ 2-fold change in expression are highlighted in blue, for down regulation and red for upregulation. Values highlighted in green indicate genes that showed a ≤ 2-fold change in expression.

Table 11: Relative expression values of selected genes in Δ*fnrP*, Δ*nnR* and Δ*narR* strains of P. denitrificans compared to the WT, determined by type II microarray technology.

Gene ID	Annotation	Δ*fnrP*,/WT	Δ*nnR* /WT	Δ*narR* /WT
Pden_1850	*fnrP*	0.19	0.70	1.67
Pden_4238	*narR*	0.94	1.14	53.80
Pden_4238	*narG*	0.14	1.30	0.66
Pden_4235	*narH*	0.08	1.19	0.17
Pden_4236	*narI*	0.06	1.11	0.16
Pden_2487	*nirS*	1.70	0.03	3.06
Pden_2483	*norB*	1.57	0.12	1.04
Pden_2484	*norC*	1.58	0.11	1.08
Pden_4219	*nosZ*	2.72	0.99	4.13

Table 11 was constructed using data from Giannopoulos 2017. The relative expression values of a number of known denitrifying genes are displayed when grown in mutant Fnrp, NNR and NarR *P. denitrificans* strains compared to the wildtype. AsFnrP and NarR are self-regulated, it was no surprise that the *fnrp* and *narR* showed a significant change in expression in Δ*fnrP*, and Δ*narR* strains respectively. Whilst *fnrp* showed a significantly reduced expression in Δ*fnrP*,indicating FnrP activates its expression in WT *P. deintrificans*, NarR showed a significantly increased expression in Δ*narR* indicating that NarR strongly represses the expression of *narR.* In Δ*nnR*, analysis showed no significant change in *fnrp* and *narR* expression between mutant and WT strains. Δ*fnrP*, and Δ*nnR* had no significant effect on the expression of *narR.* Similarly, Δ*nnR* and Δ*narR* had no significant effect on the expression of *fnrp.*

FnrP and NarR have previously been found to be important in regulating the first reduction stage of the denitrification pathway by Nar. The study conducted by Giannopoulos confirms this showing that *narGHI* and *narHI* were significantly reduced in Δ*fnrP*, and Δ*narR* respectively. Δ*nnR* was shown to have no significant change in expression of genes belonging to the *nar* operon. NNR is however, known to have significant regulation over the expression of Nir and Nor in the second and third stage of the denitrification pathway. Therefore, it was unsurprising that *nirS, norB* and *norC* were all significantly downregulated in the Δ*nnR* strain indicating that NNR upregulates their expression in WT *P. denitrificans.* NosZ is of great significance in this study as it is the catalytic subunit of Nos, responsible for the reduction of

N_2O to N_2. Interestingly, in Δ*fnrP*, and Δ*narR* strains of *P. denitrificans, nosZ* was significantly upregulated suggesting that in WT *P. denitrificans,* FnrP and NarR downregulate the expression of *nosZ*. Δ*nnR* showed no significant change in expression for *nosZ*.

3.2 FNR-regulated genes in *P. denitrificans*

The study by Giannopoulos *et al,* 2017 confirmed that the presence of FnrP, NNR and NarR have significant control of genes in denitrifying conditions (Giannopoulos *et al*., 2017). A common feature of TRs belonging to the FNR family is that they share a highly specific target sequence known as an FNR box. These FNR boxes are normally found within 200 bp upstream of the gene they regulate. Along with the study by Giannopoulos looking into the transcriptional profiles of FnrP, NNR and NarR, other studies have conducted analysis of the *P. denitrificans* genome to uncover binding motifs for FnrP, NNR and NarR upstream of genes that they are predicted to control by searching for an FNR box (Bouchal, 2010.; Van Spanning *et al*., 1995; Van Spanning *et al*., 1997). For example, a recent study undertaken by Bouchal *et al* (2010) used a proteomic approach to uncover expression differences in genes across the *P. denitrificans* genome as well as uncovering potential FNR-regulated genes (Bouchal *et al,* 2010). Data from these studies have been used to further understand the regulation of genes with the presence of FnrP, NNR NarR bunding motifs upstream of them.

In the next three sub-sections, research undertaken by previously published studies was used to collate putative motifs of FnrP, NNR and NarR which was then used to computationally characterise genes potentially controlled by these proteins later in the study.

3.2.1 Putative FnrP motifs

As has been seen from the Giannopoulos study, gene expression of genes encoding Nar and Nos were significantly downregulated and upregulated respectively by Δ*fnrP*, (Giannopoulos *et al*., 2017). Therefore, it is no surprise that a putative FnrP binding motif was found upstream of *narG, narK, narH* and *nosZ*. A putative FnrP binding motif was also found upstream of *fnrp,* the gene which self-regulates FnrP. Other genes thought to possess a putative FnrP binding motif upstream of them are listed in Table 12 with the putative FnrP sequence also listed (Bouchal, 2010).

Table 12: Genes in P. denitrificans found to possess a putative FnrP binding motif upstream of their promoter site (Bouchal, 2010).

Gene ID	Annotation	Putative FNR motif
Pden_1850	*fnrP*	TTGATTTGGGTCAA
Pden_4238	*narG*	TTGACTTAAATCAA
Pden_4237	*narK*	TTGATCTGGATCAA
Pden_4235	*narH*	TTGATCCAGATCAA
Pden_4219	*nosZ*	TTGAAGCTTAACCA TTGAGAATTGTCAA TTGACCTAAGTCAA
Pden_1848	*ccoN*	TTGATCTGCGTCAA
Pden_1844	*ccoG*	TTGATCTGCGTCAA
Pden_3636	*ompW*	TTGATCTGGATCAA
Pden_1849	*UspA*	TTGATTTGGGTCAA
Pden_4222	*pasZ*	TTGCGCCATGGCAA
Pden_5108	*Qox*	TTGATCTAGGTCAA

Using the sequences of putative FnrP binding motifs found in Table 12, an FnrP consensus sequence was created giving an indication of the most common nucleotide sequences found in FnrP binding motifs. This can be seen in Figure 12.

Figure12: FnrP binding motif logo. This logo was created based on combined sequences of putative FnrP binding motifs upstream of predicted FnrP-regulated genes: fnrp (Pden_1850), ccoG (Pden_1844), ccoN (Pden_1848), ompW (Pden_3636), pasZ (Pden_4222), narK (Pden_4237), narG (Pden_4238), narH (Pden_4235), qoxA (Pden_5108), nosZ (Pden_4219), UspA (Pden_1849). The logo was created using WebLogo (http://weblogo.berkeley.edu/logo.cgi).

3.2.2 Putative NNR motifs

Giannopoulos found that genes encoding Nir and Nor were greatly downregulated in the Δ*nnR* strain of *P. denitrificans* indicating NNR to play a key role in activation of Nir and Nor. This was backed up by the presence of putative NNR binding motifs found upstream of *nirS* and *norC.* These binding motif sequences can be seen in Table 13. The putative NNR motifs were also combined to create a consensus sequence for NNR. This can be seen in Figure 13.

Table 13: Genes in P. denitrificans found to possess a putative NNR binding motif upstream of their promoter site (Bouchal, 2010).

Gene ID	Annotation	Putative FNR motif
Pden_2487	*nirS*	TTAACAAAGGTCAA
Pden_2484	*norC*	TTGACTTTCATCAA

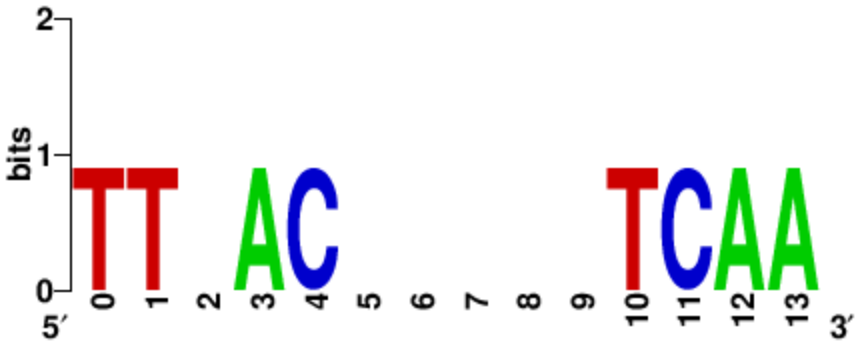


Figure 13: NNR binding motif logo. This logo was created based on combined sequences of putative NNR binding motifs upstream of predicted NNR-regulated genes: nirS (Pden_2487), norC (Pden_2484). The logo was created using WebLogo (http://weblogo.berkeley.edu/logo.cgi).

3.2.3 Putative NarR motifs

Putative NarR binding motifs were found upstream of *narK* and *narG,* following on from its predicted expression of Nar. The putative NarR binding motifs found upstream of *narK* and *narG* were used to create a consensus sequence for NarR which can be seen in Figure 14.

Table 14: Genes in P. denitrificans found to possess a putative NarR binding motif upstream of their promoter site (Bouchal, 2010).

Gene ID	Annotation	Putative FNR motif
Pden_4237	*narK*	TTGATATTTGTCAA TTGATCCAGATCAA
Pden_4238	*narG*	TTGACTTAAATCAA

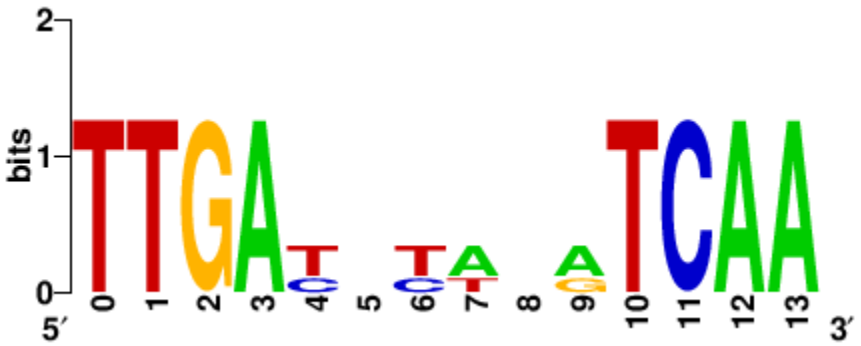


Figure 14: NarR binding motif logo. This logo was created based on combined sequences of putative NarR binding motifs upstream of predicted NarR-regulated genes: narK (Pden_4237). The logo was created using WebLogo (http://weblogo.berkeley.edu/logo.cgi).

3.3 Transcriptional Regulation of sRNAs

3.3.1 sRNAs controlled by transcriptional regulators

Although understanding of sRNAs has improved in recent years, the regulation of these sRNAs is relatively poorly understood (Eisfeld *et al*., 2021). Interestingly, studies into bacteria have shown that sRNA expression in response to the environment is largely reliant upon TRs (Brosse & Guillier, 2018). These TRs usually do not solely regulate the expression of these sRNAs, but regulate a complex regulatory pathway of which the sRNA helps to regulate. In some cases, TRs have been found to regulate sRNAs which in turn regulate the expression of that TR. This creates a feedback loop between TR and sRNA meaning the regulatory pathway under their control can be monitored more closely (Brosse & Guillier, 2018). This can be seen in Figure 15. In some cases, sRNAs have been found to be amongst the most highly regulated targets by certain TRs. For example, the sRNA RyhB was one of the most deregulated genes in a mutant *E. coli* strain that did not contain the TR Fur, which normally regulates the expression of this sRNA (Massé & Gottesman, 2002).

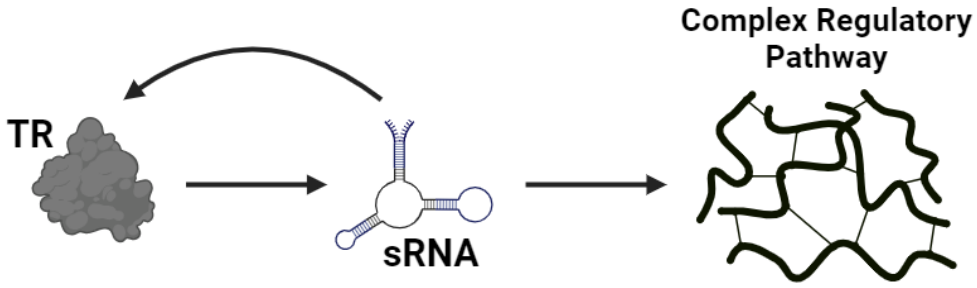


Figure 15: Transcriptional regulation of sRNAs. Transcriptional regulators can regulate sRNA expression which can in turn regulate the expression of complex regulatory pathways. These sRNAs in turn regulate transcriptional regulators creating a feedback loop.

3.3.2 FNR regulation of sRNAs

As FNR-family TRs such as FnrP, NNR and NarR are strongly regulated by the switch between oxic and anoxic environments in bacteria and are involved in large regulatory pathways such as denitrification, it is of no surprise that sRNAs have been discovered to be regulated by FNR-family TRs. One study found that a trans-encoded, Hfq-dependent sRNA named FnrS found in *E. coli* was anaerobically induced by an FNR protein (Durand & Storz, 2010). This study found that, in anaerobic conditions, FnrS would negatively regulate 32 genes, many of which are involved in energy metabolism, including *sodA, sodB, cydDC* and *metE. sodA* for example, is a gene that protects cells from oxidative damage by removing superoxide radicals (Boysen *et al*., 2010). FnrS has since been discovered to play a role in

other bacteria such as *Neisseria gonorrhoeae* where it is again induced by anaerobic conditions to up and down regulate target genes (Tanwer *et al*., 2017). Another FNR induced sRNA, AniS, was discovered in *Neisseria meningitidis.* This sRNA was found to express two mRNA targets, NMB0214 and NMB1468 which are involved in encoding PrlC oligopeptidase and a lipoprotein respectively. Interestingly, although Hfq has been reported to promote base pairing between AniS and its mRNA targets, AniS has been found to be less stable in the presence of Hfq.

Although regulation of sRNAs by FNR-family TRs is scarcely researched and currently poorly understood, it is thought that due to the nature of FNR proteins being involved in large physiological pathways, it is likely that many more sRNAs will be found to be under their influence in coming years.

3.4 Searching for FNR-regulated sRNAs

Having established an understanding of the transcriptional profile of FnrP, NNR and NarR, as well as an understanding of the transcriptional regulation of sRNAs using previously published data; it was then possible to put this knowledge into practice through a number of computational and experimental techniques. From this point onwards, all data is from original work.

The first aim of this study was to uncover sRNAs in *P. denitrificans* that are potentially regulated by FnrP, NNR and NarR. As these TRs and are known to play a key role in denitrification, it is believed that the presence of an FNR binding motifs upstream of sRNA promoter regions on the *P. denitrificans* genome means it is more likely for that sRNA to be controlled by FNR TR and more likely for that sRNA to play a role in denitrification. (Durand & Storz, 2010; Tanwer *et al*., 2017).

Artemis is a genome annotation and browser tool that allows for visualisation of whole genome sequences of a wide range of organisms using next generation data. The tool allows for identification of specific nucleotide sequences along a genome that can be input and searched for using the navigation function. Using data from already-published studies, consensus binding motif sequences for FnrP, NNR and NarR were created (Figure 12, 13, 14). These were used to to search for motifs upstream of sRNAs. Upon inspection of the consensus sequences, the first half sites found in these FNR boxes had the following sequences: TTGA, TTGT, TTGG, TTGC, TTAA. The second half sites were: TCAA, GCAA, ACCA, TCCA, TTAA. Any combination of first and second sites were searched for across the *P. denitrificans* genome. Any putative FNR box found 200bp upstream of an sRNA was identified and this sRNA would be one of the candidate sRNAs looked at in this study.

A total of 7 candidate sRNAs were found with putative FNR family binding motifs within 200bp upstream of them on the *P. denitrificans* genome. These can be seen in Table 15 along with the putative FNR sequence that may be involved in regulation of their expression. Figure 16 displays the putative FNR motifs relative to the sRNAs on the *P. denitrificans* genome.

Table 15: Candidate sRNAs found along the P. denitrificans genome. The Artemis genome browser navigate function was used to search for putative FNR family transcriptional regulators sequences upstream of sRNAs found along the P. denitrificans genome.

sRNA	**Putative FNR transcriptional regulator sequences**
sRNA11	TTGAAGGCCCGCAA
sRNA18	TTGTCGATGAACCA
sRNA31	TTGCCGAAACGCAA
sRNA36	TTGGCTCTTGGCAA
sRNA54	TTAAGATATATTAA
sRNA79	TTGGATTTTTTCCA
sRNA107	TTGCTGGATAACCA

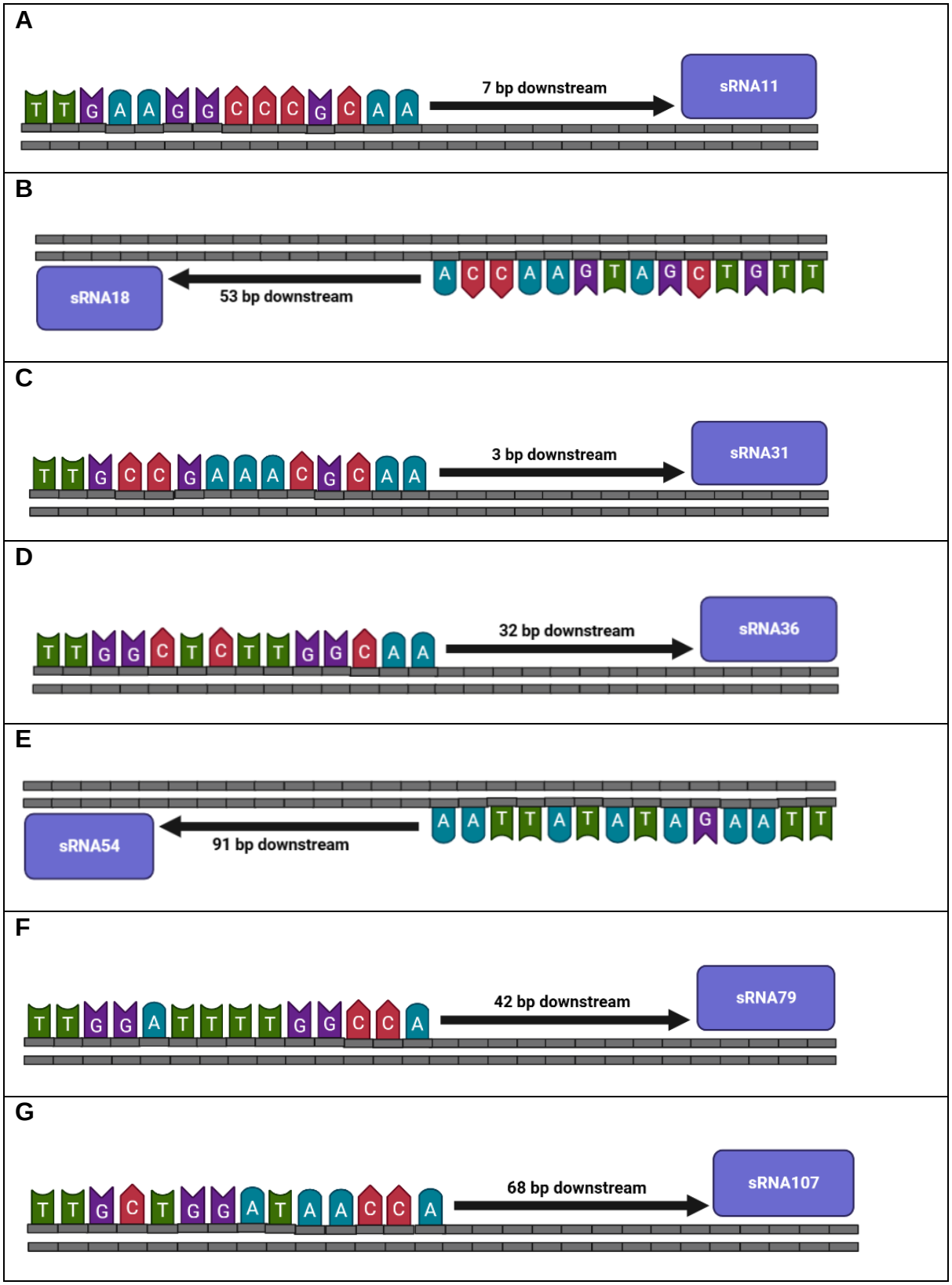


Figure 16: Putative FNR transcriptional regulator binding motifs upstream of sRNAs. The figure shows the distance in base pairs between candidate sRNAs and putative binding motifs. Candidate sRNAs: sRNA11 (A), sRNA18 (B), sRNA31 (C), sRNA36 (D), sRNA54 (E), sRNA79 (F), sRNA107 (G)

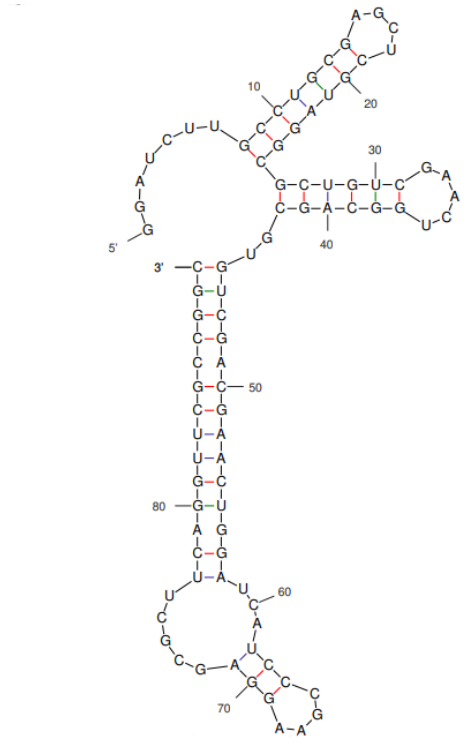
3.5 Computational characterisation of candidate sRNAs

Having established 7 candidate sRNAs in *P. denitrificans* possessed an FNR binding motif within 200 bp of their promoter region, it was necessary to characterise them in order to understand their roles in *P. denitrificans.* A number of computational steps were taken and these will be explored in the following sub-sections.

3.5.1 Prediction of candidate sRNA secondary structures

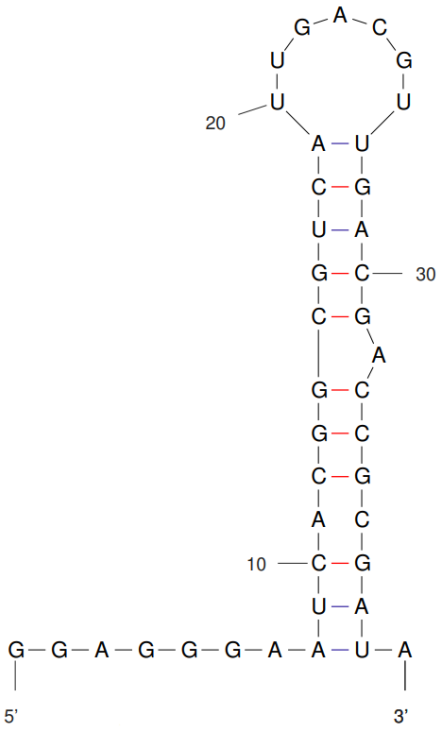
To predict the secondary structures of the candidate sRNAs, the RNA folding and hybridisation software Mfold was used (Zuker, 2003). All candidate sRNA sequences were input into the MFold database, producing an image of their secondary structures. An important structural characterisation is the existence of a hairpin loop in the sRNAs secondary structure as this indicates a potential ability of sRNAs to form complex conformations that may be necessary in order to bind with target sites. sRNA18 was found to only possess one hairpin loop. sRNA31 and sRNA54 were found to have two hairpin loops whilst sRNA11, sRNA79 and sRNA107 were all found to have three hairpin loops. sRNA36 possessed the most hairpin loops in its secondary structure with six. MFold also predicts the minimum free energy value (ΔG) which gives an indication of the thermodynamic stability of the sRNAs' secondary structures. The greater the thermodynamic energy, the more negative the ΔG value and the more specific an sRNA molecule will be to its target. sRNA107 was found to be the most stable with a ΔG value of -67.10, followed by sRNA36 with a ΔG value of -56.50. sRNA18 had the highest ΔG value of -14.00. The candidate sRNA secondary structures along with their respective ΔG values can be found in Figure 17.

A



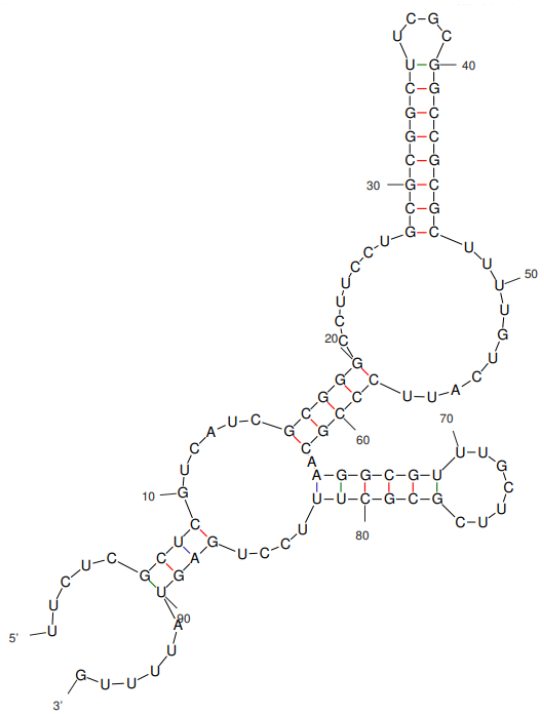
ΔG: -35.50

B



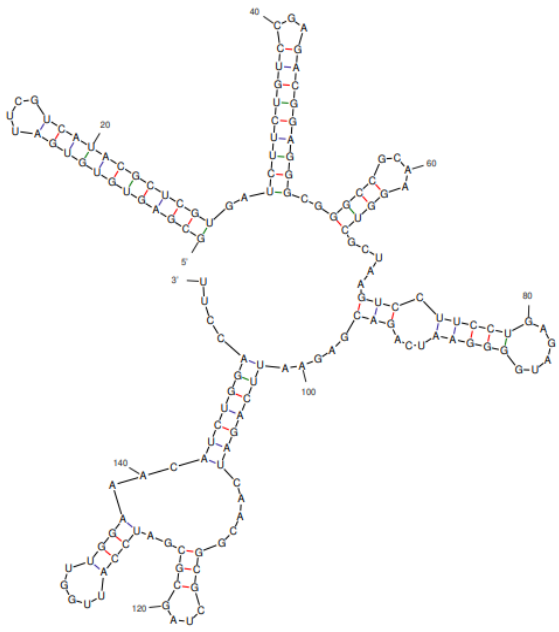
ΔG: -14.00

C



ΔG: -34.80

D



ΔG: - 56.50

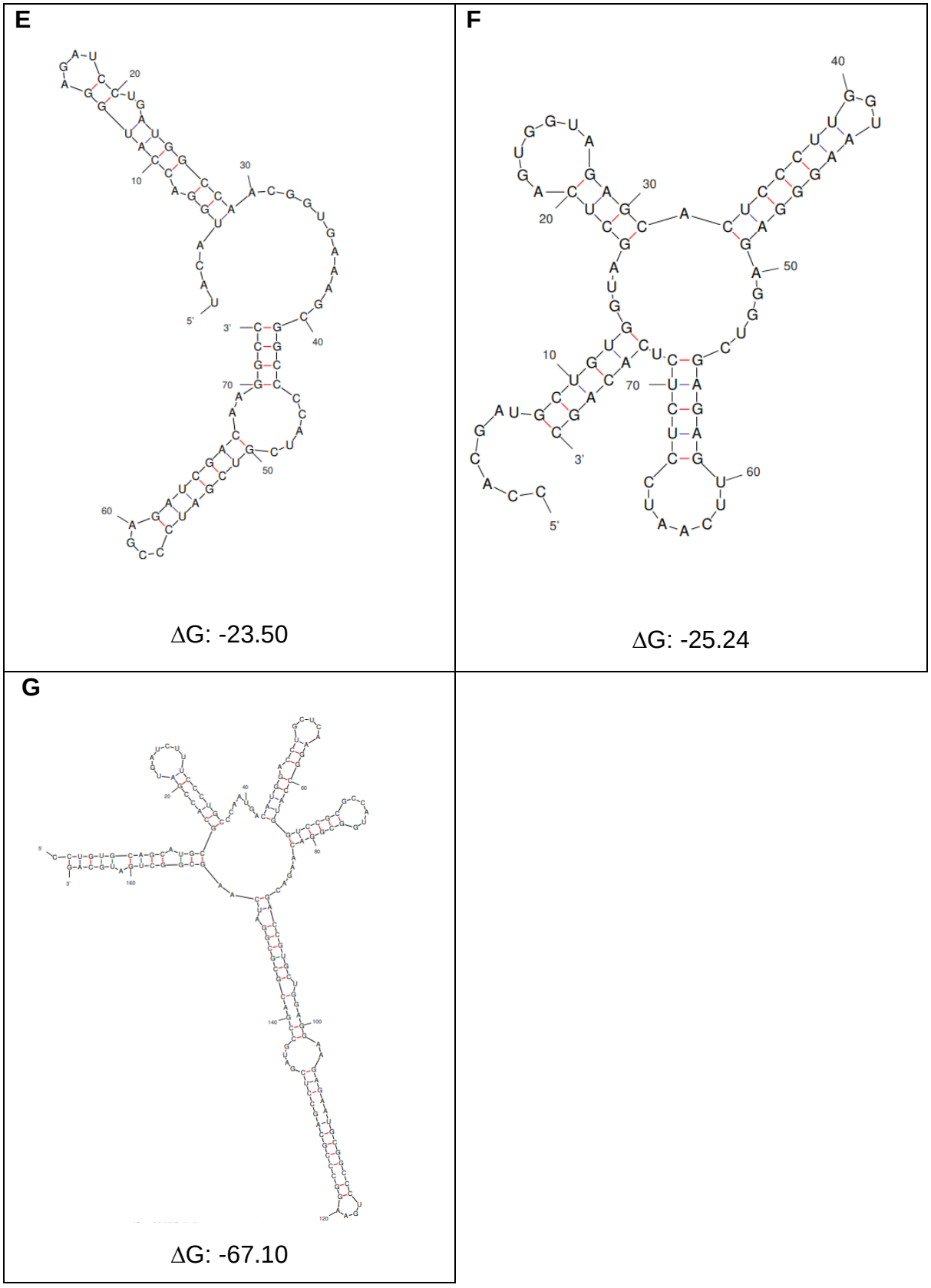


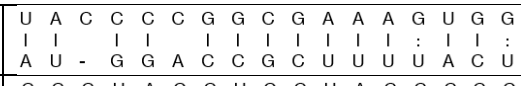
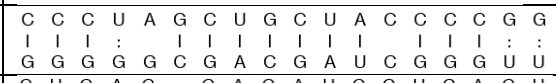
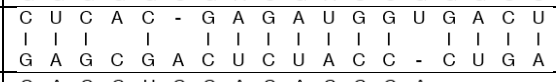
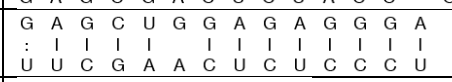
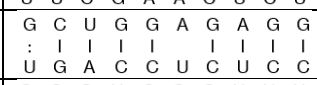
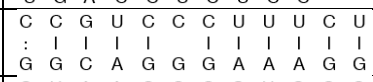
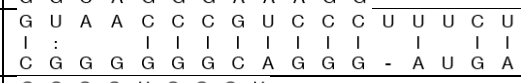
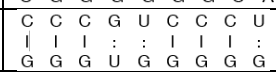
Figure 17: Secondary structures of candidate sRNAs. The secondary structures were created using MFold. The ΔG values of each structure are also given. (A) sRNA11, (B) sRNA18, (C) sRNA31, (D) sRNA36, (E) sRNA54, (F) sRNA79, (G) sRNA107.

3.5.2 Identification of potential gene targets of candidate sRNAs

To identify potential gene targets of the candidate sRNAs, TargetRNA2, a web-based tool for identifying RNA targets was used. TargetRNA2 employs four main algorithms to identify sRNA targets. 1. Conservation of the sRNA, 2. Accessibility of the sRNA, 3. Accessibility of the mRNA, 4. Energy of hybridisation - Targets with lower hybridisation energy are thought to be more likely targets of sRNAs (Kery *et al,* 2014). Each candidate sRNA nucleotide sequence was input into TargetRNA2 and potential gene targets on the *P. denitrificans* genome were found. The *P. denitrificans* genome consists of chromosome 1, chromosome 2 and a plasmid meaning all three were examined for target genes. Only targets with a P value < 0.05 were considered to be significant. The top 3 gene targets of each candidate sRNA with their complementary binding sequences are found in Table 16. All gene targets can be found in Appendix 1.

Table 16: The Top three target genes of each candidate sRNA. The table includes the binding positions on the sRNA as well as on the target mRNA. The table also displays the binding sequence of each sRNA-mRNA interaction.

sRNA	Target	sRNA binding position	mRNA binding position	Binding sequence
11	lpxC (Pden_4486)	20 – 1	-18 – 1	C U C G A G - C G U C C G U U C U A G G G A - A U C G G C A G G C A A G A - C C
11	Pden_1370	78 – 64	-24 – 10	U U C G C G A G G A A G C A A G C G C U C C U C C G
11	Pden_2562	76 – 63	9 – 20	C G C G A G G A A G C C C G U G C - C U C C U C C G
18	Pden_3255	15 – 1	-78 – -64	G G C A C U A - A G G G A G G C C - U G A U C U C C C U C C
18	Pden_4274	18 – 1	-1 – 16	U G C G G C A C U A A G G G A G G A U G C C - - G A U U C C C C C C
18	PyrE	20 – 1	1 – 20	G G C A C U A - A G G G A G G C C - U G A U C U C C C U C C
31	Pden_3891	77 – 57	-28 – -8	G C U U C G U U U G C G G A A C - G C C G A A G C G A A C G C C - U G A G G
31	Pden_4668	39 – 26	5 – 17	G C U U C G G C G C G U C G A A G C C G C - C A
31	Pden_4145	25 – 5	-11 – 10	C U U C - C G G G C G C U A - C U G C G G A G A G - A C G C G A U G G A U G
36	Pden_3492	98 – 84	1 – 16	G C A G A C U A A - G G G G U U G U C C U G A U U G C C C C G
36	Pden_2983	95 – 81	6 – 19	G A C U A A G G G G U A G C U G - U U C C C C A U C
36	Pden_3561	80 – 70	-10 – 1	U C C U U C C U G A G G A A G G A C
54	Pden_3292	44 – 31	-74 – -59	C G G C G A A A G - - U G G G C C G C U U U C U G A C C

54	Pden_2935	49 – 31	-1 – -17	
54	Pden_3810	59 – 40	-26 – -7	
79	IhfA	36 – 18	2 – 20	
79	Pden_2502	57 – 43	4 – 18	
79	Pden_1041	56 – 45	1 – 12	
107	Pden_5040	37 – 24	-46 – -33	
107	Pden_1958	42 – 24	-13 – -5	
107	Pden_1541	38 – 28	-72 – -62	

Gene targets were discovered across all candidate sRNAs. Some sRNAs displayed targets across chromosome 1, chromosome 2 and the plasmid of *P. denitrificans,* for example sRNA11. Others did not possess targets in all locations. For example, sRNA31 only displayed targets on chromosome 2. sRNA79 had the most target sites of 57 whereas sRNA36 displayed the least with 22. The majority of targets had a known function, however a total of 62/231 discovered targets were hypothetical proteins of an unknown function. The known targets had a range of roles involved in different physiological processes within *P. denitrificans.* Using data from Giannopoulos, 2017 it has been found that a number of these target genes show varied expression in Δ*fnrP*, Δ*nnR* and Δ*narR P. denitrificans* strains when compared with the WT (Giannopoulos *et al*., 2017). This may indicate a key role in the denitrification pathway. 8/231 target genes were discovered to be targets of more than one candidate sRNA, possibly indicating a conserved role for sRNAs across the *P. denitrificans* genome.

3.5.3 Conservation of candidate sRNA sequences across denitrifying bacteria

To identify whether the candidate sRNA nucleotide sequences are conserved in other denitrifying bacteria, BLAST (Basic Local Alignment Search Tool) analysis was used. This tool finds local similarities between nucleotide sequences, calculating their statistical significance. sRNA sequences were run through the Blastn database, which is used when searching for shorter queries between nucleotide sequences as well as cross-species comparisons. Conservation between sRNA sequences in *P. denitrificans* and other denitrifying bacteria would make the sRNA under investigation a more likely candidate for regulating denitrification. Bacteria that showed homology of at least 80% query cover and 60% sequence identity were investigated and the conserved species are displayed in Table 17.

Table 17: Conservation of candidate sRNA sequences across different species of bacteria. Each candidate sRNA sequence was input into Blastn and homologous bacterial species were output. Only species that showed a query cover of at least 80% and sequence identity of at least 60% are shown. Species found that contain genes involved in the denitrification pathway are highlighted in bold.

sRNA	Species of bacteria
sRNA11	N/a
sRNA18	N/a
sRNA31	***Paracoccus pantotrophus DSM 2944***
sRNA36	N/a
sRNA54	***Paracoccus aminovorans* JCM7685** ***Paracoccus methylovorus* H4-D09**
sRNA79	***Ancylobacter* sp. SL191** ***Antarctobacter heliothermus* SMS3** ***Aurantimonas* sp. HBX-1** *Bartonella henselae* ATCC49882T *Bartonella* sp. HY328 *Brevirhabdus pacifica* DY6-4 *Brucella abortus* 45597 *Brucella melitenis* TZ ***Chenggangzhangella methanolivorans* CHL1** ***Devosia* sp. D6-9** ***Gemmobacter fulva* con5** ***Hartmannibacter diazotrophicus* E19T** *Indioceanicola profundi* SCSIO 08040 ***Jiella* sp. HL-NP1** ***Ketogulonicigenium robustum* SPU_B003** *Ketogulonicigenium vulgare,* SKV ***Oceanicola* sp. D3** ***Paracoccus aminovorans* JCM7685** *Paracoccus everestensis* S8-55 *Paracoccus contaminans* RKI, sp. HNIBRBA609 ***Paracoccus marcusii* CP157** ***Paracoccus mutanolyticus* RSP-02** ***Paracoccus sanguinis* OM2164** ***Pacificitalea manganoxidans* DY2** ***Paracoccus yeei* CCUG 32053** ***Paracoccus zhejiangensis* J6** *Paradevosia shaoguenesis* J5-3 ***Pseudorhodobacter turbinis* S12M18** ***Rhodobaca barguzinensis* alga05** *Roseicitreum antarcticum* ZS2-28 ***Sulfitobacter* sp. JL08**
sRNA107	***Bradyrhizobium arachidis* SM32** ***Bradyrhizobium elkanii* USDA 61** ***Bradyrhizobium japonicum* J5** ***Bradyrhizobium vignae* ORS3257** ***Frigidbacter mobilis* cai42** *Fuscovulum blasticum* ***Paracoccus kondratievae* BJQ0001** ***Paracoccus mutanolyticus* RSP-02**

***Paracoccus pantotrophus* DSM 2944**
***Paracoccus yeei* CCUG 32053**
Pseadosulfitobacter sp. DSM 107133
***Rhodobacter* sp. LPB0142**
***Roseovarius* sp. THAF9**

sRNA31, sRNA54, sRNA79 and sRNA107 nucleotide sequences were homologous between *P. denitrificans* and other species of bacteria. sRNA11, sRNA18 and sRNA36 on the other hand did not show any homology between *P. denitrificans* and other species. All homologues found by Blastn analysis belonged to the class of Alphaproteobacteria with the majority belonging to the family Rhodobacteraceae. The most common genera was *Paracoccus* accounting for 15/47 of the conserved species found. In sRNA31 and sRNA54 1/1 and 2/2 of the conserved sRNA homologues were found to contain genes involved in denitrification respectively. In sRNA79 this was the case for 20/31 of the conserved species and for sRNA107 this was true of 11/13. All species capable of playing a role in the denitrification pathway are listed in Table 18. This table shows whether conserved species possess genes encoding for Nar, Nir, Nor and Nos, the four essential reductases in the denitrification pathway. It is also shown whether these species contain the *nosZ* gene, the catalytic subunit of Nos and an important factor in the complete reduction of N_2O to N_2. Whilst some conserved bacteria were found to contain genes encoding for all four reductases, others only possessed genes for one two or three of these reductases.

Table 18: Homologous species of candidate sRNAs that possess genes involved in the denitrification pathway. All species of bacteria that are highlighted in bold in Table 8 are shown here along with whether or not they contain genes that regulate Nar, Nir, Nor, Nos and nosZ. Ticks in each column indicate they do possess genes that regulate these reductases whereas crosses indicate they do not possess genes.

sRNA	**Organism**	**Nar**	**Nir**	**Nor**	**Nos**	***nosZ***
sRNA31	*Paracoccus pantotrophus DSM 2944*	✓	✓	✓	✓	✓
sRNA54	*Paracoccus aminovorans* JCM7685	✓	✓	✓	✓	✓
	Paracoccus methylovorus H4-D09	✓	✓	✓	✓	✓
sRNA79	*Ancylobacter* sp. SL191	X	X	✓	X	X
	Antarctobacter heliothermus SMS3	✓	✓	✓	X	X
	Aurantimonas sp. HBX-1	✓	✓	X	✓	✓
	Chenggangzhangella methanolivorans CHL1	✓	✓	✓	X	X
	Devosia sp. D6-9	X	X	✓	X	X
	Gemmobacter fulva con5	X	✓	✓	X	X
	Hartmannibacter diazotrophicus E19T	✓	✓	X	X	X
	Jiella sp. HL-NP1	X	✓	X	✓	✓
	Oceanicola sp. D3	✓	✓	✓	X	X
	Paracoccus aminovorans JCM7685	✓	✓	✓	✓	✓
	Paracoccus marcusii CP157	✓	✓	X	X	X
	Paracoccus mutanolyticus RSP-02	✓	X	✓	X	X
	Paracoccus sanguinis OM2164	✓	✓	✓	✓	✓
	Paracoccus yeei CCUG 32053	✓	✓	✓	✓	✓
	Paracoccus zhejiangensis J6	✓	✓	X	✓	X
	Pacificitalea manganoxidans DY25	✓	✓	X	X	X
	Pseudorhodobacter turbinis S12M18	X	✓	✓	✓	✓
	Rhodobaca barguzinensis alga05	✓	X	X	✓	✓
	Sulfitobacter sp. JL08	X	✓	✓	✓	✓
sRNA107	*Bradyrhizobium arachidis* SM32	✓	✓	✓	X	X
	Bradyrhizobium elkanii USDA 61	✓	✓	✓	X	X
	Bradyrhizobium japonicum J5	✓	✓	✓	✓	✓
	Bradyrhizobium vignae ORS3257	✓	✓	X	X	X
	Frigidbacter mobilis cai42	X	X	✓	✓	X
	Paracoccus kondratievae BJQ0001	✓	✓	✓	✓	✓
	Paracoccus mutanolyticus RSP-02	✓	X	✓	X	X
	Paracoccus pantotrophus DSM 2944	✓	✓	✓	✓	✓
	Paracoccus yeei CCUG 32053	✓	✓	✓	✓	✓
	Rhodobacter sp. LPB0142	✓	✓	✓	✓	✓
	Roseovarius sp. THAF9	X	✓	✓	X	X

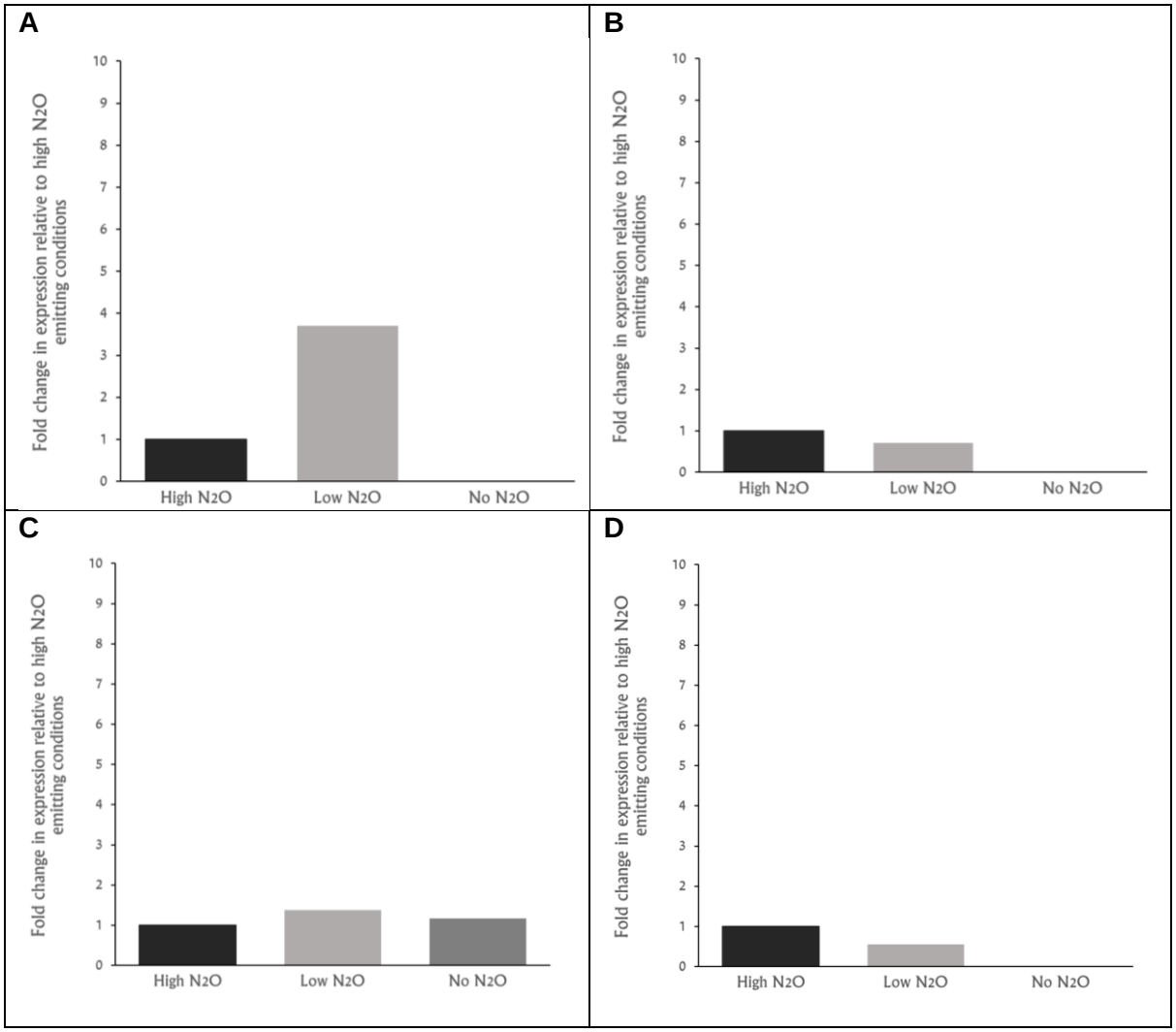
3.5.4 sRNA expression

P. denitrificans was previously grown to exponential phase in 3 different environmental conditions: “high N_2O” emitting anaerobic conditions, “low N_2O” emitting anaerobic conditions and “No N_2O” aerobic conditions (Gaimster *et al*., 2016). These environmental conditions were based on growth of *P. denitrificans* from Sullivan *et al,* (2013). This work found that whilst in “low N_2O” conditions where there was optimal Cu, N_2O did not accumulate in *P. denitrificans,* in “high N_2O” conditions 1-2 mM N_2O was emitted. Therefore, growing *P. denitrificans* under these two conditions could lead to changes in expression of sRNAs potentially indicating a regulatory role. Growth of *P. denitrificans* in “No N_2O” conditions would also help to determine whether sRNA expression changes between aerobic and anaerobic conditions.

The expression levels for each candidate sRNA under “high N_2O” emitting conditions, “low N_2O” emitting conditions and “No N_2O” emitting conditions were visualised in bar charts seen in Figure 18. Each chart displays the fold change in expression between each denitrifying condition relative to “high N_2O” emitting conditions.

Between “Low N_2O” emitting condition and “No N_2O” emitting conditions, sRNA11, sRNA18, sRNA36 and sRNA79 showed significant reduction of expression between the two conditions of between 5-fold and 10-fold. sRNA54 saw a change of expression of > 2-fold and sRNA31 and sRNA107 showed no significant change of expression between the two conditions. sRNA54 and sRNA107 showed increased expression in “No N_2O” conditions compared to “Low N_2O” conditions whereas all other candidate sRNAs showed reduced expression.

Between “High N_2O” and “Low N_2O” environments, sRNA11, sRNA54 and sRNA107 showed a change in expression between the two conditions of >2-fold. The other four candidate sRNAs did not show a significant change in expression between these two environmental conditions. sRNA18 and sRNA36 showed greater expression in “High N_2O” conditions compared to “Low N_2O” conditions whereas the other 5 candidate sRNAs had greater expression value in “Low N_2O” conditions when compared to “High N_2O” conditions.


A
Fold change in expression relative to high N2O emitting conditions
10
9
8
7
6
5
4
3
2
1
0
High N2O
Low N2O
No N2O
B
Fold change in expression relative to high N2O emitting conditions
High N2O
Low N2O
No N2O
C
Fold change in expression relative to high N2O emitting conditions
High N2O
Low N2O
No N2O
D
Fold change in expression relative to high N2O emitting conditions
High N2O
Low N2O
No N2O

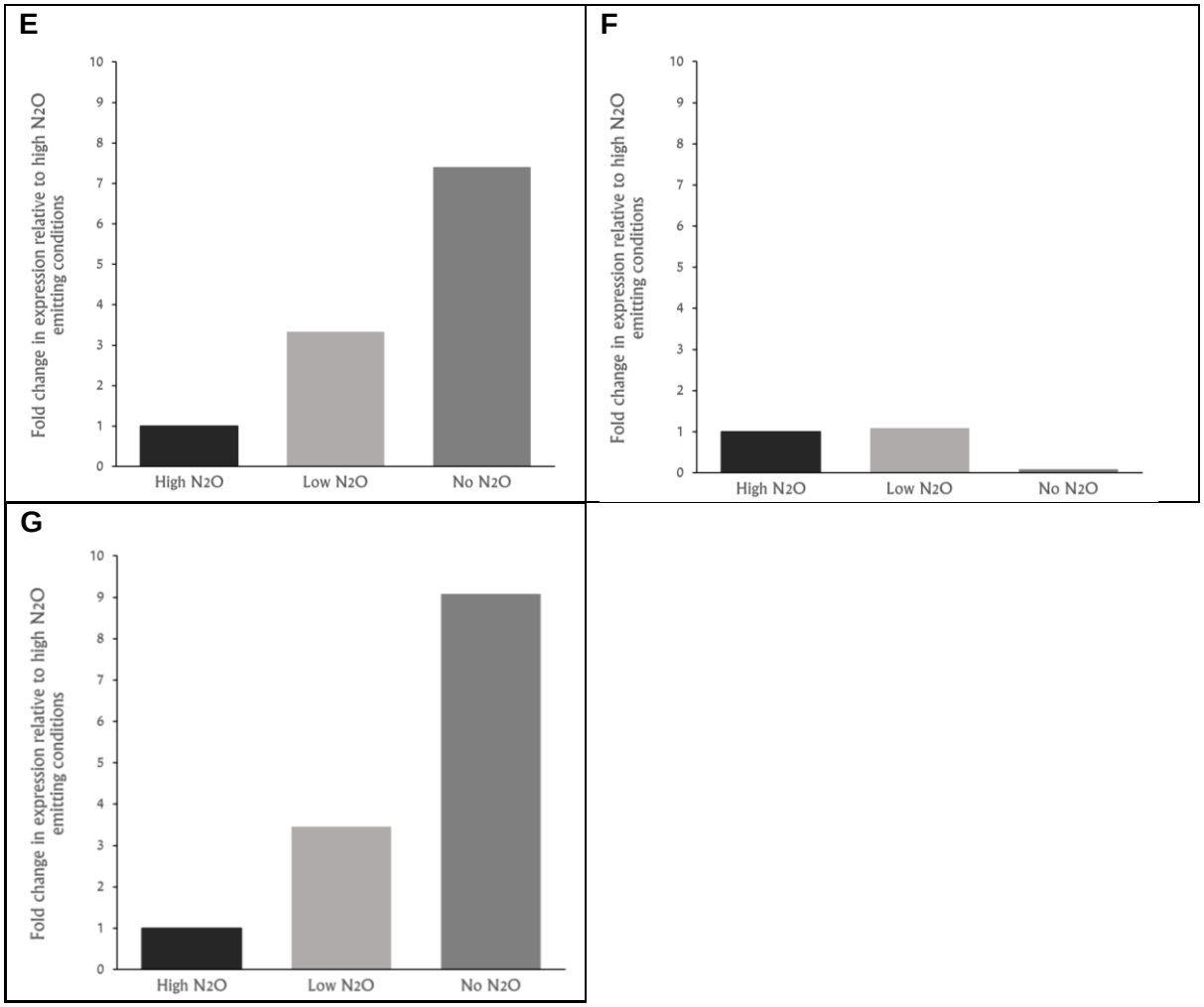


Figure 18: Expression levels of candidate sRNAs in different environmental conditions. Expression levels are relative to the levels expressed under "high N_2O" emitting conditions. (A) sRNA11, (B) sRNA18, (C) sRNA31, (D) sRNA36, (E) sRNA54, (F) sRNA79, (G) sRNA107.

Chapter 4: Experimental analysis of candidate sRNAs

4.1 Choosing 3 sRNAs for further validation

Having computationally characterised 7 candidate sRNAs with the presence of a putative FNR binding motif upstream of their promoter sequences, it was necessary to confirm whether they do in fact play a role in denitrification and potentially the release of N_2O. To do this, 3 candidate sRNAs were put through a number of experiments in a laboratory and the results of these experiments gave an indication into their physiological roles. These were chosen based on the characterisation steps followed previously.

The first of these sRNAs is sRNA18. The main reason for choosing this sRNA for further experimental validation was due to the fact that it showed a great increase in expression in anaerobic growth conditions compared to aerobic conditions indicating it could be involved with an anaerobic process such as denitrification. Additionally, it showed its greatest level of expression in "High N_2O" environmental conditions meaning it could possibly play a role in regulation of N_2O in *P. denitrificans.* Although sRNA18 was found to have the least stable structure out of all candidate sRNAs and showed no sequence conservation in other denitrifying bacteria, it was found to have a target of a GntR family transcriptional activator which have previously been found to play a role in denitrification (Gaimster *et al,* 2019).

The second candidate sRNA chosen for further research by experimental validation was sRNA36. The predicted secondary structure of sRNA36 showed the most hairpin loops out of all of the candidate sRNAs and the second lowest energy of hybridisation value showing how its stability and potential for forming complex conformations with a number of target sites. Like sRNA18, it had a GntR TR target. It also had a target of pseudoazurin which is known to be an electron donor for Nir, Nor and Nos in the denitrification pathway. sRNA36 targets a nitrogen-regulatory protein responsible for activating and repressing genes involved in nitrogen metabolism and regulation of nitrogen compounds throughout bacterial cells. Like sRNA18, sRNA36 did not show any sequence homology with any other bacterial species however its expression was also greatly increased in anaerobic environmental conditions, especially that of "high N_2O".

The final candidate sRNA chosen for further experimental validation was sRNA79. This sRNA had a relatively complex predicted secondary structure with three hairpin loops. sRNA79 had the most sequence homology with other bacterial species out of all seven candidate sRNAs. This included 19 species found to encode denitrifying reductases, 8 of which encoded *nosZ.* sRNA79 had a target gene of a nitrate transport system substrate binding protein which is involved in the transport of nitrate into cells, possibly used in the denitrification pathway.

sRNA79 expression was also found to be significantly upregulated in anaerobic conditions compared to aerobic conditions.

Having identified that sRNA18, sRNA36 and sRNA79 as strong potential candidates of having involvement in the denitrification pathway, it was necessary for them to undergo laboratory analysis to confirm whether they do play a role in denitrification or not.

4.2 sRNA Growth curves

Mutant *P. denitrificans* strains containing candidate sRNAs and an empty vector WT strain were overexpressed from the taurine-inducible promoter in the vector pLMB509. As has previously been discovered, the addition of 10 mM taurine induces a 15-fold to 20-fold increase in expression of pLMB509 (Pohlmann *et al*., 2000). Figure 16 displays the growth rates of the bacterial strains in a CuL environment with and without the presence of taurine. As can be seen by all growth curves in Figure 19, the addition of taurine to the bacterial strains caused no significant change in growth rate. This observation was also apparent in Figure 20, which displays bacterial growth in a CuH environment with and without the addition of taurine. Both figures display similar growth rates in all graphs across a 30-hour growth period, normally displaying a significant increase in growth around hour 15, and a plateau in growth around 24 hours.

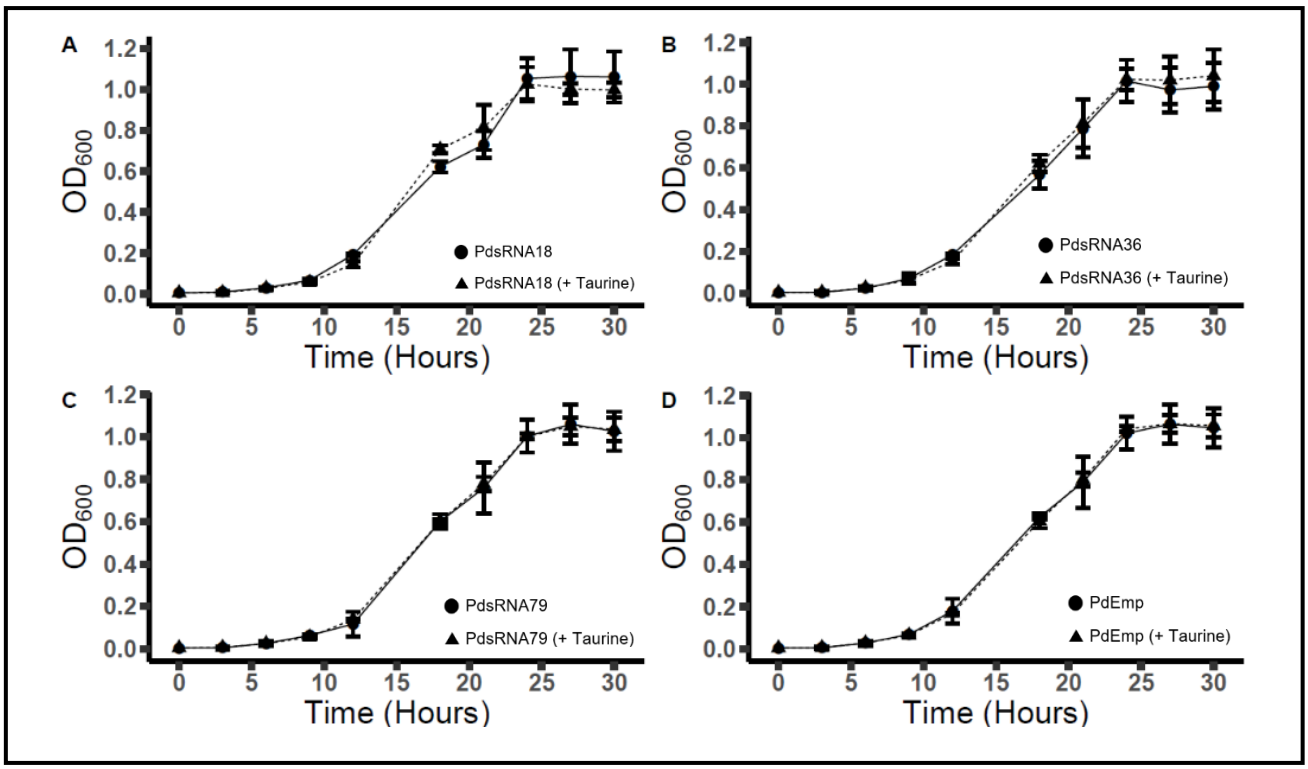


Figure 19: Overexpression of PdEmp, PdsRNA18, PdsRNA36 and PdsRNA79 in CuL conditions. PdEmp (A), PdsRNA18 (B), PdsRNA36 (C) and PdsRNA79 (D) showed no significant change in growth rate in copper low conditions when grown with and without taurine. The growth curves of each strain were created over a 30-hour period and growth measurements were taken at OD_{600} using a spectrometer. In each graph, the strain grown without the presence of taurine acted as the control variable. Error bars represent the standard deviation (SD) of the mean growth measurements obtained from triplicate experiments.

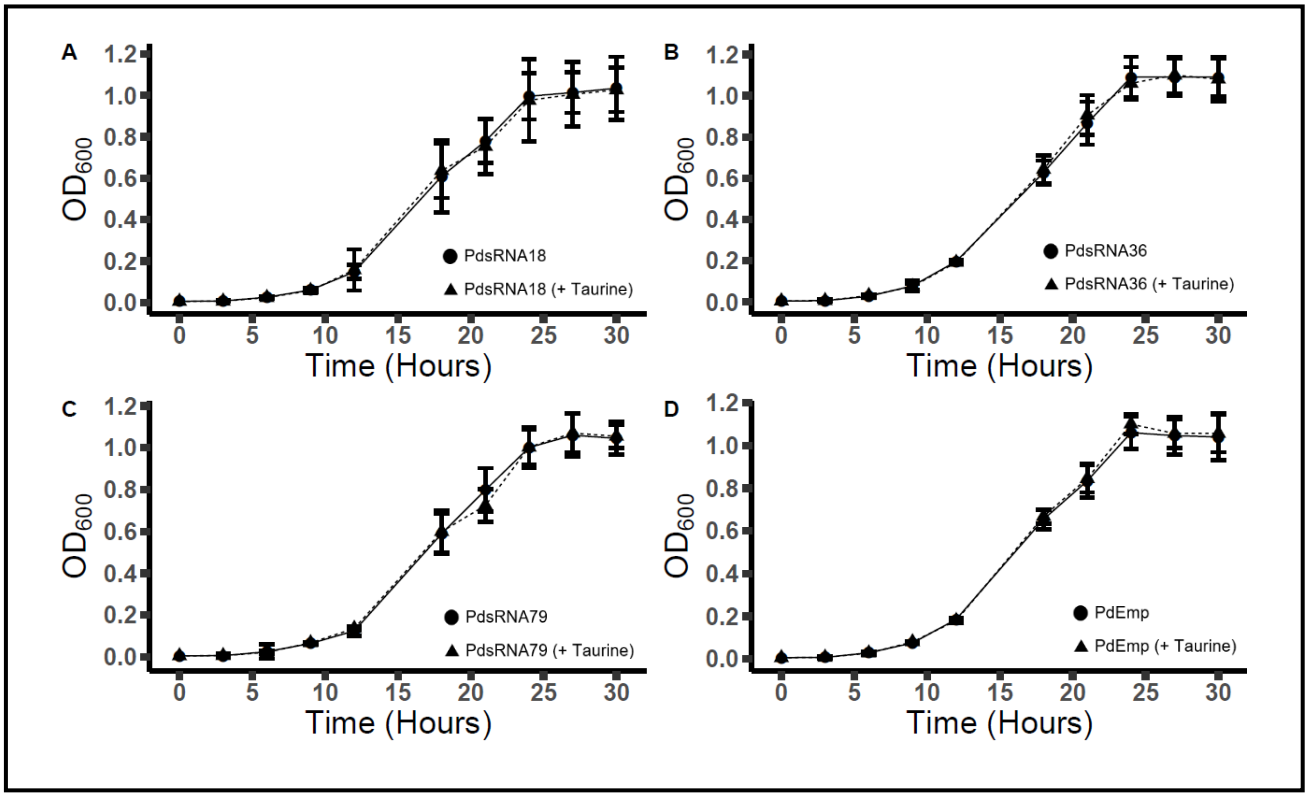


Figure 20: Overexpression of PdEmp, PdsRNA18, PdsRNA36 and PdsRNA79 in CuH conditions. PdEmp (A), PdsRNA18 (B), PdsRNA36 (C) and PdsRNA79 (D) showed no significant change in growth rate in copper high conditions when grown with and without taurine. The growth curves of each strain were created over a 30-hour period and growth measurements were taken at OD_{600} using a spectrometer. In each graph, the strain grown without the presence of taurine acted as the control variable. Error bars represent the standard deviation (SD) of the mean growth measurements obtained from triplicate experiments.

Figure 21 displays growth in the bacterial strains comparing their growth in CuH and CuL environments, in the presence of taurine. Once again, there was no significant difference in growth between bacterial strains grown in CuH and CuL environments meaning a change in Cu concentration had no significant impact on the growth of the bacterial strains.

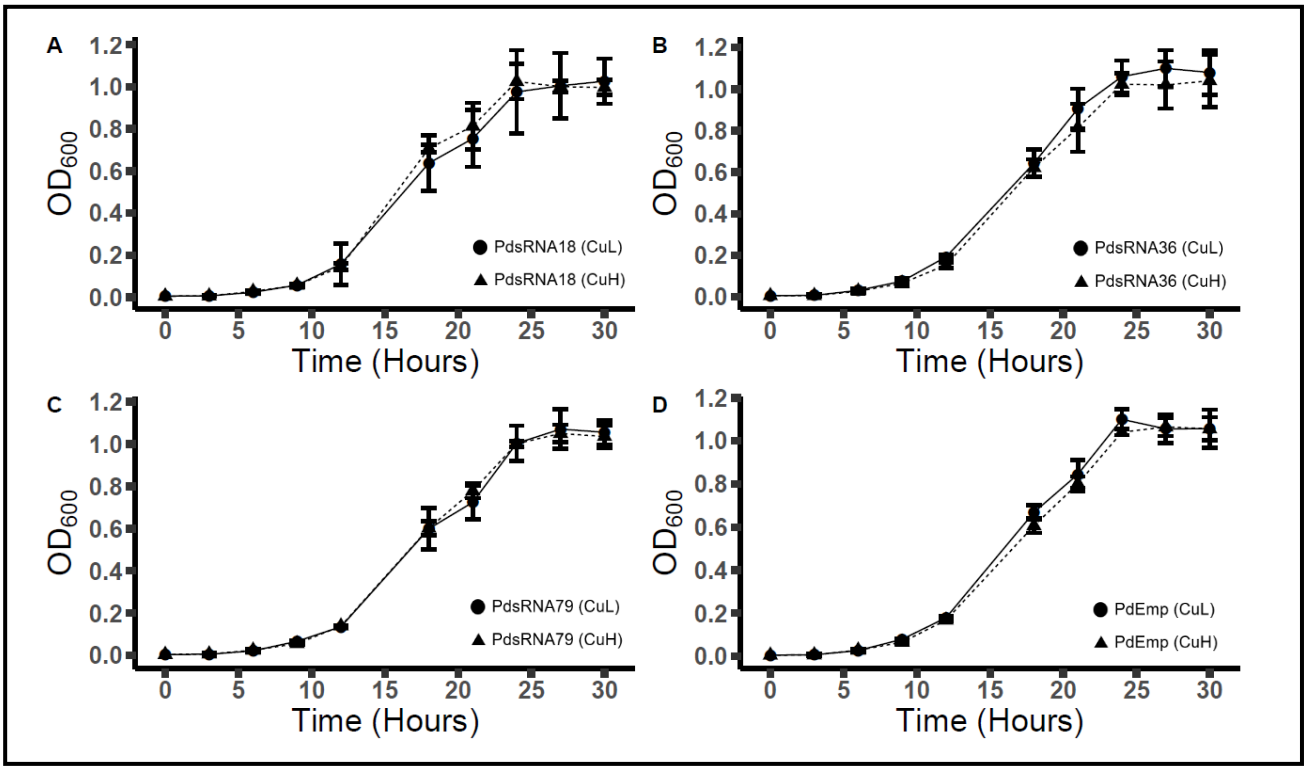


Figure 21: Comparing the overexpression of PdEmp, PdsRNA18, PdsRNA36 and PdsRNA79 in CuL and CuH conditions. Showing overexpression of PdEmp (A), PdsRNA18 (B), PdsRNA36 (C) and PdsRNA79 (D) in a copper high compared to a copper low environment with the addition of taurine to both samples. The growth curves of each strain were created over a 30-hour period and growth measurements were taken at OD_{600} using a spectrometer. The dotted line on the graph represents growth in a copper low environment whereas the solid line represents growth in a copper high environment. In each graph, the strain grown in CuH environmental conditions acted as the control variable. Error bars represent the standard deviation (SD) of the mean growth measurements obtained from triplicate experiments.

4.3 Measurement of N_2O in culture

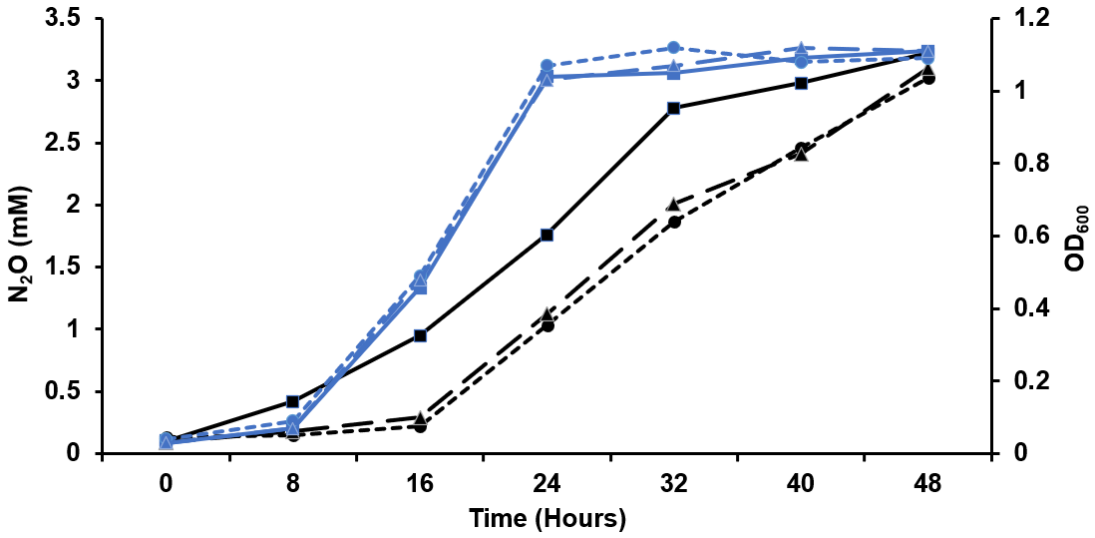


Figure 22: Overexpression of sRNA36 causes N_2O accumulatic ■ PdsRNA36 ed by P. denitrificans. 3 cultures of P. denitrificans; PdsRNA36, PdsRNA79 ● PdsRNA79 np were grown under CuL denitrifying conditions (20 mM nitrate as electron accept ▲ PdEmp e addition of 10 mM taurine to induce overexpression. N_2O levels were measured every 8 hours over a 48-hour period. Growth rates of all three PdsRNA36, PdsRNA79 and PdEmp are shown in blue and correspond with the Y axis showing OD_{600}; whilst the correlating N_2O emission rates are shown in black and correspond with the Y axis showing N_2O (mM). In each case, PdEmp is the control variable.

Figure 22 displays the level of N_2O emissions released by PdsRNA36, PdsRNA79 and PdEmp when grown in denitrifying CuL environmental conditions. When overexpressed in these conditions, PdsRNA36 produced a significantly greater emission rate of N_2O throughout growth when compared to PdsRNA79 and PdEmp. As PdEmp is wildtype *P. denitrificans,* it signifies the rate of N_2O emissions that should be seen in *P. denitrificans* in environmental denitrifying conditions of low Cu. PdEmp therefore acted as the control variable in this experiment. Overexpression of PdsRNA79 was found to emit a similar rate of N_2O as PdEmp, therefore indicating that sRNA79 does not have a significant effect on the release of N_2O from the denitrification pathway. On the other hand, when overexpressed, PdsRNA36 produced N_2O at a much faster rate than PdsEmp and PdsRNA79. Although the total N_2O produced by each culture was relatively similar after 48 hours, sRNA36 had more than double the N_2O concentration of sRNA79 and the empty vector after 8 hours and more than triple after 16 hours. The fact that sRNA36 produced a N_2O at a faster rate indicates that sRNA36 may play a significant role in the regulation of N_2O in the denitrification pathway. The reason as to why the N_2O concentration in all three strains was similar at the 48 hour mark could be attributed to the fact that the growth cycle of these bacterial strains

were towards the end of the stationary phase and sRNA36 no longer being expressed to such a high level; therefore not having such a great effect on N_2O emission rate. From this experimental analysis, it can be concluded that sRNA36 has a negative impact on the rate of N_2O reduction.

4.4 Confirmation of sRNA regulation by FNR

4.4.1 sRNA promoter region PCRs

PCR products of sRNA18, sRNA36 and sRNA79 were run along an agarose gel alongside a 1Kb Bioline hyperladder. The PCR products were 200bp in length as can be seen by Figure 23.

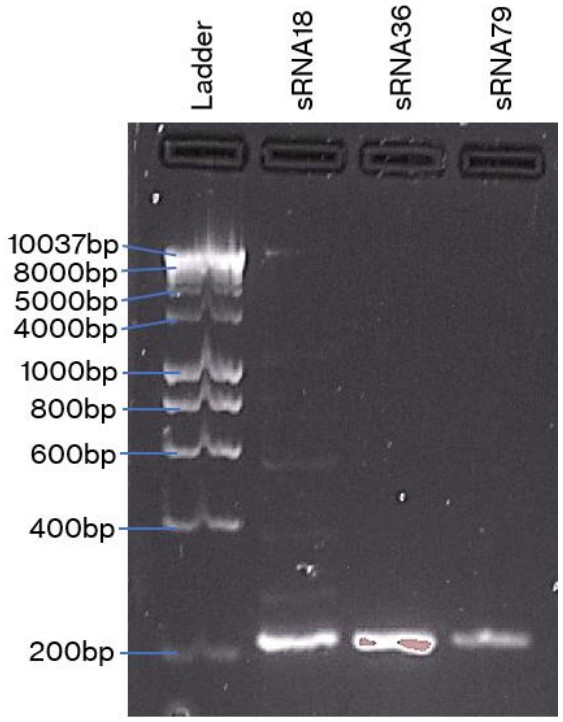


Figure 23: DNA products of a PCR of candidate sRNA promoter regions. The gel above shows 5 µl of product run along a 2% agarose gel next to a 1Kb DNA hyperladder.

4.4.2 PCR gel extraction

Figure 24 displays the products of a DNA gel extraction of the PCR products in Figure 23. This shows that the DNA had been successfully extracted and remained the correct length of 200bp.

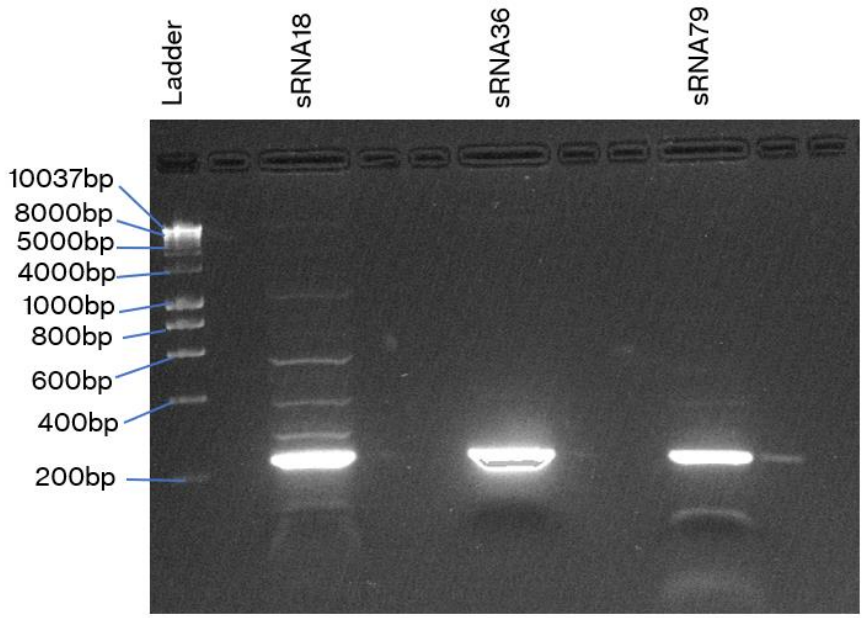


Figure 24: DNA products of a gel extraction on the sRNA PCR products extracted from the gel seen in Figure 21. The gel above shows 95µl of product run along a 2% agarose gel next to a 1Kb DNA hyperladder.

4.4.3 Restriction enzyme digests

The gel extracted sRNA promoter regions and the plasmid vector pmP220 went through restriction enzyme digest reactions using the enzymes EcoR1 and Pst1. This allowed for digested sRNA promoter regions to be inserted into the vector DNA. The DNA from the sRNA promoter region's restriction enzyme digest and the plasmid vectors restriction enzyme digest can be found in Figure 25 and Figure 26 respectively.

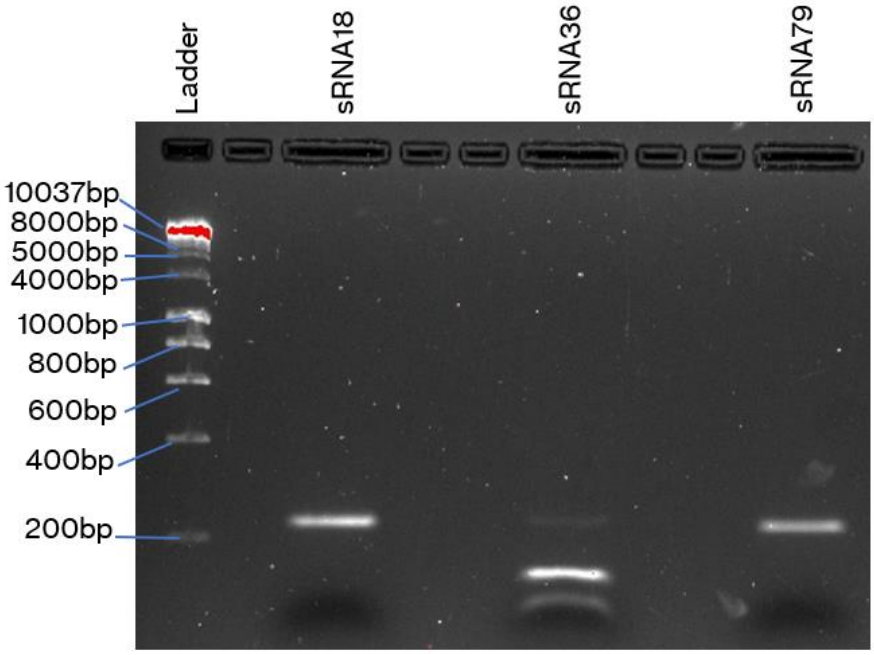


Figure 25: DNA products of a restriction enzyme digest on the sRNA promoter region DNA. The gel above shows 10µl of restriction enzyme digest reactions run along a 2% agarose gel next to a 1Kb DNA hyperladder.

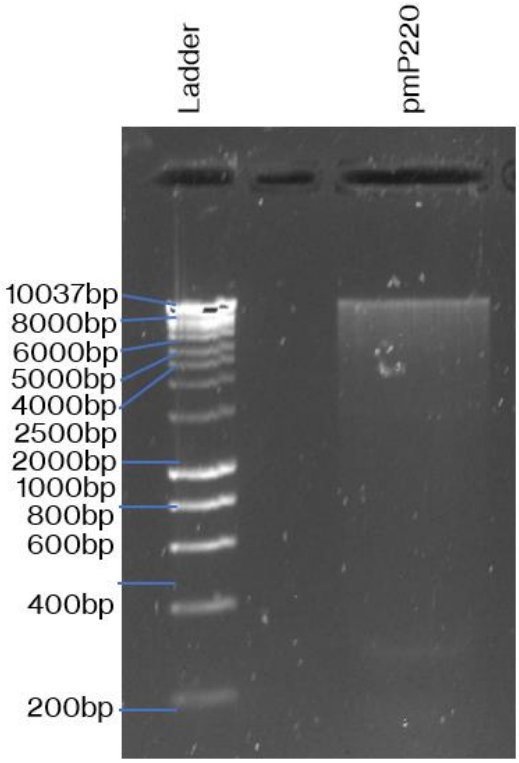


Figure 26: DNA products of a restriction enzyme digest on pmP220. The gel above shows 5µl of product run along a 1% agarose gel next to a 1Kb DNA hyperladder

4.4.5 Beta-galactosidase assay

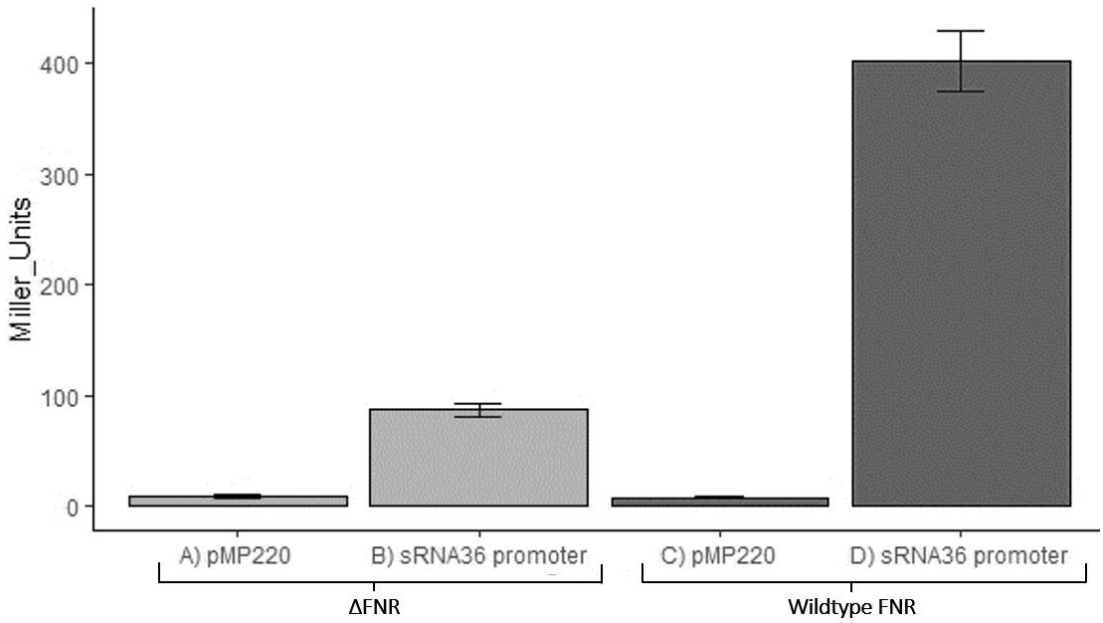


Figure 27: B-galactosidase activity directed by sRNA36-lacZ fusions in P. denitrificans PD1222 and the isogenic FNR mutant. The activity levels of the sRNA36 promoter and empty pMP220 are shown. Activity level was measured in Miller Units at an OD_{600} of 1.0. Error bars represent the standard deviation (SD) of the mean growth measurements obtained from triplicate experiments.

Figure 27 displays the activity level of the sRNA36 promoter when grown in wildtype *P. denitrificans* compared to when grown in an FNR mutant strain. A paired T test was conducted and it was found that there was a very statistically significant (P value < 0.01) change in activity of the sRNA36 promoter between growth in wildtype and FNR mutant strains of *P. denitrificans*. When grown in wildtype *P. denitrificans,* the sRNA36 promoter showed a mean activity level of 401.67 Miller units. This was significantly higher than in FNR mutant conditions which showed a mean of 87 Miller units. This therefore shows that the presence of FNR greatly increases the activity level of the sRNA36 promoter in *P. denitrificans* and it is likely that FNR is working as an activator under these conditions. The empty pMP220 vector showed no significant change in expression between mutant and wildtype FNR *P. denitrificans.*

Chapter 5: Discussion

5.1 Introduction

This study has achieved its aims of discovering putative FNR-regulated sRNAs and analysing them in a laboratory setting to uncover how they may affect the pressing issue of harmful N_2O emissions. Having provided an in-depth analysis into potential FNR-regulated sRNAs found in *P. denitrificans,* it is now possible to take a step back and provide a greater understanding of what these results mean and take a look into the future of sRNA research including how it could be improved.

5.2 Candidate sRNA secondary structures

The function of sRNAs depends greatly on its tertiary structure. However, tertiary structures of sRNAs are complex and are not represented well in previous research. Therefore, the secondary structures of sRNAs have become an important area of sRNA function research in recent years and one focussed on in this study (Zhang *et al*., 2019). Secondary structures can give indications about the functions of sRNAs and their interactions with other RNAs.

One key feature of an sRNAs secondary structure is the presence of hairpin loops, a section of RNA where a single stranded sequence folds back on itself forming an unpaired loop. The stem of a hairpin loop is made up of complementary RNA nucleotides that have formed strong hydrogen bonds leading to greater stabilisation (Svoboda & Di Cara, 2006). Hairpin loops increase stabilisation and can create specific binding sequences that are complementary to mRNA targets. The stem of a hairpin loop is made up of complementary RNAs nucleotides that have formed strong hydrogen bonds with one another leading to greater stabilisation in the sRNA structure. They are also important in recognising and binding to target mRNAs (Svoboda & Di Cara, 2006)The minimum free energy value predicted by MFold is also important to sRNAs as it helps express the stability of an sRNA and its ability to bind with mRNA targets. (Murakami *et al*., 2016).

Looking at the MFold outputs of the candidate sRNAs, sRNA36 had the greatest number of hairpin loops with 6, 3 more than any other candidate sRNA possibly indicating a high level of stability in its secondary structure and specificity to its target sites. sRNA36 also had the second lowest minimum free energy value of -56.50 again indicating its structural stability. sRNA107 had the lowest minimum free energy value of -67.10 and 3 hairpin loops indicating a high level of structural stability in this sRNA secondary structure. sRNA18 had just one hairpin loop and the lowest minimum free energy value of -14.00 indicating this sRNA to have

the lowest level of stability and possibly a lack of specificity when it comes to mRNA target sequences.

5.3 Candidate sRNA target sites

Since the discovery of sRNAs, there has been a large focus onthe identification of sRNAs using modern high-throughput sequencing technology. However, a major lack of understanding still remains surrounding the regulatory responses of these sRNAs in relation to target genes. Research has therefore started to focus on the discovery of sRNA gene targets and their regulatory roles to improve overall understanding of an sRNAs global response.

Computational approaches to sRNA target identification have been at the forefront of this research due to their efficiency in producing results compared to experimental techniques. One of these computational characterisation software is TargetRNA2 (Kery *et al*., 2014). TargetRNA2 is the first target identification software used specifically for identification of bacterial sRNA targets building upon previously available software including TargetRNA, IntaRNA and RNApredator (Busch *et al*., 2008; Eggenhofer *et al*., 2011; Tjaden *et al*., 2006). TargetRNA2 employs four main algorithms to identify sRNA targets. 1. Conservation of the sRNA – The sRNA nucleotide sequence that is input into TargetRNA2 is compared with every sequence available in GenBank and those that show greater conservation are more likely to be target sites of RNAs. 2. Accessibility of the sRNA – The structure of each sRNA is examined for stability with those that are more stable with regions accessible to targets are more likely to be considered as target-interacting regions. 3. Accessibility of the mRNA – Much like the examination of the sRNA structure, the target mRNA structure is examined for stability and identification of regions where an sRNA could bind. Those with greater stability and accessibility to sRNAs are considered more likely targets. 4. Energy of hybridisation - Targets with lower hybridisation energy are thought to be more likely targets of sRNAs (Kery *et al,* 2014).The large majority of predicted target sites of the candidate sRNAs were not known to have any direct impact on N_2O regulation or the denitrification pathway in general. However, many targets could indirectly affect the denitrification pathway. For example, metabolic processes within bacteria can affect availability of substrates and electron donors that may be linked to the denitrification process (Iyer *et al*., 2021).

Many target sites were hypothetical proteins. Hypothetical proteins are still of interest in studies into target genes as, although their function is unknown due to no previous characterisation, it is possible that these proteins could play a key role in the denitrification pathway. In all candidate sRNAs, TargetRNA2 displayed hypothetical proteins with relatively

high energy values as one of the sRNAs' top targets. In order to fully understand the regulatory profiles of the candidate sRNAs, future research should aim to uncover the roles of these hypothetical proteins. Hypothetical protein in sRNA31 (Pden_4145, Pden_2890) and sRNA54 (Pden_4223, Pden_4316) showed a significant change in expression between WT and mutant strains of *P. denitrificans* (Giannopoulos *et al*., 2017). Interestingly, in sRNA31, the 2 hypothetical protein targets both showed a reduction in expression in all mutant strains whereas the hypothetical protein targets of sRNA54 showed increased expression in mutant strains. This indicates that the hypothetical protein targets belonging to sRNA31 are regulated in some way by the presence of FnrP, NNR and NarR.

TR targets were found to be targets in all candidate sRNAs apart from sRNA54. These involved families of TRs such as AraC, TetR and GntR. TRs are known to play roles across many different physiological pathways including the denitrification pathway such as the GntR regulator found to be controlled by DenR (Gaimster *et al,* 2019). GntR family transcriptional regulators were found to be targets in sRNA18 (Pden_4274, Pden_3944) and sRNA36 (Pden_4274). If sRNA18 or sRNA36 are found to be involved in the denitrification pathway, then further research may involve experimental validation of the roles of these GntR regulators to understand whether they could also have a role in denitrification.

sRNA11 had a target of cytochrome-c-oxidase (Pden_4321) and sRNA36 had a target of pseudoazurin (Pden_2983). Cytochrome c and pseudoazurin are important in the transfer of electrons from the cytochrome bc_1 complex to Nir, Nor and Nos to carry out their functions (Spiro, 2012).

Targets relating to different stages of the nitrogen cycle aside from the denitrification pathway indicated potential role for candidate sRNAs in various stages of this cylce. sRNA36 had a predicted target of a nitrogen regulatory protein P-II (Pden_4461). These proteins are responsible for monitoring nitrogen-containing compounds within bacterial cells such as NO_3. They are also involved in activating or repressing genes that are involved in nitrogen metabolism. This is significant as these proteins are able to regulate the expression of genes involved in denitrification such as Nar, Nir, Nor and Nos depending on the level of nitrogen-containing compounds available to a bacterial cell. (Huergo *et al*., 2013). sRNA11, sRNA31 and sRNA79 had a predicted target of a NO_3 transport system binding protein. In fact, sRNA11 and sRNA79 target the same binding protein (Pden_4169). NO_3 transport system binding proteins are important as they are involved in the uptake of NO_3 into bacterial cells. They bind to NO_3 molecules before transporting them across the cell membrane to be used in physiological pathways such as denitrification. They are part of the family of ABC transporters which are involved in uptake of a lot of essential nutrients to cells (Koropatkin *et al*., 2006). As

NO_3 acts as an electron acceptor in the denitrification process, the presence of a high concentration of NO_3 in denitrifying bacterial cells is important. Glutamine synthase, Type 1 (Pden_4462) was a target of sRNA18. This enzyme is essential in assimilation and the conversion of NH_3 and glutamine to glutamate, an essential amino acid. Therefore, although not directly related to denitrification, glutamine synthase has a role in the assimilation of nitrogen into bacterial cells. Therefore, although this target does not directly affect the denitrification pathway, it can affect the efficiency of the process by controlling the availability of nitrogen sources in bacterial cells (Cruzat *et al*., 2018).

A number of targets were conserved in more than one sRNA. This could indicate a conserved role between sRNAs. However, none of these conserved targets had any common physiological role meaning no specific conserved roles between sRNAs could be deduced. Each candidate sRNA showed at least one target that had been found to be significantly up or down regulated between Δ*fnrP*, Δ*nnR* Δ*narR* and wildtype strains of *P. denitrificans.* Although most of these targets do not have a known role in denitrification as of yet, this discovery may indicate they could play a role and further experimental analysis in the future would confirm this.

5.4 Conservation of sRNA sequences in other denitrifying species

Homology amongst bacterial species is strong evidence that those bacterial species are related in some way, whether that be structurally or metabolically for example. Discovery of conserved sRNA sequences in other denitrifying bacteria aside from *P. denitrificans* may mean that sRNA is more likely to have a conserved denitrifying role over an sRNA that is not conserved in other denitrifying species.

The fact that sRNA11, sRNA18 and sRNA36 showed no sequence homology to other bacteria aside from *P. denitrificans* does not mean they definitely do not play a role in denitrification. It may be the case that they do play a role in regulation of the denitrification pathway in *P. denitrificans* and are in fact species-specific (Liu *et al*., 2023).sRNA79 was found to share sequence homology with the most species of denitrifying bacteria with 19 homologues found species belonging to 14 different generaindicating sRNA79 to be most likely to have a conserved denitrifying role. Of these 8/19 were found to possess *nosZ* like *P. denitrificans* indicating a potential conserved role in the final reduction stage of the denitrification pathway of N_2O to N_2. sRNA107 was found to share sequence homology with 11 denitrifying species of bacteria belonging to five different genera. This would suggest that sRNA107 is also likely

to have a conserved denitrifying role. Five of these species possessed the *nosZ* gene. sRNA31 and sRNA54 sequences were found in just one and two conserved species of denitrifying bacteria respectively. These were *Paracoccus pantotrophus DSM 2944, Paracoccus aminovorans JCM7685* and *Paracoccus methylovorus H4-D09*. These three species are all complete denitrifiers. As can be seen, all three of these species belong to the *Paracoccus* genera meaning these candidate sRNAs may have a role in denitrification within the *Paracoccus*.

Paracoccus was unsurprisingly the genera that the most conserved species belonged to with ten species found to have a conserved nucleotide sequence for sRNA79 and four to have a conserved nucleotide sequence for sRNA107. The *Paracoccus* genus comprises of at least 40 known species that have been isolated over a wide range of habitats. *Paracoccus* have an ability to adapt metabolically in these environments often utilising denitrification as a key respiratory process for their survival. Although it is known that *Paracoccus* are able to show versatility in environments and carry out global biochemical cycles such as denitrification, only the genome of *P. denitrificans* can be found in the NCBI database (Dziewit *et al*., 2014). As can be seen from Table 18, some conserved species of *Paracoccus* had genes involved in each stage of the denitrification pathway, possibly indicating them to be complete denitrifiers; whereas others had genes only partially involved the pathway. However, aside from *P. denitrificans,* none of these species of *Paracoccus* have been experimentally validated and subsequently confirmed as being able to carry out denitrification. Due to the success of *P. denitrificans* as a model denitrifier and other members of the *Paracoccus* genus possessing denitrifying genes, future research should investigate how denitrification differs between species of *Paracoccus.*

Reading into the literature surrounding conserved species found in Table 18, I discovered that just three of them had been studied for their roles in denitrification and subsequently confirmed as being able to complete partial or complete denitrification.

P. pantotrophus DSM 2944, found to have conserved sequences of sRNA31 and sRNA107, has been found to be able to utilise denitrification in anaerobic environments and is closely related to *P. denitrificans* sharing a NarR homologue (Bockwoldt *et al*., 2020). Interestingly, although Table 18 shows *P. denitrificans* to possess genes involved in the expression of Nar, Nir, Nor and Nos possibly suggesting that *P. pantotrophus* could be a complete denitrifier; research has found that *P. pantotrophus* is a partial denitrifier, only able to complete the first stage of the denitrification pathway reducing NO_3 to NO_2 (Gates *et al*., 2008). *P. pantotrophus* expresses two NO_3 reductases, Nap and Nar which have previously been mentioned as having key roles in the nitrogen cycle as well as in denitrification. This means that even though

some bacteria possess genes involved in encoding the key reductases of the denitrification pathway, it does not necessarily mean they have all the genomic material required to complete denitrification.

Gemmobacter fulva con5 wasfound to have a conserved nucleotide sequence belonging to sRNA79. Bacteria belonging to the *Gemmobacter* genera are methanotrophs capable of denitrifying methane oxidation under anaerobic conditions. The genome strain *Gemmobacter fulva* con5 was found to contain *nirB, nirK, nirQ norB, norC* and *norC,* all involved in denitrification and essential genes of Nir and Nor (Jin *et al,* 2021). However, no genes belonging to Nar or Nos were found meaning the bacteria was unable to complete the first and last stage of the denitrification pathway (Jin *et al*., 2021).

Bradyrhizobium japonicum, a homologue of the nucleotide sequence of sRNA107. This species has been found to possess the denitrifying genes: *napEDABC*, *nirK*, *norCBQD* and *nosRZDFYLX* (Mesa *et al*., 2003).

Some conserved bacterial species encoded genes involved in different stages of the nitrogen cycle aside from denitrification. sRNA107 was found to be conserved in four species of bacteria belonging to the genus *Bradyrhizobium.* Bacteria belonging to the *Bradyrhizobium* genus are generally known to be having an endosymbiotic N_2-fixing relationship with leguminous crops in which they fix N_2 in exchange for essential nutrients needed for growth (Argaw, 2014). *Bradyrhizobium* contain a wide range of genes involved in regulation of N_2-fixation such as regulators for nitrogenase, the enzyme necessary for N_2-fixation. *Fuscovulum Blasticum* which also contained a conserved nucleotide sequence for sRNA107 was also found to contain genes involved in the regulation of N_2-fixation and possessed no genes involved in denitrification. The fact that the nucleotide sequence of sRNA107 was found in five different species of bacteria involved in N_2-fixation could suggest that this sRNA could play a key role in N_2-fixation.

Looking at Table 17, some conserved species were found to have no relevancy to the nitrogen cycle whatsoever. For example, homologues of sRNA79 included opportunistic pathogens belonging to the *Bartonella* and *Brucella* genera. This shows how sRNAs are capable of potentially being involved in a wide range of different physiological pathways.

5.5 sRNA Expression

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An understanding of sRNA expression in different environmental conditions is important to understand how they respond to varied environmental forces. Using knowledge that Cu is of great importance to Nos and the reduction of N_2O to N_2, growth of sRNA strains in anaerobic

conditions of optimal Cu concentrations (low N_2O) compared to anaerobic conditions of low Cu (high N_2O) were analysed. Significant changes in sRNA expression between these two conditions would indicate that sRNA to respond to the presence of Cu meaning a potential role Nos regulation. sRNA expression was also analysed in aerobic conditions (No N_2O) to understand how the presence of O_2 could affect sRNA expression.

sRNA54 and sRNA107 were most greatly expressed in “No N_2O”, aerobic conditions. In fact, sRNA54 saw an increase of >2-fold between “No N_2O” and “Low N_2O” environmental conditions. As denitrification is an anaerobic process, it would be unexpected for sRNAs involved in denitrification to be significantly expressed in aerobic conditions. This could indicate that sRNA54 and sRNA107 may not have regulatory functions involved in denitrification. sRNA31 showed no significant change in expression between aerobic and anaerobic conditions also indicating that this sRNA may also not be involved in an anaerobic process such as denitrification. On the other hand, sRNA11, sRNA18, sRNA36 and sRNA79 showed a fold increase in expression of between 5-fold and 10-fold in “low N_2O” anaerobic conditions compared to “No N_2O” aerobic conditions. This indicates that these four sRNAs are more likely to be involved in regulating anaerobic biological pathways.

sRNA expression comparisons between “high N_2O” and “low N_2O” conditions indicates whether or not candidate sRNAs may play a role in the final reduction stage in the denitrification pathway. An increase in sRNA expression in “high N_2O” conditions over “low N_2O” conditions could indicate that sRNA is being overexpressed to respond to low Cu conditions and high N_2O levels; therefore, indicating a role in the final denitrification reduction stage of N_2O to N_2. This was the case in sRNA18 and sRNA36. On the other hand, sRNA11, sRNA54 and sRNA107 showed a >2-fold increase in expression in “low N_2O” conditions compared with “high N_2O” conditions. This may suggest that these sRNAs are not being expressed as highly in conditions of high N_2O as they do not play a role in reducing N_2O to N_2. sRNA31 and sRNA79 did not show a significant difference between either environmental condition, however, they too showed greater expression in “low N_2O” conditions.

5.6 Growth curve analysis

As there was no significant change in growth patternsbetween PdsRNA18, PdsRNA36, PdsRNA79 and PdEmp when grown in CuH and CuL conditions, no indication that any of the candidate sRNAs were involved in the reduction of N_2O to N_2 could be concluded. These strains also showed no change in expression when grown in the presence of taurine between CuH and CuL, showing overexpression did not affect their growth..

5.7 Gas analysis

Although there was no change to bacterial growth in varied environmental conditions such as low copper, it was possible that these conditions could affect N_2O rates. Gas analysis of PdsRNA36, PdsRNA79 and PdsEmp was undertaken under CuL environmental conditions. As can be seen in Figure 22, PdsRNA79 and PdsEmp cultures showed no significant difference in the rate at which each emitted N_2O. On the other hand, the culture containing PdsRNA36 showed a significantly faster rate of N_2O emissions. This is of great significance as it is therefore more likely that sRNA36 plays a denitrifying role, directly affecting N_2O emission rate. Interestingly, whereas overexpression of the previously studied sRNA DenR caused a significant reduction in N_2O, sRNA36 showed a significant increase in N_2O emissions. It is already known that DenR shows this as its overexpression strongly downregulated Nir and Nor and subsequently reduced N_2O emissions; whereas the significant increase in N_2O emissions seen by overexpression of sRNA36 may indicate this sRNA to strongly activate the activity of Nor which reduces NO to N_2O, or equally it could work by repressing the activity of Nos which reduces N_2O to N_2. Similar to DenR, analysis of the exact genes activated and repressed by sRNA36 would present a clearer understanding of its role in denitrification, and the reasoning behind a relatively high N_2O emission rate when overexpressed in a denitrifying culture.

Although growth of PdsRNA18 was normal, it did not show any consistent results in terms of N_2O production and therefore, these results were not plotted in Figure 22. This experiment will therefore need to be repeated in the future to understand whether sRNA18 can change N_2O emission rates during denitrification.

Having established that sRNA36 has a potentially important role in the regulation of N_2O in *P. denitrificans,* it was necessary to once again analyse the predicted targets of sRNA36 to understand whether any could explain the reasoning for an increased rate of N_2O reduction. As has been previously mentioned, pseudoazurin plays an important role in denitrification whereby it transfers electrons to Nos in order to aid the reduction of N_2O to N_2 (Spiro, 2012). Therefore, as a predicted target of sRNA36, sRNA36 may act to repress the activity of pseudoazurin meaning N_2O production in cells could increase. sRNA36 also had a predicted target of a nitrogen regulatory protein P-II. These proteins are involved in activating or repressing genes that are involved in nitrogen metabolism. Activation or repression of this protein could affect the expression of Nor or Nos by sRNA36 possibly explaining the N_2O accumulation (Huergo *et al*., 2013). sRNA36 was also found to target 3 hypothetical protein targets. Although, the function of these targets is not yet known, it is possible that they could play a role in the regulation of N_2O during denitrification. As is has now been discovered that

sRNA36 impacts N_2O emissions during denitrification, it would be interesting to fully understand the roles of these targets.

Further studies will be required to understand how the overexpression of candidate sRNAs effect the rate of N_2O produced in denitrifying conditions. Future experiments into N_2O production by these sRNAs should focus on examining the emission rates in cultures with and without the addition of taurine to compare how greatly overexpression of sRNAs changes the rate of N_2O emitted. It would also be interesting to compare how emission rates vary between CuL and CuH environments.

5.8 Confirmation of FNR regulation

Having understood that sRNA36 significantly increases N_2O emissions when overexpressed in denitrifying conditions, this strongly indicates that sRNA36 has a role in regulation of N_2O by influencing the denitrification pathway in some way. As sRNA36 was chosen to be studied based on the presence of an FNR TR motif found upstream of it on the *P. denitrificans* genome, it was necessary to investigate whether sRNA36 was in fact FNR-regulated.

B-Galactosidase is an enzyme that is encoded by the *lacZ* gene of the *lac* operon in *E. coli.* The function of B-Galactosidase is to cleave lactose, forming galactose and glucose to be used as energy sources (Juers *et al*., 2012). O-nitrophenyl-B-D-galactosidase (ONPG) is a substrate that is cleaved to galactose and o-nitrophenol which is yellow in colour. ONPG can be used to determine the concentration of B-galactosidase in a reaction whereby the production of o-nitrophenol is proportional to the presence of B-galactosidase; therefore, producing a yellow colour proportional to the concentration of B-galactosidase. Jeffrey Miller published “Experiments in Molecular Genetics” in 1972, which contained the protocol for using ONPG to determine the concentration of B-galactosidase. This led to the standardised level of B-galactosidase activity to be referred to as “Miller Units” (Miller, 1972).

As can be seen in Figure 27, the sRNA36 promoter region showed a significant change in activity level when grown in wildtype and FNR mutant *P. denitrificans* under anaerobic conditions. The FNR mutant strain displayed a mean of 87 Miller units, whereas B-galactosidase activity in the wildtype strain displayed a mean of 401.67 Miller units. This confirms that the presence of FNR greatly increases the expression of the sRNA36 promoter region and strongly suggests that sRNA36 is FNR-regulated.

FNR can act as either a repressor or an activator of transcription. From this experiment, the increase in the expression of sRNA36 makes it is clear that FNR acts as an activator of sRNA36. However, as this experiment was run under anaerobic conditions, it would be of

interest in the future to determine whether aerobic conditions would alter the regulation of sRNA36 by FNR.

5.9 The Impact of this Study, Advancements in sRNA Discovery and Novel sRNA Applications

5.9.1 The impact of this study

When DenR was discovered in 2019 as being the first known sRNA to have an effect of the rate of N_2O emissions, it signified a huge step forward in mitigation strategies against N_2O as sRNA research is a novel and undiscovered area (Gaimster *et al,* 2016). Therefore, over recent years, there has been an increase in research into how sRNAs function and how they fit into various regulatory pathways. This study has discovered a second sRNA, sRNA36, in *P. denitrificans* known to have an effect on the rate of N_2O emissions. This is another step forwards into understanding environmental regulators involved in the switch between complete and incomplete denitrification.

5.9.2 Future steps for sRNA36

Having been discovered as an sRNA that could potentially affect the rate of N_2O emissions in the denitrification pathway, sRNA36 should undergo further experimental validation steps to fully understand its role in the denitrification pathway. One step that should be taken is to measure all denitrification intermediates emitted in *P. denitrificans* growth culture when sRNA36 is overexpressed. This will give an improved understanding of the exact position along the denitrification pathway that is being affected by sRNA36. A second experiment that could be conducted on sRNA36 is real-time quantitative reverse transcription PCR (qRTPCR). This technique uses reverve transcription to produce a DNA template from an RNA which can then be amplified. These DNA templates could then be analysed to understand which genes sRNA36 transcribes. A final experimental technique that could be employed would be to mutate the secondary structure of sRNA36 and analyse how N_2O emission rate changes. By doing this, it will be possible to determine whether N_2O emission rate changes in the mutant strain.

5.9.2 Advancements in sRNA discovery and analysis

Although research into sRNAs and their applications in complex regulatory pathways such as denitrification has greatly advanced in recent years; these discoveries have only scratched the surface of a wide array sRNAs and pathways yet to be discovered. Advancements in techniques used to uncover these sRNAs will be of vital importance in coming years.

A number of computational characterisation techniques were used throughout this study to identify candidate sRNAs. However, whilst software is relatively easy to use, the majority of information available on sRNAs is restricted to model organisms that have been well-characterised previously. This has made the discovery of sRNAs using computer software alone a difficult task; especially as sRNAs are so diverse such as their structure and conservation in various bacterial species. High throughput RNA-seq has meant sRNAs can be more easily identified, however, again this technology is restricted to well-studied model organisms. Further technological advancements will be necessary in the future to make sRNA identification a more efficient and accurate process.

In this study, the identification of sRNA targets was vital in identifying putative roles of candidate sRNAs. Through identifying mRNA targets, it is possible to place sRNAs into biochemical pathways such as denitrification. The use of computation tools in this instance is highly desirable over the time-consuming approach of experimental target identification using techniques such as genetic knockouts, microarray analysis and qRT-PCR. However, many of these online tools including TargetRNA2, which was used in this study, can be inaccurate. Therefore, it is necessary to establish a novel method of identifying sRNA targets as such an important factor in sRNA studies.

This study along with many other studies focusses on denitrification using a model denitrifier. Although this is accurate as these model denitrifiers are usually well characterised and highly tractable, it does not give an accurate depiction of how denitrification would work in the natural environment. In the environment, bacteria exist alongside each other in large communities and they often work together in complex biological pathways. To progress research in this field, it will be necessary to look on a metagenomic scale to understand how various denitrifiers interact and how they all may favour common sRNAs in relation to denitrification and the reduction of N_2O. Novel sequencing methods have been found to be able to analyse metagenomic profiles in humans and are able to display the sRNA content of these samples (Mielle et al 2020). A study looking into the microbial metatranscripts from the ocean have discovered an abundance of sRNAs known to be involved in nutrient acquisition

and carbon metabolism (Shi et al 2009). A similar study looking at metatranscriptome data from the desert identified a large number of sRNAs involved in the uptake of water from the soil (Gelsinger et al 2020). Studies like these which are performed on a wider metatranscriptome scale will be important to the future of sRNA research.

5.9.3 Novel sRNA Applications

Some recent advancements in sRNA applications in 'real-life' scenarios have displayed how sRNA research can progress. A relatively recent area has been in a medical sense. Analysis of human microbiomes in colorectal cancer patients found an altered sRNA abundance in samples of ill patients when compared to healthy patients (Tarallo et al 2019). In this instance, it is possible for sRNAs to act as 'biomarkers' for identifying the early stages of a disease in medical studies. The pathogen *Fusarium nucleatum* is often associated with colorectal cancer. Studies have found that the secretion of an sRNA by the colon has been linked to depletion of this pathogen. Therefore, it is though that in future medical practices, sRNAs such as this could be administrated as 'sRNA antibiotics' to help combat diseases (Vogel, 2020).

Applying these two novel sRNA applications back to this study, it is possible that in the future, sRNAs with a known function could act as biomarkers in denitrification studies. For example, the use of sRNAs as 'biomarkers' could indicate agricultural soils which are releasing a greater level of N_2O if an sRNA known to repress N_2O reduction is found there. Steps could be taken to change the sRNA profile of this soil. One of these steps could take into consideration the novel approach of using 'sRNA antibiotics'. For example, the implementation of sRNAs that are known to increase reduction of N_2O.

5.9 Concluding remarks

After a number of computational characterisation and experimental validation steps, sRNA36 joins DenR as the second sRNA found in *P. denitrificans* known to affect the rate of N_2O emissions. The discovery of sRNAs that can affect denitrification rate and N_2O emissions rate is important to improve understanding in this field and it is likely that there are many other significant sRNAs yet to be discovered.

Future research should focus on improving computational techniques to discover sRNAs and sampling a wide metatranscriptome of microbes for sRNA profiles to gather as much information on sRNAs as possible. Promising advancements in sRNA applications in recent

years such as their potential use as ‘biomarkers’ and ‘antibiotics’ are positive in the fight against global N_2O emissions.

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Appendix

Table 1: Target genes of sRNA11 found on chromosome 1. Including the energy value between sRNA and target genes as well as any changes in expression of targets between FNR family mutant strains and wildtype *P. denitrificans.*

Chromosome 1	**Energy value**
Pden_1370 hypothetical protein	-12.90
Pden_2562 hypothetical protein	-12.69
Pden_0183 D-amino acid dehydrogenase small subunit	-11.89
Pden_2103 ureidoglycolate hydrolase	-10.79
Pden_0539 signal transduction histidine kinase regulating citrate/malate metabolism	-10.52
Pden_0071 hypothetical protein	-9.50
Pden_1613 acyl-CoA dehydrogenase	-9.28
Pden_0919 hypothetical protein	-9.07
Pden_0406 hypothetical protein	-8.97
Pden_1259 periplasmic solute binding protein	-8.70
Pden_0033 binding-protein-dependent transport system inner membrane protein	-8.54

Table 2: Target genes of sRNA11 found on chromosome 2. Including the energy value between sRNA and target genes as well as any changes in expression of targets between FNR family mutant strains and wildtype *P. denitrificans.*

Chromosome 2	**Energy**
lpxC (Pden_4486) UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase	-13.11
Pden_2958 BadM/Rrf2 family transcriptional regulator	-12.29
Pden_4321 cytochrome-c oxidase	-11.02
Pden_3398 twin-arginine translocation pathway signal	-10.75
Pden_3297 heavy $_{metal}$ translocating P-type ATPase	-10.15
Pden_4398 FAD linked oxidase domain-containing protein	-9.73
Pden_4169 sulfonate/nitrate transport system substrate-binding protein	-9.53
Pden_4201 TonB-dependent heme/hemoglobin receptor family protein	-9.42
Pden_2943 RND efflux system outer membrane lipoprotein	-9.26
Pden_3587 hypothetical protein	-9.15
Pden_3933 AraC family transcriptional regulator	-8.91
Pden_3164 DNA methylase N-4/N-6 domain-containing protein	-8.44

Table 3: Target genes of sRNA11 found on the plasmid. Including the energy value between sRNA and target genes as well as any changes in expression of targets between FNR family mutant strains and wildtype *P. denitrificans.*

Plasmid	**Energy**
Pden_4854 hypothetical protein	-10.84
Pden_5040 glycosyl transferase, group 1	-10.42
Pden_4775 hypothetical protein	-9.66
Pden_4631 major facilitator transporter	-8.82
Pden_4816 TRAP C4-dicarboxylate transport system permease DctM subunit	-8.72
Pden_4614 hypothetical protein	-8.54

Table 4: Target genes of sRNA18 found on chromosome 2. Including the energy value between sRNA and target genes as well as any changes in expression of targets between FNR family mutant strains and wildtype *P. denitrificans.*

Chromosome 2	**Energy**
Pden_3255 Cupin 2, conserved barrel domain protein	-17.85
Pden_4274 GntR family transcriptional regulator/ MocR family aminotransferase	-16.46
pyrE orotate phosphoribosyltransferase	-16.10
Pden_4394 hypothetical protein	-14.57
Pden_3816 F0F1 ATP synthase subunit alpha	-13.38
Pden_3778 metallophosphoesterase	-12.11
Pden_4226 UbiD family decarboxylase	-11.39
Pden_2945 exopolysaccharide synthesis, ExoD	-10.48
Pden_3802 binding-protein-dependent transport systems inner membrane component	-10.06
Pden_3944 GntR family transcriptional regulator	-9.91
Pden_3208 hypothetical protein	-9.65
Pden_3198 conjugal transfer protein TrbJ	-9.34
Pden_4462 glutamine synthetase, type 1	-9.33
Pden_3866 disulphide bond formation protein DsbB	-9.26
Pden_4268 ABC transporter	-8.89
Pden_4365 AraC family transcriptional regulator	-8.51

Table 5: Target genes of sRNA18 found on the plasmid. Including the energy value between sRNA and target genes as well as any changes in expression of targets between FNR family mutant strains and wildtype *P. denitrificans.*

Plasmid	**Energy**
Pden_5016 Serine O-acetyltransferase	-14.69
Pden_4998 ABC transporter related	-14.01
Pden_4748 ABC transporter related	-12.11
Pden_4904 bifunctional aldehyde dehydrogenase	-10.81
Pden_4610 FAD dependent oxidoreductase	-9.91
Pden_4582 hypothetical protein	-9.13
Pden_4850 ABC transporter related	-8.56

Table 6: Target genes of sRNA31 found on chromosome 2. Including the energy value between sRNA and target genes as well as any changes in expression of targets between FNR family mutant strains and wildtype *P. denitrificans.*

Chromosome 2	**Energy**
Pden_3891 pyruvate dehydrogenase subunit beta	-16.46
Pden_4145 hypothetical protein	-14.83
Pden_3026 hypothetical protein	-13.89
Pden_4214 ApbE family lipoprotein	-11.81
Pden_2890 hypothetical protein	-11.68
Pden_3841 helicase	-10.79
Pden_3275 thiamine pyrophosphate binding domain-containing protein	-10.66
Pden_4083 NAD-dependent DNA ligase	-10.40
Pden_3426 LamB/YcsF family protein	-10.31
Pden_4337 hypothetical protein	-10.09
Pden_3416 acetamidase/formamidase	-9.44
Pden_4347 radical SAM domain-containing protein	-9.31
Pden_3705 extracellular solute-binding protein	-9.24
Pden_3958 uroporphyrin-III C/tetrapyrrole methyltransferase	-9.11
Pden_4356 cytochrome B561	-8.96
Pden_3670 HflK protein	-8.80
Pden_3999 surface antigen (D15)	-8.79
Pden_3991 helix-turn-helix domain-containing protein	-8.70
Pden_3830 CopG/Arc/MetJ family transcriptional regulator	-8.69

Table 7: Target genes of sRNA31 found on the plasmid. Including the energy value between sRNA and target genes as well as any changes in expression of targets between FNR family mutant strains and wildtype *P. denitrificans.*

Plasmid	**Energy**
Pden_4668 UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase	-15.05
Pden_4851 BadM/Rrf2 family transcriptional regulator	-11.99
Pden_5019 cytochrome-c oxidase	-11.39
Pden_4748 twin-arginine translocation pathway signal	-11.30
Pden_4683 heavy metal translocating P-type ATPase	-10.24
Pden_4952 FAD linked oxidase domain-containing protein	-9.81
Pden_4757 sulfonate/nitrate transport system substrate-binding protein	-9.09
Pden_4788 TonB-dependent heme/hemoglobin receptor family protein	-8.94
Pden_4907 RND efflux system outer membrane lipoprotein	-8.90
Pden_5030 hypothetical protein	-8.84
Pden_4524 AraC family transcriptional regulator	-8.47

Table 8: Target genes of sRNA36 found on chromosome 2. Including the energy value between sRNA and target genes as well as any changes in expression of targets between FNR family mutant strains and wildtype *P. denitrificans.*

Chromosome 2	**Energy**
Pden_3492 4-carboxymuconolactone	-15.51
Pden_2983 pseudoazurin	14.70
Pden_3561 transposase IS116/IS110/IS902 family protein	14.05
Pden_4461 nitrogen regulatory protein P-II	-13.82
Pden_3957 hypothetical protein	-13.73
Pden_4274 GntR family transcriptional regulator	-11.89
Pden_2836 adenine deaminase	-11.53
Pden_4241 Bcr/CflA subfamily drug resistance transporter	-10.46
Pden_2845 glutamate-cysteine ligase	-10.26
Pden_3854 hypothetical protein	-8.99
Pden_4123 small GTP-binding protein	-8.87
Pden_3933 AraC family transcriptional regulator	-8.49
Pden_4036 rod shape-determining protein RodA	-8.41
Pden_3319 hypothetical protein	-8.29

Table 9: Target genes of sRNA36 found on the plasmid. Including the energy value between sRNA and target genes as well as any changes in expression of targets between FNR family mutant strains and wildtype *P. denitrificans.*

Plasmid	**Energy**
Pden_5127 Fis family transcriptional regulator	-13.84
Pden_5002 rhodanese domain-containing protein	-10.03
Pden_4919 methionine aminopeptidase	-9.85
Pden_5103 poly-beta-hydroxybutyrate polymerase domain-containing protein	-9.65
Pden_4894 FAD dependent oxidoreductase	-8.98
Pden_5125 monooxygenase	-8.48
Pden_4681 TetR family transcriptional regulator	-8.46
Pden_5051 polysaccharide deacetylase	-8.38

Table 10: Target genes of sRNA54 found on chromosome 2. Including the energy value between sRNA and target genes as well as any changes in expression of targets between FNR family mutant strains and wildtype *P. denitrificans.*

Chromosome 2	**Energy**
Pden_3295 heavy metal translocating P-type ATPase	-16.17
Pden_2935 phosphatidate cytidylyltransferase	-14.43
Pden_3810 hypothetical protein	-14.34
Pden_3942 hypothetical protein	-14.27
Pden_3447 2-octaprenyl-6-methoxyphenyl hydroxylase	-12.73
Pden_3242 RND family efflux transporter MFP subunit	-12.15
Pden_3574 aldo/keto reductase	-10.87
Pden_3218 hypothetical protein	-10.65
Pden_3863 beta-lactamase domain-containing protein	-10.64
Pden_4351 hypothetical protein	-10.61

Pden_2947 cytosine/purines uracil thiamine allantoin permease	-10.54
Pden_4223 hypothetical protein	-10.21
Pden_4442 hypothetical protein	-10.03
Pden_4317 extracellular solute-binding protein	-9.74
Pden_4094 cytochrome c oxidase subunit III	-9.70
Pden_3570 hypothetical protein	-9.25
Pden_4316 AmiS/UreI transporter	-9.00
Pden_4162 SURF1 protein	-8.73
Pden_3246 hypothetical protein	-8.55

Table 11: Target genes of sRNA54 found on the plasmid. Including the energy value between sRNA and target genes as well as any changes in expression of targets between FNR family mutant strains and wildtype *P. denitrificans.*

Plasmid	**Energy**
Pden_4923 regulatory proteins, IclR	-11.98
Pden_4871 pyrroline-5-carboxylate reductase	-10.36
Pden_4694 FAD-dependent pyridine nucleotide-disulphide oxidoreductase	-9.75
Pden_4663 hypothetical protein	-9.70
Pden_4892 MmgE/PrpD family protein	-9.69
Pden_4966 hypothetical protein	-8.99
Pden_4771 inner-membrane translocator	-8.58

Table 12: Target genes of sRNA79 found on chromosome 1. Including the energy value between sRNA and target genes as well as any changes in expression of targets between FNR family mutant strains and wildtype *P. denitrificans.*

Chromosome 1	**Energy**
IhfA Integration host factor subunit alpha	-17.86
Pden_2502 hypothetical protein	-17.67
Pden_1041 aspartate dehydrogenase	-16.98
Pden_1988 bifunctional folylpolyglutamate synthase/dihydrofolate synthase	-16.45
Pden_2773 group 1 glycosyl transferase	-15.36
Pden_0243 succinate-semialdehyde dehydrogenase (NAD(P)(+))	-15.31
Pden_0580 hypothetical protein	-14.42
pheT phenylalanyl-tRNA synthase subunit beta	-14.33
Pden_0509 hypothetical protein	-13.38
Pden_1829 hypothetical protein	-12.65
Pden_0901 YjgP/YjgQ family permease	-12.30
Pden_0198 butyryl-CoA dehydrogenase	-12.18
Pden_1536 hypothetical protein	-11.99
Pden_1313 hypothetical protein	-11.93
Pden_2525 cobalt transporter subunit CbtA	-11.28
Pden_0351 hypothetical protein	-10.90
Pden_0998 hypothetical protein	-10.64
Pden_0604 hypothetical protein	-10.53
Pden_1215 activator of Hsp90 ATPase 1 family protein	-10.46
Pden_2029 methyltransferase small	-10.37

Pden_0524 purine nucleoside phosphorylase	-10.36
Pden_0463 recombinase	-10.23
Pden_2134 hypothetical protein	-9.97
Pden_0195 L-carnitine dehydratase/bile acid-inducible protein F	-9.70
Pden_0675 aminohydrolase	-9.64
Pden_0340 hypothetical protein	-9.36
Pden_0846 lipopolysaccaride biosynthesis protein	-9.15
Pden_0690 ATP-dependent metalloprotease FtsH	-8.92
Pden_1915 hypothetical protein	-8.55
Pden_1267 NADH dehydrogenase	-8.30

Table 13: Target genes of sRNA79 found on chromosome 2. Including the energy value between sRNA and target genes as well as any changes in expression of targets between FNR family mutant strains and wildtype *P. denitrificans.*

Chromosome 2	**Energy**
Pden_4169 sulfunate/nitrate transport system substrate-binding protein	-15.58
Pden_4147 SoxS	-15.39
Pden_4422 alkylhydroperoxidase	-15.16
Pden_3541 short-chain dehydrogenase/reductase SDR	-14.44
Pden_3255 cupin	-13.69
Pden_3138 hypothetical protein	-13.39
Pden_2846 hypothetical protein	-13.23
proA gamma-glutamyl phosphate reductase	-13.02
Pden_2841 hypothetical protein	-12.98
Pden_4123 small GTP-binding protein	-12.90
Pden_3184 hypothetical protein	-12.10
Pden_3340 arsenical-resistance protein	-11.37
Pden_4224 hypothetical protein	-11.28
Pden_3243 aldo/keto reductase	-11.18
Pden_3515 allantoate amidohydrolase	-11.01
Pden_2855 formate dehydrogenase family accessory protein FdhD	-10.99
hemC porphobilinogen deaminase	-10.97
Pden_4036 rod shape-determining protein RodA	-10.46
Pden_3812 ATP-dependent Clp protease ATP-binding protein ClpA	-10.45
Pden_3801 binding –protein-dependent transport system inner membrane protein	-10.40
Pden_3848 hypothetical protein	-9.96
Pden_3099 HupE/UreJ protein	-9.17
Pden_4112 L-carnitine dehydrogenase/bile acid-inducible protein F	-8.70

Table 14: Target genes of sRNA79 found on the plasmid. Including the energy value between sRNA and target genes as well as any changes in expression of targets between FNR family mutant strains and wildtype *P. denitrificans.*

Plasmid	**Energy**
Pden_5127 Fis family transcriptional regulator	-15.21
Pden_4724 nucleoside diphosphate kinase regulator	-11.71

Pden_4798 phenylacetic acid degradation protein paaN	-11.64
Pden_4730 methylamine dehydrogenase heavy chain	-11.14

Table 15: Target genes of sRNA107 found on chromosome 1. Including the energy value between sRNA and target genes as well as any changes in expression of targets between FNR family mutant strains and wildtype *P. denitrificans.*

Chromosome 1	**Energy**
Pden_1958 hypothetical protein	-16.36
Pden_1541 parB-like partition proteins	-14.78
Pden_1217 hypothetical protein	-11.87
Pden_1067 OsmC family protein	-11.36
Pden_0387 hypothetical protein	-11.35
Pden_0072 CHAD domain-containing protein	-10.95
Pden_0587 UDP-N-acetylmuramoylalanyl-D-glutamyl-2,6-diaminopimelate--D-alanyl-D-alanyl ligase	-10.85
clpP (Pden_1265) ATP-dependent Clp protease proteolytic subunit	-10.68
Pden_0192 short-chain dehydrogenase/reductase SDR	-10.68
Pden_0633 thiamine pyrophosphate protein	-10.62
Pden_1780 RND family efflux transporter MFP subunit	-10.38
Pden_2696 hypothetical protein	-10.24
Pden_2276 pyruvate kinase	-10.19
Pden_0495 response regulator receiver protein	-9.53
Pden_1195 MarR family transcriptional regulator	-9.52
Pden_2540 precorrin-4 C(11)-methyltransferase	-9.43
Pden_2643 hypothetical protein	-9.35
Pden_0935 glyoxalase/bleomycin resistance protein/dioxygenase	-8.75
Pden_0109 AraC family transcriptional regulator	-8.73
Pden_0258 hypothetical protein	-8.67
rpsR (Pden_0890) 30S ribosomal protein S18	-8.50

Table 16: Target genes of sRNA107 found on chromosome 2. Including the energy value between sRNA and target genes as well as any changes in expression of targets between FNR family mutant strains and wildtype *P. denitrificans.*

Chromosome 2	**Energy**
Pden_4174 hypothetical protein	-14.26
Pden_3408 parB-like partition proteins	-13.86
Pden_3462 hypothetical protein	-13.36
Pden_3919 OsmC family protein	-13.31
Pden_3652 hypothetical protein	-11.92
Pden_4206 CHAD domain-containing protein	-10.78
Pden_4428 UDP-N-acetylmuramoylalanyl-D-glutamyl-2,6-diaminopimelate--D-alanyl-D-alanyl ligase	-10.72
Pden_2922 ATP-dependent Clp protease proteolytic subunit	-10.70
guaA (Pden_3696) short-chain dehydrogenase/reductase SDR	-10.69
Pden_3650 thiamine pyrophosphate protein	-8.99
Pden_3290 RND family efflux transporter MFP subunit	-8.81
Pden_3314 hypothetical protein	-8.44

Table 17: Target genes of sRNA107 found on the plasmid. Including the energy value between sRNA and target genes as well as any changes in expression of targets between FNR family mutant strains and wildtype *P. denitrificans.*

Plasmid	**Target**
Pden_5040 hypothetical protein	-17.45
Pden_5101 parB-like partition proteins	-13.52
Pden_4645 hypothetical protein	-12.57
Pden_4986 OsmC family protein	-12.17
Pden_4531 hypothetical protein	-10.47
Pden_4852 CHAD domain-containing protein	-10.27
Pden_4695 UDP-N-acetylmuramoylalanyl-D-glutamyl-2,6-diaminopimelate--D-alanyl-D-alanyl ligase	-10.24
Pden_4519 ATP-dependent Clp protease proteolytic subunit	-9.85
Pden_4909 short-chain dehydrogenase/reductase SDR	-9.35
Pden_5117 thiamine pyrophosphate protein	-8.87
Pden_4723 RND family efflux transporter MFP subunit	-8.58