Transcriptional Regulation of Bacterial Nitrous Oxide Emissions

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A Thesis submitted for the degree of Doctor of Philosophy

School of Biological Sciences

University of East Anglia

July 2024

Abstract

Nitrous oxide (N_2O) is a potent greenhouse gas and a major threat to the ozone layer. It is primarily produced during the final stage of microbial denitrification through the enzymatic reduction of nitric oxide (NO). This process mainly happens in terrestrial environments, with agricultural soils being dominant N_2O emitters due to the large quantities of nitrogen added as fertilisers. These fertilisers, containing ammonium and nitrate, provide soil microorganisms with sufficient nitrogen species to denitrify at an elevated rate. Each stage of denitrification requires unique enzymes that have been well-characterised, however, much less research has been done on the regulation of gene expression for each process. Further investigating transcriptional regulation could help unearth why incomplete denitrification occurs.

Small RNAs (sRNAs) are small, non-coding RNA molecules which posttranscriptionally regulate gene expression, yet their role in regulating the denitrification pathway is not well understood. Recent studies have identified sRNAs and their chaperones as potentially crucial to the pathway's regulation. In this study, we used the model soil denitrifier *Paracoccus denitrificans* to characterise the role of two sRNAs and the RNA chaperone Hfq on N_2O emissions when overexpressed under denitrifying conditions. The overexpression of these regulators caused varying impacts on N_2O production and consumption. Overexpression of *P. denitrificans*' sRNA11 resulted in an 80% reduction in N_2O emissions and a significantly impaired cell growth rate. Hfq overexpression also substantially reduced N_2O emissions, showing a potential overarching role of Hfq in sRNA denitrification regulation. sRNA Pda200 overexpression also decreased N_2O emissions, experimentally confirming hypotheses from previous studies.

These findings show that the sRNAs and chaperone examined here are relevant to the denitrification pathway in *P. denitrificans*. Therefore, sRNAs and their chaperones should be further considered in environmental bacteria when developing strategies to reduce N_2O emissions.

Acknowledgements

First of all, I will start by thanking the other students I worked with at UEA for adding a good atmosphere and much fun to my PhD; Sophie, Anabela, and Dan from our lab and Colleen, Joe and Hayley from the next-doors lab. Especially for the ease of chatting with when anything was not going as hoped. Also to ARIES for their funding and administrative support throughout. As well as to Gary for his understanding approach of supervision throughout and unwavering support of my ability to put this document together, even if I would need every last day.

To my friends from home; Jas, Mike, Stu, Josh and Henry, you all gave me what I have needed most these last 5 years, a place to go where the stress of this endeavour could not follow me. Also the ego boost of feeling like the smartest in the room whenever we would get a chance to get together!

My housemates Liv, Jack and Jo for being there every day that I would need them and to give me the sympathy I was looking for when heading into the lab at midnight for months on end.

The Aye-Aye frisbee team for the fun and memories of all we achieved together. I will never be able to think of this degree without remembering how important everyone I played with was to my time in Norwich.

To my sister Annette, and Rich, who kept me somewhat sane through lockdown, and to my amazing niece Evie who never failed to put a smile on my face even when times were tough.

And finally, to the best thing to come out of the PhD, my incredible partner Soph, without whom I would likely never have submitted my thesis, thank you endlessly for helping me see how much I wanted this and giving me the inspiration and support to do it, even if I never did stop complaining.

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ABC	ATP-binding cassette.
ANR	Anaerobic regulator of arginine deiminase and nitrate reductase.
ATP	Adenosine triphosphate.
BLAST	Basic local alignment search tool.
CFU	Colony forming units.
DNA	Deoxyribonucleic acid.
DNRA	Dissimilatory nitrate reduction to ammonium.
DOH	Doxycycline hydrochloride.
ECD	Electron capture detection.
eDNA	Environmental DNA.
EMSA	Electrophoretic mobility shift assay.
eukNR	Eukaryotic nitrite reductase.
FnrP	Fumarate and nitrate reduction protein.
FQ	Flumequine.
GC	Gas chromatograph.
GDS	Glutamate decarboxylase system.
GHG	Greenhouse gas.
IPTG	Isopropyl B-D-1-thiogalactopyranoside.
LB	Luria-Bertani.
lncRNA	Long non-coding RNA.
MAPs	Microtubule-associated proteins.

mRNA	Messenger RNA.
Nap	Periplasmic nitrite reductase.
Nar	Membrane-bound nitrite reductase.
NarR	Nitrate reductase regulator.
Nas	Assimilatory nitrite reductase.
NCBI	National Center for Biotechnology Information.
Nif	Nitrogenase enzyme complex.
Nir	Nitrite reductase.
NNR	Nitrite reductase and nitric oxide reductase regulator.
Nor	Nitric oxide reductase.
NS	Neomycin sulfate.
OD	Optical density.
OMV	Outer membrane vesicle.
PCR	Polymerase chain reaction.
ppm	Parts per million.
PSI	Pound per square inch.
RFM	Random forest model.
RNA	Ribonucleic acid.
RNS	Reactive nitrogen species.
ROS	Reactive oxygen species.
RPM	Revolutions per minute.
SM	Sulfadimidine.
sRNA	Small RNA.
TBE	Tris-borate EDTA.
TCA	Tricarboxylic acid.
UTR	Untranslated region.
UV-Vis	Ultraviolet–visible.

WMO	World Meteorological Organisation.
WT	Wild-type.

Contents

1 Introduction

1.1 Introduction to the nitrogen cycle

Nitrogen is the most abundant gas on our planet, making up 78% of the Earth's atmospheric gases. The nitrogen cycle is a collection of natural processes whereby nitrogen, an essential element for all life, is converted between many chemical forms via various biological, chemical and physical processes (Stein and Klotz, 2016). Each of these chemical forms is a molecule that contains nitrogen in a different oxidation state. Ranging from atomic charges of -3 to 5 allows for nitrogen to exist in many forms whilst still forming stable molecules. Nitrogen is needed for the production of amino acids, nucleic acids, proteins and ATP, all of which are considered the essential building blocks of biology. As such all life requires some aspect of nitrogen retrieval to survive. These various stable nitrogen-based molecules are reduced, oxidised or assimilated by various organisms to either harness the potential energy held within the molecule (dissimilatory), or to make use of it as a building block (assimilatory). Understanding how nitrogen is altered and subsequently moved through various stages of its biogeochemical cycle is critical to being able to later consider how the transcriptional regulation of the nitrogen cycle functions. Because of its crucial role in regulating primary productivity, it is arguably the most important nutrient cycle (Vitousek et al., 2002). Here we will present an overview of the nitrogen cycle and its key pathways to provide context for the research presented in later chapters.

The nitrogen cycle is defined by seven key processes: nitrogen fixation, nitrification, assimilation, ammonification, anammox, dissimilatory nitrate reduction to ammonium (DNRA) and denitrification (Fig. 1.1). These manipulate the oxidation states of nitrogen as it moves through various forms of biological matter. Together, they direct the flow of nitrogen within the Earth's systems such as the atmosphere, biosphere, hydrosphere and lithosphere. Overall, these form the global nitrogen budget, which defines how nitrogen inputs and outputs balance between these systems. Understanding the budget is crucial when assessing how anthropogenic activities are impacting the nitrogen cycle, and to develop strategies for managing nitrogen in industrialised environments such as agriculture.

The budget is made up of reservoirs of varying nitrogen species, each having sources and sinks determining their size. The largest reservoir of nitrogen as mentioned earlier is in the atmosphere, where $\sim 4 \times 10^9$ Tg N is stored in the form of dinitrogen gas (N_2) (Sorai et al., 2007). The next largest reservoir is from near-surface geological formations such as the Earth's crust and ocean sediments, these combine to form a reservoir of approximately $\sim 2 \times 10^9$ Tg N (Wedepohl, 1995). Nitrogen here is in an organic form within sedimentary rocks or as ammonium in silicate minerals. Beyond these, nitrogen is also stored in the oceans, freshwater systems and in soils. Generally, these reservoirs are expected to remain stable, with changes only perceived over geologically relevant time periods. However, anthropogenic activities have led to fluctuations within the nitrogen budget, significantly impacting nitrogen availability, ecosystem health and

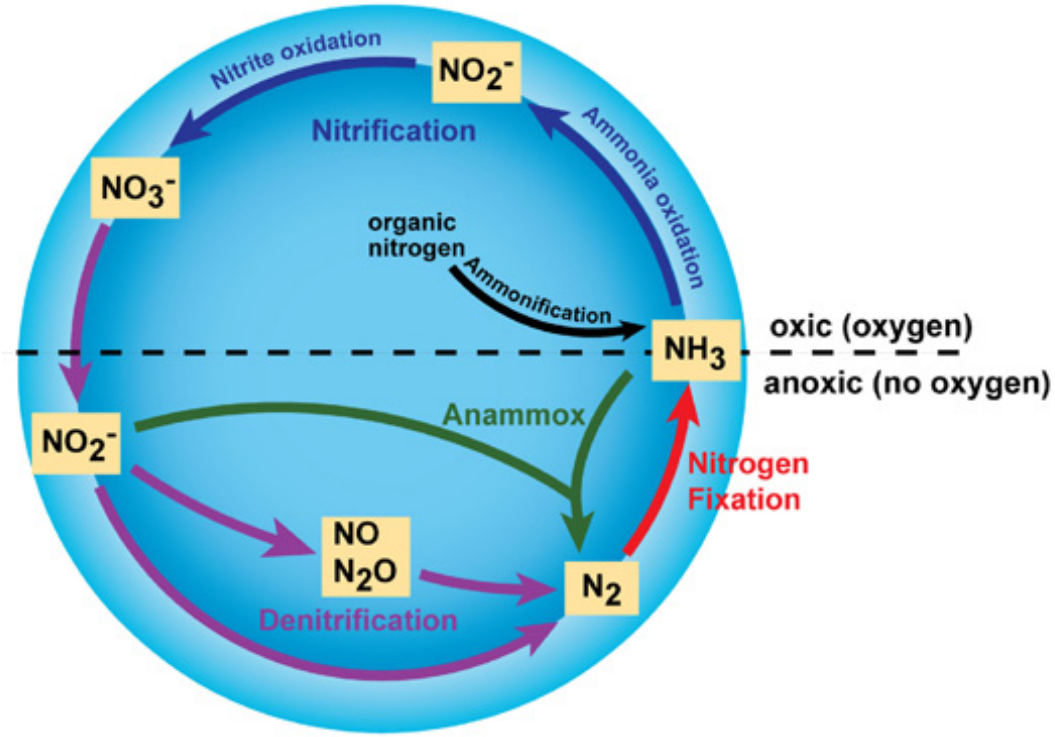

Figure 1.1: The major pathways of the nitrogen cycle (arrows) and the nitrogen species produced by an entering pathway (squares). The top hemisphere includes processes that occur in oxic environments whilst the bottom hemisphere requires anoxic conditions. Figure taken from Bernhard (2010).

global warming (Schlesinger, 2009). To fully recognise how anthropogenic activities are impacting the nitrogen cycle, we first need to understand how they naturally function.

1.2 Key processes of the nitrogen cycle

1.2.1 Nitrogen fixation

Nitrogen fixation is a chemical process which converts the inert gas dinitrogen (N_2) into ammonia (NH_3), a bioavailable nitrogen form which organisms can assimilate. This step of the nitrogen cycle is crucial for incorporating atmospheric nitrogen into biological systems, supporting microbial and plant growth. The biological form of nitrogen fixation is exclusively carried out by bacteria and archaea, living in symbiosis with plant roots or as free-living bacteria. To complete the process of converting N_2 to NH_3, the reaction must be catalysed by the enzyme nitrogenase. The reaction for biological nitrogen fixation is shown in Equation 1.

$$N_2 + 16\,ATP + 16\,H_2O + 8\,e^- + 8\,H^+ \longrightarrow 2\,NH_3 + H_2 + 16\,ADP + 16\,P_i \tag{1}$$

N_2, adenosine triphosphate (ATP), water, electrons and protons are catalysed by nitrogenase to form ammonia (NH_3), hydrogen gas, and ATP derivatives. The process of biological nitrogen fixation is usually happening within topsoil, where microorganisms living on plant roots can interface with the atmosphere. This process is strongly influenced by soil conditions such as temperature, moisture, pH, and oxygen content as well as other factors (Mohammadi, 2012).

The reduction of N_2 to NH_3 is a remarkable biological achievement, considering the stability of the triple bond holding together N_2. To break down N_2, the nitrogenase enzyme complex (Nif) is made of two metalloproteins, dinitrogenase reductase (Fe-protein) and the α-subunit dinitrogenase (MoFe-protein), which work together to catalyse this stage of the nitrogen cycle (Eady, 1996). These proteins are encoded by the *nifH* and *nifD* genes respectively. The *nifH* gene is often used as the biological marker for determining whether a microorganism can fix nitrogen. This is primarily due to the *nifH* gene sequence being well conserved across species, but it is also the most thoroughly studied gene of the *nif* operon (Dedysh et al., 2004). The *nifH* gene has been found in a wide range of environments, from marine sediments to Antarctica (Zehr et al., 1995; Eckford et al., 2002), however, it is most commonly found in forest and agricultural soils (Ueda et al., 1995; Shaffer et al., 2000). This is likely due to the symbiotic nature of most nitrogen-fixing bacteria, whereby they provide bioavailable N in the form of NH_3 to the plants in these environments and in return, receive carbon compounds such as malate (Khamar et al., 2010), and crucial mineral nutrients such as phosphorus and sulphur (Al-Niemi et al., 1997; Krusell et al., 2005). Known as endophytic diazotrophs, these bacteria reside within the roots of

their host plants where competition is reduced, exchanging nutrients for mutual benefit.(James et al., 2000).

Alongside biological nitrogen fixation, there is also a pathway which can convert N_2 to NH_3. This derives from an anthropogenic source known as the Haber-Bosch process (Haber and Bosch, 1905).

$$N_2 + 3H_2 \longrightarrow 2NH_3 \tag{2}$$

The Haber-Bosch process describes a relatively simplistic reaction allowing for the synthesis of NH_3 from N_2 and H_2 (Eq. 2). It requires high temperature and pressure, as well as an iron metal catalyst. Its discovery revolutionised agriculture and industry as it simplified the large-scale production of ammonia, a crucial ingredient in fertilisers and ensured sufficient crops could be grown to feed billions of people (Erisman et al., 2008). It is now estimated that the Haber-Bosch process is responsible for the production of approximately 120×10^9 kg ammonia per year (Razon, 2018). In comparison, non-anthropogenic sources account for 203×10^9 kg. This industrial process for producing fertiliser allowed global agriculture to dramatically increase productivity, enhancing crop yields and allowing for the feeding of much larger populations.

Overall, nitrogen fixation is responsible for fixing the abundant atmospheric nitrogen into a bioavailable form that can be used by plants. We have described the primary route of this process; the reactions both biotic and abiotic that turn N_2 to NH_3, however, there are other ways to fix atmospheric nitrogen, such as the energy produced from a lightning strike, breaking the bonds of dinitrogen and forming nitrate which in turn falls to the Earth as rain (Section 1.2.3).

1.2.2 Ammonification

Ammonification is the process by which organic nitrogen from dead organisms and waste products is converted into either ammonia (NH_3^+) or ammonium (NH_4^+). It is a key process of the nitrogen cycle as it facilitates the recycling of nitrogen from organic matter back into a form more readily available within ecosystems. This natural process fertilises the soil and increases ecosystem productivity when efficiently completed. Ammonification is completed by bacterial or fungal decomposer microorganisms, which break down organic matter. There are many types of enzymes responsible for this as it involves the recycling of proteins, nucleic acids and other nitrogenous compounds into ammonia. Proteases, nucleases and deaminases are the main catalysts of these reactions, with decomposers able to produce some or all of these enzymes. Amongst bacteria, genera like *Bacillus*, *Clostridium*, *Pseudomonas* and *Proteus* are the most thoroughly researched for their diverse range of ammonifying enzyme activity. Whilst fungi such as *Aspergillus* and *Penicillium* are found to focus mostly on proteases and ureases (Tiquia et al.,

2002; Hao et al., 2008). Unsurprisingly, these decomposers are found to be most abundant where dead organic matter or waste products build up. This makes them most common in habitats such as livestock manure, however, due to their role, they are found in the same locations that macro-organisms are, making them some of the most ubiquitous microorganisms on Earth.

Ammonification as a process is influenced by environmental conditions such as temperature, pH, moisture and oxygen availability. Unlike nitrogen fixation, ammonification is most efficient in oxic environments, as well as being enhanced in soils of pH 4.5 - 5.5 (Ste-Marie and Paré, 1999). pH is the determining factor of whether this process will form NH_3 or NH_4^+, as under alkaline conditions the equilibrium between the two forms shifts towards the formation of NH_3 (Eq. 3).

$$NH_3 + H^+ \longleftrightarrow {NH_4}^+ \tag{3}$$

This is because at higher pH levels there are fewer protons available, which are required to convert ammonia into ammonium. Therefore, under acidic to neutral conditions (pH <7), ammonium is the primary molecule of the pair. Depending on the pH and other conditions such as temperature this equilibration will balance differently, meaning that whenever NH_3 is nitrified and converted to other molecules, some NH_4^+ will convert to NH_3 to maintain the balance.

1.2.3 Nitrification

Nitrification is a microbial process by which ammonia is sequentially oxidised to (NO_2^-) and then to (NO_3^-). This pathway usually requires aerobic conditions (Di et al., 2013) and is carried out by both ammonia-oxidising bacteria and archaea, which can carry out the first steps of nitrification, the oxidation of ammonia to nitrite. These ammonia-oxidisers initially form hydroxylamine as an intermediate (Eq. 4), which is then catalysed by the ammonia monooxygenase enzyme to form NO_2^- (Eq. 5). Only certain bacterial species can complete the oxidation of NO_2^- to NO_3^- as it requires the hydroxylamine oxidoreductase enzyme (Eq. 6).

$$NH_3 + O_2 + 2\,e^- \longrightarrow NH_2OH + H_2O \tag{4}$$

$$NH_2OH + H_2O \longrightarrow {NO_2}^- + 5\,H^+\,4\,e^- \tag{5}$$

$$2\,{NO_2}^- + O_2 \longrightarrow 2\,{NO_3}^- \tag{6}$$

Nitrification is an extremely important biogeochemical process as the production of nitrite and nitrate is a staple source of nitrogen for most of the Earth's plant life. The process of oxidising these compounds is not very energy-rich for the cells resulting in nitrifying bacteria needing to process many molecules of ammonia to power cellular functions. As shown in Equations 4-6 these reactions are highly dependent on oxygen availability, and therefore will not occur in

anoxic environments. The ideal nitrifying conditions require an O_2-rich environment, a pH of 7.3 to 8 and a temperature of 20 °C, assuming there is sufficient decompostable organic matter available (Amatya et al., 1970). This is because ammonia oxidation is generally less efficient than in neutral or slightly alkaline soils due to acidic conditions limiting most ammonia oxidisers' activity (Boer and Kowalchuk, 2000). Although several nitrifying bacterial and archaeal species have been identified to prefer acidic soils, these are the minority (He et al., 2012). The nitrification process also lowers pH as hydrogen ions are released during the oxidation of hydroxylamine, resulting in acidification (Equation 5).

Nitrification is the only biotic source of nitrate, however, nitrate can also enter the nitrogen cycle from another natural source, the atmospheric fixing of nitrogen by lightning. This process requires lightning with high enough energy and heat to break the strong triple bonds holding together N_2 as well as a supply of oxygen (Tuck, 1976). One flash of lightning is predicted to form on average 3.5 kg N (Schumann and Huntrieser, 2007). Once the N_2 molecules are split, the nitrogen and oxygen atoms act as free radicals, binding together and forming a variant of nitrous oxide (NO_x). These compounds are of no use to plants, however, once the molecule cools it can react with oxygen to form nitrous dioxide, which can then react with water to form nitrous, or nitric acid (Levine et al., 1984). These acids then seep into substrates and degrade into nitrate, a process which is seen as being of more relevance to nitrogen cycling over oceans than on land (Gallardo and Rodhe, 1997).

The continued production of nitrite and nitrates by these processes is essential to the final pathway of the nitrogen cycle, denitrification. The impacts of the previously mentioned Haber-Bosch process are not only seen in the nitrogen fixation stage of the cycle but continue throughout, leading to drastic impacts on climate change due to the global warming potential of the intermediate, nitrous oxide.

1.2.4 Anammox

Bacteria able to perform the anammox pathway, which is anaerobic ammonium oxidation, are capable of converting ammonium and nitrite to form nitrogen gas. Common in marine environments and first discovered in wastewater (Mulder et al., 1995), anammox bacteria are often slow-growing and complex to culture (Kartal et al., 2012). It is estimated that of the dinitrogen gas released into the environment, 50% of this was produced through the anammox process (Arrigo, 2005). Equation 7 describes the production of anammox gas.

$$NH_4{}^+ + NO_2{}^- \longrightarrow N_2 + 2\,H_2O \tag{7}$$

1.2.5 DNRA

The dissimilatory nitrate reduction to ammonium (DNRA), is similar to denitrification with the first step being the reduction of nitrate to nitrite, however, this is followed by the reduction to ammonium, rather than nitric oxide. The *nrfA* gene is often used as the molecular marker for DNRA activity in a community, as the *nirB* gene which can also reduce nitrite to ammonium can also mediate the assimilatory reduction of nitrite (Wang et al., 2018; Pandey et al., 2020). The two formulas which describe the DNRA pathway are shown in Equations 8 & 9.

$$NO_3{}^- + 2e^- + 2H^+ \longrightarrow NO_2 + H_2O \tag{8}$$

$$NO_2{}^- + 6e^- + 8H^+ \longrightarrow NH_4{}^+ + 2H_2O \tag{9}$$

1.3 Denitrification

Denitrification is the reduction of nitrate through a stepwise pathway to N_2. This process is performed by a wide range of microorganisms, often when under anaerobic conditions thus allowing the utilisation of these nitrogen intermediates as alternative electron acceptors. Prokaryotes are seen as the primary facilitator of microbial denitrification, whilst archaea (Cabello et al., 2004), and even less so eukaryotes (Woehle et al., 2018), also contribute to this pathway.

Denitrification begins with the reduction of NO_3^- to NO_2^-, catalysed by the nitrate reductase (Nar/Nap) enzyme (Eq. 10). NO_2^- is then catalysed by nitrite reductase (Nir) to form nitric oxide (NO) (Eq. 11). NO reduces to nitrous oxide (N_2O) when catalysed by nitric oxide reductase (Nor) (Eq. 12). Lastly, N_2O is catalysed by the nitrous oxide reductase (NosZ) enzyme to form N_2 (Eq. 13).

$$2NO_3{}^- + 4H^+ + 4e^- \xrightarrow{\text{Nar/Nap}} 2NO_2{}^- + 2H_2O \tag{10}$$

$$2NO_2{}^- + 4H^+ + 2e^- \xrightarrow{\text{Nir}} 2NO + 2H_2O \tag{11}$$

$$2NO + 2H^+ + 2e^- \xrightarrow{\text{Nor}} N_2O + H_2O \tag{12}$$

$$N_2O + 2H^+ + 2e^- \xrightarrow{\text{Nos}} N_2 + H_2O \tag{13}$$

1.3.1 Nitrate reductase

Focusing on the first stage of this pathway, the reduction of nitrate to nitrite brings the primary focus on nitrate reductases. There are four main groups of nitrate reductases, all of which are molybdenum dependent: eukNR, Nas, Nap and Nar. eukNR (eukaryotic nitrate reductase) is the only known nitrate reductase found in eukaryotic organisms, identified in both plants and

fungi (Slot and Hibbett, 2007). Nas, (assimilatory nitrate reductases) are found in both bacteria and archaea and form an anabolic pathway, reducing NO_3^- to later transform the subsequent NO_2^- to form and assimilate the produced NH_4^+. Conversely, the Nar enzyme, which is bound to the cytoplasm-facing side of the cytoplasmic membrane, and the Nap enzyme, which reduces NO_3^- in the periplasm rather than the cytoplasm, are both used for dissimilatory purposes in the cell. However, this is slightly different in Archaea, where Nar is instead facing the periplasm (Martinez-Espinosa et al., 2007). This involves reducing NO_3^- not to incorporate the nitrogen into the cell, but instead following a catabolic pathway to use the NO_3^- as a source of energy and ATP generation. Nar is found in both gram-positive and gram-negative bacteria, whilst Nap is absent in gram-positive microorganisms as they lack a periplasmic space. Both the eukNR and Nas are cytoplasmic proteins, as the cytoplasm is where biosynthesis is predominantly carried out by dissimilatory nitrate reductases.

The Nar enzyme is encoded for by the *nar* operon, which contains *narGHJI*. *narG* is responsible for the synthesis of the nitrate reductase α-subunit. This is the catalytic part of the Nar enzyme and is responsible for reducing NO_3^- to NO_2^-. Leading up to reduction, nitrate is drawn to NarG due to it containing a molybdenum cofactor. *narH* encodes the β-subunit of Nar and acts as the electron transfer component for the enzyme. This protein contains an iron-sulfur cluster that facilitates its role by transferring electrons to NarG. *narJ* encodes for a chaperone protein which is essential in the proper assembly and maturation of the Nar complex. NarJ does this by inserting the molybdenum cofactor into NarG and ensuring the enzyme is correctly folded and functional. *narI* encodes the gamma subunit of the Nar enzyme, anchoring the enzyme complex to the cell membrane and assisting in electron transfer. In addition, there are several regulatory genes closely linked to nitrate and nitrite reduction. In *P. denitrificans*, both *narX* and *narQ* encode for sensor kinases which detect nitrate and nitrite in the environment. When detected, they will phosphorylate NarL and NarP, activating these proteins to regulate the *nar* operon and other related genes (Darwin and Stewart, 1995).

1.3.2 Nitrite reductase

As discussed, the reduction of nitrate is often completed as an alternative source of energy and ATP production, producing nitrite as a byproduct. An issue with this is that nitrite can be toxic and will inhibit bacterial activity at sufficient concentrations (Baumann et al., 1997). Therefore, it is in the interest of the cell to remove nitrite by either expelling it from the cell or reducing it further, forming nitric oxide (NO). To do the latter, bacteria will encode for either NirK or NirS, both of which are nitrite reductase enzymes, however, they differ in their cofactor. NirK is a copper-containing nitrite reductase which is built from three identical subunits which surround the catalytic centre of several copper centres. NirS is a cytochrome cd_1 nitrite reductase formed

of two identical proteins with each subunit containing a *c*-type heme, which functions as the electron acceptor of the enzyme and a d_1 heme, which forms the catalytic centre (Besson et al., 2022). Despite these structural differences, both reductases are functionally and physiologically equivalent (Glockner et al., 1993). Therefore, their differences come primarily from their genetic lineages. *nirS* is most commonly found in bacteria such as *Pseudomonas*, *Paracoccus* and *Shewanella*, whilst *nirK* is typically present in *Alcaligenes*, *Bradyrhizobium* and some strains of *Achromobacter* (Heylen et al., 2006).

1.3.3 Nitric oxide reductase

The production of nitric oxide during this stage of denitrification contains a similar issue to the previous step, in that NO is highly cytotoxic. NO is far more aggressive than NO_2^-, partly due to it being a highly active free radical and oxidant, fitting within the category of reactive nitrogen species (RNS). It is capable of reacting with several biological targets such as superoxide (O_2^-), forming peroxynitrite: an RNS that can oxidise lipids, proteins and DNA (Eiserich et al., 1996; Vliet et al., 1997). NO is such a powerful RNS that it can even cause mitochondrial dysfunction in eukaryotes by binding to and inhibiting the mitochondrial cytochrome *c*-type oxidase, impairing ATP production (Stewart and Heales, 2003). Therefore, much like the reduction of nitrite, cells will benefit from being capable of removing NO as it reduces the likelihood of cellular damage, but also for the ATP generation its reduction to N_2O facilitates.

The third stage of denitrification is the reduction of nitric oxide to nitrous oxide (N_2O), as described in Equation (Eq. 12). This process is primarily catalysed by nitric oxide reductase (Nor), an iron-containing enzyme that is integrated into the cell membrane. There are two main families of Nor enzymes, one being the quinone-dependent Nor (qNor), found in both non-denitrifying pathogens and denitrifiers (Hendriks et al., 2000; Zumft, 2005). Pathogenic species benefit from synthesising qNor as it protects them against NO produced by a host's macrophages during an immune response (Stevanin et al., 2007). qNor is a single subunit enzyme containing a binuclear active site. The other family is cytochrome *c*-dependent Nor (cNor), its active site is structurally similar to qNor however, cNor is formed of two subunits, NorB and NorC. NorC, the smaller of the subunits, is a membrane-anchored cytochrome *c*-type protein, whilst NorB is a *b*-type cytochrome protein (Hendriks et al., 2000). NorB acts as the catalytic subunit and NorC acts as its electron transfer partner, transferring electrons from donor molecules to NorB. In addition, the *nor* operon contains several other genes responsible for the optimal functioning of the Nor enzyme. *norD* encodes for an accessory protein involved in the assembly and stabilisation of the nitric oxide reductase complex. *norE* also encodes for an accessory protein however it is thought to be involved in both the maturation of Nor and the regulation of the *nor* operon. NorQ acts in cooperation with NorD, activating Nor at the protein level (Kahle et al., 2023). The role of the *nor*

operon is to synthesise and ensure the catalytic function of the NorB subunit for the reduction of NO to the greenhouse gas N_2O. This is why this part of the denitrification pathway has been of heightened interest to researchers over the past few decades, as bacterial catalysis of NO by NorB is the main source of the potent greenhouse gas N_2O on Earth (Thomson et al., 2012).

1.3.4 Nitrous oxide reductase

As the final stage of denitrification, the biological reduction of N_2O to N_2 requires the *nos* operon (Eq. 13). The primary catalytic unit encoded for by the *nos* operon is NosZ, NosZ is a homodimeric metalloprotein which contains two copper centres per monomer (Cu_A and Cu_Z), these are oriented head-to-tail, whereby the Cu_A site of one monomer is in close proximity to the Cu_Z monomer of the other (Zhang et al., 2019). As a homodimer, one of these domains is responsible for transferring electrons to the copper-containing centre (Cu_A) and the other is the catalytic domain unique in biology known as the Cu_Z centre. Cu_Z is based on a 4Cu-2S structure that is crucial for the enzyme's catalytic activity (Dell'Acqua et al., 2011). In addition to *nosZ*, there are several other key genes for the functioning of the nitrous oxide reductase enzyme. NosDFY is a purported ABC transporter complex hypothesised to assist in the binding of sulfur to the Cu_Z structure (Zumft, 2006). *nosR* is a regulatory gene that controls the expression of nosZ and its associated genes, responding to environmental signals such as the presence of NO to regulate the *nos* operon (Wunsch and Zumft, 2005). *nosL* encodes for a membrane-anchored lipoprotein that assists in chaperoning copper to NosZ for Cu_Z assembly (Bennett et al., 2019). The latter two of these genes, *nosRL* are found primarily in clade I denitrifiers (*nosZI*).

A decade ago, a study by Jones et al. (2013) discovered a clade of previously unaccounted-for denitrifiers. This clade (*nosZII*), differs in several ways from the original *nosZI* clade (Sanford et al., 2012). As mentioned before, the *nosZII* clade lacks the *nosR* and *nosL* genes, but it also differs in enzyme phylogeny. Other differences include the organisation of the *nos* gene cluster, their NosZ translocation pathway and most importantly, the frequency of co-occurrence of other denitrification genes. This final point refers to how the majority of nosZI species contain the genes required for complete denitrification, converting NO_3^- to N_2 through four stages of enzymatic reduction. In contrast, *nosZII* species are usually partial denitrifiers, capable of only certain steps in the denitrification pathway. For instance, the *nosZII* species *Dyadobacter fermentans* can only produce the NosZ enzyme and is therefore incapable of reducing any other components of the denitrification pathway. This common lack of the *nar*, *nir*, or *nor* genes in *nosZII* organisms makes this clade a potential N_2O sink, as some species lack the apparatus to produce N_2O, but can still reduce it. This theory was tested by Domeignoz-Horta et al. (2016), in this study agricultural soil samples from several European sites were inoculated with *D. fermentans*. They found that in a third of the soils N_2O consumption increased by an average of 189%. Implying that some soil

types could have N_2O consumption drastically increased by inoculation of this *nosZII* denitrifier. This information is made even more relevant as the NosZ enzyme has been found to not always be active in the environment, resulting in enhanced emissions of N_2O into the atmosphere. Several regulators of NosZ activity have been found, such as the previously described regulation by NosR. Similarly to the other denitrification genes, the presence of O_2 also negatively regulates the expression of the *nos* operon. This process is often regulated through the fumarate and nitrate reduction regulator (FNR), a transcriptional regulator that reacts to O_2 levels and either activates or represses genes in response (Aral et al., 1997). The nitric oxide response regulator (NnrR) impacts NosZ activity by responding to NO concentrations and regulating various genes involved in denitrification, including *nosZ*.

The reason why N_2O is often not reduced in the environment remains uncertain, although several key environmental factors are considered relevant. For example, the pH of the soil is understood to impact the N_2O/N_2 product ratio of denitrification, with acidic soils leading to more N_2O being produced (Stevens and Laughlin, 1998; Šimek and Cooper, 2002; Uversky, 2003). A result of the suboptimal pH interfering with protein synthesis and protein assembly rates, rather than the transcription of *nosZ* (Bergaust et al., 2010).

However, when it comes to understanding why N_2O is often not reduced in the environment, the response of the *nosZ* to extracellular levels of copper could be another avenue of regulation. This was initially identified in *P. denitrificans* when cultured anaerobically on minimal media, as a lack of copper in the media resulted in the reduced expression of the *nosZ* gene (Sullivan et al., 2013). Additionally, Shen et al. (2020) found that agricultural soils supplemented with a Cu-fertiliser can enhance denitrifiers ability to reduce N_2O to N_2. A question which is still in need of answering though is what concentration of copper is required for efficient denitrification to take place in the environment.

Denitrifying bacteria are found in abundance in cropland soils, utilising the huge amounts of nitrogen added to farmed soils as fertiliser through the Haber-Bosch process (Wang et al., 2018). This over-abundance of nitrogen has led to changes in the bacterial ecology and N_2O emissions of these habitats (Smith et al., 1997; Sarathchandra et al., 2001). In fact, since the 1860s, 82% of the increase in N_2O emissions is attributed to originate from croplands (Tian et al., 2019). Likely a result of increasing fertiliser application on croplands. One study found that of 190 soil samples taken from across 15 countries, 14% of these were found to be Cu-deficient (Sillanpaa, 1990). Cu-limitation in soils was defined previously as $<12\,mg\,kg^{-1}$ Cu in soil (Bradford et al., 1967). This has been expanded on in a study that determined that 20% of Europe's arable soils are considered Cu-deficient for efficient crop yields (Alloway, 2008). However, it is debated whether these levels of Cu-limitation in soils impact the efficiency of soil microorganisms as they do for crops (Dell'Amico et al., 2008; Brandt et al., 2010; Fernández-Calviño et al., 2010).

1.3.5 Other forms of denitrification

The described step-wise reduction of NO_3^- to N_2 is the most common non-anthropogenic method of the denitrification pathway. However, aerobic denitrification is a process which was first identified in aerobic cultures of *Thiosphaera pantotropha* (Robertson and Kuenen, 1984), a legacy species which has since been reclassified as *Paracoccus denitrificans* (Ludwig et al., 1993). Aerobic denitrification as an enzymatic process hardly differs from anaerobic denitrification, however, its rate is heavily affected by dissolved oxygen concentration, carbon/nitrogen load ratio, temperature and pH (reviewed in Ji et al. (2015). This process contradicts the established theory that denitrification can only occur under anaerobic conditions although the proportion of total denitrification done aerobically has yet to be determined. However, a study investigating a subtropical acidic forest soil found that denitrification was the main source of measured N_2O emission despite the high oxygen content of the soil (Xu et al., 2013). They identified that anaerobic microsites had formed within the soil matrix, and that despite these soils being considered aerobic, these sites were sufficient for measurable changes in N_2O emissions.

There is also chemodenitrification, an abiotic process in which N_2O is produced from the reaction between ferrous iron (Fe(II)) and NO_2^- in the presence of protons (Eq. 14).

$$3\,Fe^{2+} + NO_2{}^- + 4\,H^+ \longrightarrow 3\,Fe^{3+} + N_2O + 2\,H_2O \tag{14}$$

Fe(II) is most commonly produced in the environment by Fe(III)-reducing microorganisms; however, this reaction does not require enzymatic catalysis. This is potentially environmentally relevant in marine sediments where chemodenitrification may play a major role in N_2O emissions (Wankel et al., 2017). Later research of these environments showed that 15-25% of their N_2O emissions are formed by chemodenitrification, with the rest from microbial denitrification (Otte et al., 2019). With these alternative denitrification pathways, it is clear that bacterial denitrification is not only the primary source of N_2O emissions, but it is also heavily influenced by anthropogenic actions.

1.3.6 N_2O in the context of global warming

The reason that N_2O emissions are of such interest is that it is considered the third most impactful greenhouse gas (GHG) behind carbon dioxide (CO_2) and methane (CH_4). Molecule for molecule, N_2O has a global warming potential 298-fold greater than CO_2 (Lashof and Ahuja, 1990). N_2O contributes 6% to global radiative forcing (Butler and Montzka, 2015) and can remain stable in the atmosphere for 114 years before degrading (IPCC, 2007). To further enhance its role as a GHG, it is also the dominant gas that depletes the ozone layer (Ravishankara et al., 2009), and its atmospheric concentration continues to increase from 2024 levels of 337.7 ppb by approximately

1 ppb per year (Lan et al., 2024). N_2O emissions enter the atmosphere from only a few sources; 5% of emissions are from atmospheric reactions, 35% originate from ocean systems, whilst the majority (60%) are emitted from soil environments, particularly agricultural (Ko et al., 1991). The World Meteorological Organisation (WMO) found that 60% of all N_2O emissions are from natural sources, with the other 40% from anthropogenic sources (WMO, 2015).

Based on this information, further studies looking into the source of cropland N_2O emissions should focus on bacterial species, especially considering that NosZ is the only biological sink for N_2O and bacteria make up the majority of NosZ activity in soils (Simon, 2020). A recent study used biogas waste as a growth substrate and vector to bioengineer the microbiota of agricultural soils resulting in N_2O emissions being reduced by up to 95% (Hiis et al., 2024). The N_2O reducing bacterium *Cloacibacterium* sp. CB-01 was added at high cell densities to these soils, a process that if scaled up, is projected to reduce global N_2O emissions by up to 20%. Techniques like these are now thought to be one of the main ways to reduce N_2O emissions in many habitats. However, this will not provide a solution to all environments and as such other methods must be employed. One such is to focus on the transcriptional regulation of denitrification genes, furthering our understanding of the complex regulatory processes that activate or repress bacterial N_2O emissions.

1.4 Regulation of denitrification processes

As mentioned in the descriptions of each section of the denitrification pathway, both environmental conditions and transcriptional regulators are relevant regulators of denitrification and N_2O emissions (reviewed in Gaimster et al. (2018).

1.4.1 Transcriptional regulators

Most environmental signals affect the expression or activity of a transcriptional regulator (Balázsi and Oltvai, 2005). Transcriptional regulators are proteins that control the rate of transcription of genetic information from DNA to RNA. They usually do this by binding to specific DNA sequences and either repressing or activating their expression (Browning and Busby, 2004). Transcriptional regulators that are activators of gene expression often bind to promoter regions of certain genes, facilitating the recruitment of RNA polymerase which in turn accelerates gene expression (Weidemüller et al., 2021). Repressors decrease the rate of transcription, often by binding to operator regions or other specific DNA sequences. This blocks access for RNA polymerase and hinders transcription machinery (Reynolds et al., 2013). Other mechanisms of regulation exist in eukaryotic organisms, such as chromatin modification whereby transcriptional regulators influence the structure of the DNA-protein complex in eukaryotic cell nuclei. However, the previously

mentioned mechanisms of acting dominate prokaryotic transcriptional regulators. When it comes to denitrification and N_2O emissions, several transcriptional regulators have been identified as having a regulatory function in these processes. Three of these belong to the fumarate and nitrate reduction (FNR) cyclic-AMP receptor protein (CRP) superfamily of transcriptional regulators (Fig. 1.2). The FNR/CRP superfamily is a group of proteins that play an important role in bacterial gene expression, particularly in response to varying environmental conditions, both FNR and CRP are well-characterised members of this group (Green et al., 2001). Members of the FNR/CRP superfamily typically have a conserved N-terminal DNA-binding domain and a C-terminal domain that is involved in dimerization and sensing environmental signals. This C-terminal domain usually contains a helix-turn-helix motif, which facilitates specific binding to DNA sequences in promoter regions (Körner et al., 2003). Members of this superfamily have been found to commonly regulate various metabolic pathways such as carbon utilisation (Zhang et al., 2005), anaerobic respiration (Unden and Trageser, 1991) and stress response (Elvers et al., 2005). However, much research has also been conducted on the three members of the FNR/CRP superfamily that are involved in the denitrification pathway.

One of these three is the fumarate and nitrate reduction protein (FnrP). FnrP was identified as a transcriptional activator in *Paracoccus denitrificans* of both the *nar* and *nos* operons when NO or oxygen were present. FnrP contains a 4Fe-4S cluster which undergoes conversion to a 2Fe-2S cluster when in the presence of oxygen or NO. This leads to the separation of the active dimer into monomers, thereby inactivating it (Crack et al., 2016). FnrP is very important to *P. denitrificans* survival when oxygen levels deplete, as found when a FnrP deletion mutant had a 5-fold reduction to anaerobic growth rate than the control (Spanning et al., 1997). This was due to the 3-fold reduction in Nar enzyme activity seen in the mutant strain, limiting ATP production for the bacteria. FnrP is also found in other bacteria, for instance, *Pseudomonas aeruginosa* contains a similar anaerobic regulator of arginine deiminase and nitrate reductase (ANR) transcription factor. ANR functions very similarly to FnrP, however, its primary function is to activate genes responsible for nitrate transporters and reductases when oxygen levels are low (Schreiber et al., 2007). FnrP is also deemed relevant to N_2O reduction, as it is thought to activate the *nos* operon in response to NO. However, an FnrP mutant did not significantly affect N_2O emissions from *P. denitrificans*, whilst a double mutant of both FnrP and NNR lacked the potential to reduce N_2O (Bergaust et al., 2012).

NNR (nitrite and nitric oxide reductase regulator) is homologous to FnrP, however, it lacks N-terminal cysteines. NNR targets the *nir* and *nor* operons for its regulation, primarily reacting to oxygen concentrations, but also involved in NO response (Spanning et al., 1997). When cultured in denitrifying conditions as an NNR deletion strain, *P. denitrificans* growth rate was halved and nitrite was accumulating, the latter being a direct result of Nir activity completely halting (Spanning et al., 1995). As mentioned before, NNR has also been found to regulate the *nos* operon,

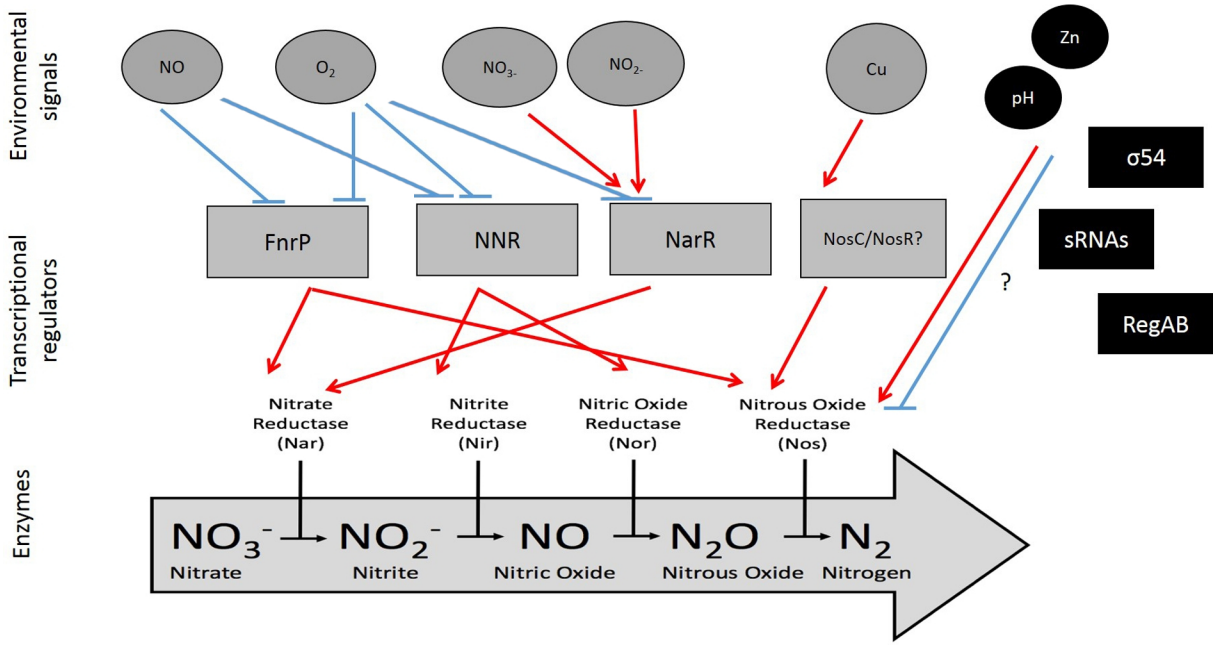

Figure 1.2: An overview of the known (grey) and potential (black) regulators of *P. denitrificans*' denitrification pathway. The arrows linking environmental signals to transcriptional regulators show signalling events, whilst arrows from the middle layer to the enzymes indicate regulation of gene expression. Blue arrows represent repressive effects and red arrows activating effects. Figure updated from Gaimster et al. (2018).

but when in the presence of NO, FnrP is necessary for induction (Bergaust et al., 2012).

The last of these FNR/CRP-type transcriptional regulators is the nitrate reductase regulator (NarR), the least extensively researched transcriptional regulator of the three, it acts in response to both nitrate and nitrite and has the primary responsibility of switching the bacteria to denitrifying pathways when nitrate is available. When detecting nitrate, NarR will respond by activating the expression of Nar, allowing the cell to generate ATP and reduce it to nitrite (Bergaust et al., 2012).

Beyond the FNR-CRP superfamily, there are also the GntR-type transcription factors. These were first identified in *Bacillus subtilis* as regulators of the gluconate operon *gntRKPZ* (Fujita and Fujita, 1986; Haydon, 1991). GntR in *E. coli* was found to have a similar function, however, it also regulated the *eda* gene, responsible for growth on several mucus-based sugars (Murray and Conway, 2005). Since then, GntR has been identified in many other species, including *Sinorhizobium meliloti* (Steele et al., 2009), *Vibrio cholerae* (Roy et al., 2016) and *Paracoccus denitrificans* (Gaimster et al., 2019). GntR-type regulators have an N-terminal with a helix-turn-helix DNA-binding domain, this is then linked to a C-terminal signalling domain. Their role in metabolic processes makes them a critical component of many species' regulatory systems.

1.4.2 Environmental factors affecting denitrification

As discussed earlier, copper is important when it comes to NosZ activity as it is required to form the catalytic subunit of the protein (Sullivan et al., 2013). However, the presence of copper also impacts the functional activity of NosC and NosR, regulatory proteins which are responsible for regulating other sections of the *nos* operon (Fig. 1.2). Similarly, zinc has been found to regulate other sections of the denitrification pathway. When *P. denitrificans* is cultured anaerobically in zinc-replete media the *norBC*, *nirS* and *nosC* genes were all upregulated (Neupane et al., 2017).

In addition to trace minerals, pH is also a very important factor affecting denitrification. For example, the total gaseous emissions of NO, N_2O and N_2 are lower from acidic soils than from neutral or alkaline soils (Šimek and Cooper, 2002). The N_2O/N_2 product ratio has also been found to be higher in acidic soil, compared to alkali soils (Zaman et al., 2007). Previous understanding was that the acidic conditions were not negatively affecting the denitrification enzymes, instead, it was a result of the reduced amounts of carbon and nitrogen in bioavailable forms.

Denitrifiers have also been observed bet-hedging their denitrification pathway during certain environmental conditions. Bet-hedging is a biological process whereby organisms will reduce their fitness in optimal conditions to optimise their fitness should they enter into stressful conditions (Ripa et al., 2010). This was observed in *P. denitrificans*, whereby when cultured in conditions replicating a switch from an anoxic to an oxic environment, most cells would limit their synthesis of the Nir and Nor enzymes, but all retained synthesis of Nos (Lycus et al., 2018). Therefore, under changeable conditions, this population becomes a net-N_2O sink. This was

determined to be a method of protecting the population from sudden anoxia as N_2O depletion in the soil is less likely than nitrate depletion. This is because N_2O is sometimes passively produced in the soil matrix from nitrifier nitrification, ammonia oxidation and chemodenitrification (Guo et al., 2018), and it can diffuse faster than nitrates due to it being a gas. Therefore, in these liminal oxic conditions, denitrifiers with early and strong expression of NosZ may be acting as N_2O sinks.

1.4.3 Sigma factors

Sigma factors are crucial regulatory proteins that function similarly to transcription factors, controlling the expression of functionally similar sets of genes. They differ in their process of regulation, as sigma factors will form a σ-bound RNA polymerase holoenzyme complex, enabling the transcriptional activation of specific gene promoters (Helmann and Chamberlin, 1988). In bacterial cells σ^{70} is the primary sigma factor and is responsible for transcribing most essential growth-related genes (reviewed in Feklístov et al. (2014)). However, in terms of the denitrification pathway, σ^{54} is deemed the most important.

σ^{54} is encoded for by the *rpoN* gene and is particularly important for the expression of nitrogen-regulated genes, among other physiological functions (Kustu et al., 1989). *Ralstonia eutropha* was found to be entirely dependent on σ^{54} for anaerobic growth when cultured on nitrate. This has been explained as being a result of *R. eutropha* relying on the σ^{54}-dependent NorR regulator, which regulates expression of the *nor* operon (Pohlmann et al., 2000). However, this is not always the case for other bacteria, as an *rpoN* mutant of *P. aeruginosa* can grow anaerobically on nitrate (Totten et al., 1990). The model soil denitrifier *Paracoccus denitrificans* contains a homologue for σ^{54}, however, little is known about its regulatory function in this organism (Gaimster et al., 2018).

Transcriptional regulators like FnrP, NNR and NarR and to a lesser degree σ-factors are hugely important in switching cells to anaerobic conditions and for promoting the transcription of denitrifying apparatus, however, many other environmental factors regulate the denitrification pathway.

1.5 *Paracoccus denitrificans*

The model soil denitrifier *Paracoccus denitrificans* is often used in experiments due to its ability to synthesise a range of biological apparatus for utilisation of a range of electron acceptors. It has also been very well characterised in its ability to switch between aerobic and anaerobic respiration (Baumann et al., 1996; Giannopoulos et al., 2017). It has a fully sequenced genome, assisting in understanding the genetic regulation of various processes (NCBI, 2006), and its denitrification genes have been well characterised. *P. denitrificans* is a gram-negative α-proteobacteria first

isolated as *Micrococcus denitrificans* in 1910 before being reclassified (Beijerinck and Minkman, 1910). Its genetic material is stored on two chromosomes and a plasmid, and its denitrification genes are found spread out between them. *nir* and *nor* operons are on chromosome 1, *nar* and *nos* on chromosome 2 and *nap* is on the plasmid. As such, *P. denitrificans* is capable of performing complete denitrification, using both the membrane-bound (Nar) and periplasmic nitrate reductases (Nap) for the reduction of NO_3^-. For the reduction of NO_2^- it has the cytochrome *c*-type nitrite reductase (NirS) and NO is catalysed by the NorBC enzyme. Lastly, it has the *nosZI*-clade enzyme for the reduction of N_2O. Combined, these reductases allow *P. denitrificans* to react to oxygen limitation by changing its respiration pathway and survive on nitrogen compounds. *P. denitrificans* has become very relevant in the examination of N_2O reduction and was the organism used to show that N_2O emissions accumulate when copper is exempt from the growth media (Sullivan et al., 2013). *P. denitrificans* N_2O emissions have also been observed to increase under decreased pH, where NosZ synthesis and intracellular Cu content were negatively affected, causing the increase in N_2O emissions (Olaya-Abril et al., 2021). Furthermore, it was the study organism used to identify the first small RNA (sRNA) to regulate the denitrification pathway (*denR*), whose overexpression resulted in reduced N_2O emissions (Gaimster et al., 2019).

1.6 Small RNAs

sRNAs are non-coding RNA molecules approximately 50-200 nucleotides in length, with a common function of regulating gene expression at the posttranscriptional level. They are found in all three domains of life and have roles regulating a wide range of cellular processes. sRNAs are either *cis*-, or *trans*-encoded, depending on where they are positioned in the genome compared to their target. *Cis*-encoded sRNAs, often referred to as antisense sRNAs, are located in the genome opposite to the gene responsible for their target mRNA, usually resulting in the complete complementarity of base pairs (Gottesman and Storz, 2011a).

Trans-encoded sRNAs are transcribed from distant sections of the genome to their targets, as a result, they often have limited complementarity and rely on a seed region to initiate binding with their mRNA. *Trans*-encoded sRNAs are generally repressive regulators (Aiba, 2007), often functioning as modulators of gene expression in response to environmental stress. These negatively acting sRNAs have several different mechanisms for reducing the translation of their target mRNA.

One prevalent method is direct base-pairing with target mRNAs within the boundary of a ribosomal binding site, Shine Dalgarno sequence or start codon, blocking ribosomal access and inhibiting translation initiation, leading to RNase-mediated decay (Udekwu et al., 2005) (Fig. 1.3A). The sRNA RyhB in *Escherichia coli* uses this mechanism by binding to the mRNAs of iron

storage proteins, preventing translation under iron-limited conditions (Dutta and Srivastava, 2018). Some sRNAs also rely on Hfq, an RNA chaperone to provide stability to the sRNA-mRNA complex and ensure the mRNA is degraded before translation can occur (Section 1.6.1).

As for positively acting sRNAs, one method involves the stabilization of target mRNAs, protecting them from degradation by RNases (Wadler and Vanderpool, 2007). For instance, the sRNA SgrS in *E. coli* and *Salmonella* binds to its target mRNA *pldB-yigL*, this masks an RNase E cleavage site on pldB, facilitating the production of YigL phosphatase (Papenfort and Vanderpool, 2015). Also, sRNAs can bind to hairpin structures in mRNAs, altering the secondary structure and exposing a previously inaccessible ribosomal binding site which opens up translation (Dutta and Srivastava, 2018). This is seen with the *Pseudomonas aeruginosa* sRNA PhrS which causes a conformational change to the *ufo* mRNA, opening the ribosomal binding site to translation (Sonnleitner et al., 2011). There are other methods of acting for sRNAs, however, those mentioned are the most thoroughly researched (reviewed in Moeller et al. (2021)).

Much of the early research into sRNAs focused on their roles as virulence factors in pathogens such as *E. coli*, and *Salmonella*. Here they were found to be critical regulatory units for the survival of cells under extreme stress conditions both outside of, and within a host (Waters and Storz, 2009). As sRNAs are small pieces of regulatory machinery working at the posttranscriptional level, they allow cells to respond rapidly to adaptive environments, a necessary quality for pathogens (Johansson and Cossart, 2003). There has even been proof of sRNAs being transported via outer membrane vesicles (OMVs) into host cells, modulating the host's immune response (Koeppen et al., 2016). Usually, however, sRNAs impact bacterial pathogenesis by modulating gene expression of virulence-relevant genes. Pathogenic strains of *E. coli* have been found to have an extremely high density of sRNAs within the genetic sequence of their pathogenicity islands (39 sRNAs/Mb), then compared to the total genome (23 sRNAs/Mb) (Raghavan et al., 2011). A regular theme of these pathogenic sRNAs is their reliance on RNA chaperones to ensure their regulatory functions.

1.6.1 RNA chaperones

As mentioned for some negatively acting sRNAs, RNA chaperones can be necessary for the regulatory effects of sRNAs to activate. This is because RNA chaperones play critical roles in ensuring various RNA molecules achieve and maintain their intended secondary and tertiary structures, differing from traditional protein chaperones by not requiring ATP to function (Rajkowitsch et al., 2007).

Many RNA chaperones aim to fold RNA molecules into their correct structures by preventing misfolding and resolving any misfolding mistakes. This is crucial for RNA's function in processes such as translation, splicing, and ribosome assembly. RNA chaperones can also stabilise struc-

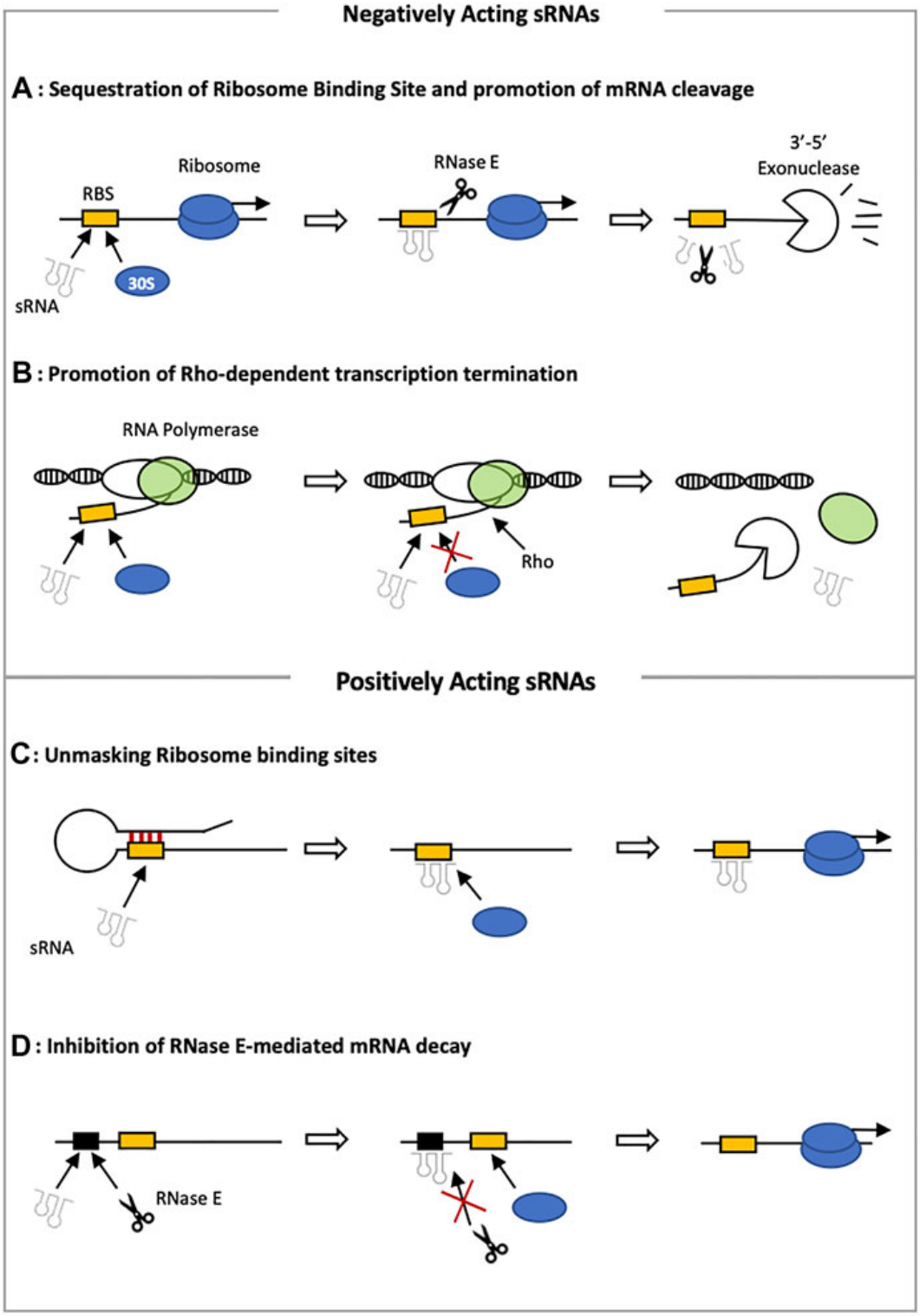

Figure 1.3: Mechanisms of sRNA activation of repression. **(AB)** shows two mechanisms used by negatively acting sRNAs to lower the translation of their target mRNA. **(CD)** shows two methods of positively acting sRNAs increasing the stability of their target mRNA and therefore enhancing translation rate. Figure taken from Moeller et al. (2021).

tures during the folding process, a function that can also extend to ensuring sRNA lifespans increase sufficiently for them to complete their function (Russell, 2008).

Hfq is a good example of a well-characterised RNA chaperone that performs a variety of functions for differing sRNAs. The majority of this research has been conducted within *Escherichia coli*, a model organism for investigating RNA interactions and functions (reviewed in Cech et al. (2016)). These studies have highlighted the wide-ranging functionality of the Hfq chaperone, particularly when interacting with sRNAs. However, the presence of Hfq in *P. denitrificans* has not been fully confirmed. *P. denitrificans* gene Pden_4124 has a 95% similarity to the Hfq protein in *Rhodobacter sphaeroides*, indicating a likely chance that Hfq is a functional RNA chaperone for *P. denitrificans* (Gaimster et al., 2016). This is interesting as this same study identified 167 sRNAs throughout the *P. denitrificans* genome when cultured in anaerobic denitrifying conditions. 59 of these sRNAs were differentially expressed by 2-fold either higher or lower between high N_2O, and low N_2O emitting conditions. This identification of many sRNAs with possible roles in the denitrification pathway makes research on the Hfq chaperone in *P. denitrificans* of even greater interest.

Despite this seeming wealth of knowledge on sRNAs and RNA chaperones, the majority of the research has been conducted on pathogenic species such as *E. coli*, *Salmonella* and *P. aeruginosa*. Very little is known about the role of sRNAs in environmental microbiology despite their seemingly broad reach across many cellular processes. For instance, a study looked at the model marine heterotroph *Ruegeria pomeroyi* and identified 99 putative sRNAs, 14 of which were differentially expressed between carbon and nitrogen limitation conditions (Rivers et al., 2016). This shows that sRNAs in these environmental bacteria may be quite important in responding to specific nutrient limitations affecting the strain, and their chaperones may be relevant for processes beyond just virulence.

1.6.2 sRNAs impacting denitrification in *P. denitrificans*

The same research group that identified a possible Hfq in *P. denitrificans* later classified *denR*, an sRNA that causes the downregulation of nitrite reductase and limits both NO and N_2O emissions when overexpressed (Gaimster et al., 2019). It does this by stabilising the expression of the previously unknown GntR-type transcriptional regulator (NirR). *denR* shares a computationally predicted 7-bp seed region located within the coding sequence of the *nirR* mRNA. This is hypothesised to allow the stabilisation of *nirR* and inhibit RNase E decay of the mRNA, increasing the concentration of NirR in the cell. Further experiments with *nirR* being overexpressed in *P. denitrificans* found that NO_2^- accumulated much quicker in the cell than the control as a result of *nirS* being downregulated in its presence. This reduction in activity of the denitrification pathway at the nitrite reduction stage resulted in decreased concentrations of NO and N_2O in the culture

media. This was an incredibly important discovery as *denR* shows that sRNAs form a critical part of this previously unappreciated regulatory pathway. A pathway that may point towards methods of reducing N_2O emissions from bacteria.

In addition to *denR*, a recent study identified Pda200 from *P. denitrificans* that was cultured under antibiotic stress, intended to simulate environments in aquaculture systems (Wang et al., 2021). It was found to be differentially expressed by >10-fold when cultured under antibiotic pressure of florfenicol. A later study focused on characterising Pda200 found that overexpressing Pda200 upregulated *napA*, *napB* and *norB* expression (Wang et al., 2022). Furthermore, they computationally predicted that Pda200 has close complementarity with the *nosZ* mRNA and that there was a correlation between the presence of Pda200 and the *nosZ* mRNA when under florfenicol stress. They hypothesised that Pda200 could therefore be of particular interest in these aquaculture environments that require nitrate removal and a reduction in N_2O emissions.

1.7 Aims

The aim of this thesis is to investigate how sRNAs interact with the denitrification pathway of the model denitrifier *P. denitrificans* and lead to changes in cellular N_2O emissions. This will involve focusing on a previously identified *P. denitrificans* sRNA (sRNA_11) that has been shown to be differentially expressed under denitrifying conditions (Chapter 3) (Gaimster et al., 2016). We will also present our findings on how the RNA chaperone Hfq can regulate N_2O emissions (Chapter 4), before investigating an sRNA that has been claimed to directly interact with the *nosZ* mRNA (Chapter 5).

2 Materials & Methods

2.1 Culture Conditions

2.1.1 Media

All strains were stored at −80 °C in a freezer, using Microbank™ Vials (Pro-Lab Diagnostics, Bromborough, UK) containing porous beads coated in cryopreservative, allowing for safe storage and retrieval for several years. Strains were transferred from storage to solid Luria-Bertani (LB) media containing 1.5% agar (Formedium™) using a pipette tip and a heat-sterilised inoculation loop. The appropriate antibiotic to select for the desired strain as listed in Table 1 was added to the flask before pouring. LB broth contained 10 g tryptone (Formedium™), 10 g NaCl (Sigma™) and 5 g yeast extract powder (Formedium™) per litre de-ionised water (Bertani, 1951; Bertani, 2004). Components were combined using a magnetic stirrer and stirring bar before 200 mL were aliquoted into 500 mL conical flasks. 1.5% agar was added only to flasks required to be solid LB. Flasks had air-permeable bungs inserted into the neck and the top of the flasks were covered in aluminium foil to reduce evaporation during autoclaving. Once sterilised, flasks were stored at room temperature until required. When preparing solid LB-agar to make plates, flasks were microwaved until LB was entirely liquid. Necessary antibiotics were added to the LB once cooled to the touch, followed by pouring approximately 65 mL into Petri dishes (Thermo Scientific) in a sterile environment using aseptic technique. Plates were left to solidify before either being stored at 4 °C for up to 4 weeks, or for strains to be streaked onto the agar using a pipette tip and inoculation loop. Once streaked, plates containing environmental strains were placed in the 30 °C regulated temperature room to incubate, whilst pathogenic strains were incubated at 37 °C in an incubator. Inoculated plates were left to incubate until individual colonies were visible and transferable. Once incubated, plates were stored at 4 °C for up to 2 weeks before being disposed of.

When preparing overnight growths, single colonies of the desired bacterial strain were transferred from the agar plate to 10 mL LB-broth containing the required antibiotics. Overnight cultures were incubated at 30 °C for up to 10 hours to an optical density (OD_{600}) of 1. Optical density was measured using a spectrophotometer, an instrument which quantifies how much light of varying wavelengths is absorbed or transmitted by the sample, used as a proxy for cell growth. 1 mL of the sample was transferred to a cuvette (Fisherbrand) and analysed on the spectrophotometer. The OD_{600} was calculated by comparing the results to a blank cuvette sample containing 1 mL sterile LB media. The overnight bacterial cultures were used from this stage to inoculate the relevant experiment.

Table 1: Characteristics of each strain used during this study and the antibiotic and its concentration used for each strain or plasmid selected for.

Strain	**Description**	**Antibiotic** (μg/mL)
P. denitrificans		
PdWT	Wild-type strain of PD1222.	Rifampicin (25)
Pd_empty	PdWT containing pLMB509 plasmid with an empty overexpression vector.	Gentamicin (25)
Pd_11	PdWT containing pLMB509 plasmid with sRNA11 cloned into the NdeI site of plMB509.	Gentamicin (25)
PdΔ*nosZ*	*nosZ* deletion mutant of PdWT containing pLMB509 plasmid with an empty overexpression vector.	Gentamicin (25)
Pd_hfq	PdWT containing pLMB509 plasmid with *hfq* cloned into the NdeI site of plMB509.	Gentamicin (25)
Pd_Pda200	PdWT containing pLMB509 plasmid with Pda200 cloned into the NdeI site of plMB509.	Gentamicin (25)
Eschericia coli		
DH5α pRK2013	DH5α strain containing helper plasmid prk2013.	Kanamycin (20)
Top10	Transformation strain used as donor strain for pLMB509 conjugation to PdWT.	Kanamycin (20)

Table 2: Characteristics of each plasmid used during this study.

Plasmid	**Description**
pLMB509	Taurine inducible expression vector
Overexpression constructs	
pLMB509_sRNA11	*P. denitrificans* sRNA11 inserted into pLMB509
pLMB509_hfq	*P. denitrificans hfq* gene inserted into pLMB509
pLMB509_Pda200	*P. denitrificans* Pda200 inserted into pLMB509

2.2 Media

To study the nitrogen cycle, and specifically the denitrification pathway of *P. denitrificans*, a defined minimal medium was prepared as done in Sullivan et al. (2013), based on the original recipes devised by Taylor and Hoare (1971); Robertson and Kuenen (1983). This medium was designed to contain the essential salts for *P. denitrificans* growth - such as magnesium, potassium, sodium, and chlorine -, a carbon source in succinate, and nitrate (NO_3) as an electron acceptor. Specifically, this medium contained: 29 mmol/litre Na_2HPO_4; 11 mmol/litre KH_2PO_4; 10 mmol/litre NH_4Cl; 0.4 mmol/litre $MgSO_4$; 30 mmol/litre sodium succinate dibasic; and 20 mmol/litre $NaNO_3$. These components were all sourced from FisherScientific. Minimal media was prepared by mixing the required ingredients with deionised water, mixing on a magnetic stirrer with a stirring bar and transferring to a Duran bottle (DWK Life Sciences, Mainz, Germany). The media would then be sterilised by autoclaving before use.

A trace element supplement (Vishniac & Santer trace metal solution) was added to all anaerobic *P. denitrificans* cultures to allow the production of enzymes necessary for complete denitrification. This trace element solution contained: 129.9 mmol/litre EDTA; 13.59 mmol/litre $ZnSO_4 \cdot 7\,H_2O$; 41.99 mmol/litre $MnCl_2 \cdot 4\,H_2O$; 33.85 mmol/litre $FeSO_4 \cdot 7\,H_2O$; 4.2 mmol/litre $(NH_4)_6Mo_7O_{24} \cdot 4\,H_2O$; 5.39 mmol/litre $CuSO_4 \cdot 5\,H_2O$; 13.13 mmol/litre $CoCl_2 \cdot 6\,H_2O$; and 49.54 mmol/litre $CaCl \cdot 2\,H_2O$. When preparing copper low media (Cu_L), $CuSO_4 \cdot 5\,H_2O$ was omitted from the solution. Once prepared, the trace element solution was filter sterilised using 0.22 μm Millex Millipore filters (Merck, Darmstadt, Germany) into 15 mm Falcon tubes (Corning, Corning, USA) and stored at 4 °C to complete maturation over several months. The trace element solution was classed as matured once it had changed colour from a freshly prepared green to a dark purple. The freshly made Cu_low trace elements solution changed from orange to dark red as it matured. The maturation and colour changing of these solutions is a result of the metals oxidating, a process which can be accelerated by adding hydrogen peroxide or sparging the solu-

tion with compressed air (Laguna, 2018). This stage is necessary as for instance *P. denitrificans* can only produce the NosZ enzyme in the presence of oxidised copper (Moffett et al., 2012). When producing the trace element solution, maturation was determined by UV-Vis spectroscopy, 1 mL trace element solution was aliquoted into a cuvette and its absorbance was measured in a spectrophotometer. This was continued over several months until there was no change in the absorbance spectrum of both solutions. Figure 2.1 shows that the absorbance spectrum of a freshly prepared Vishniac and Santer trace elements solution is noticeably different than that of a fully matured solution. The peak at a wavelength of 530 nm for both copper levels shows a significant increase in absorbance for the matured solution, compared to the fresh. There is also an increased absorbance present between 650 nm and 850 nm due to the presence of copper in the solution (Figure 2.1 A). Once matured, the trace elements solution was stored at 4 °C.

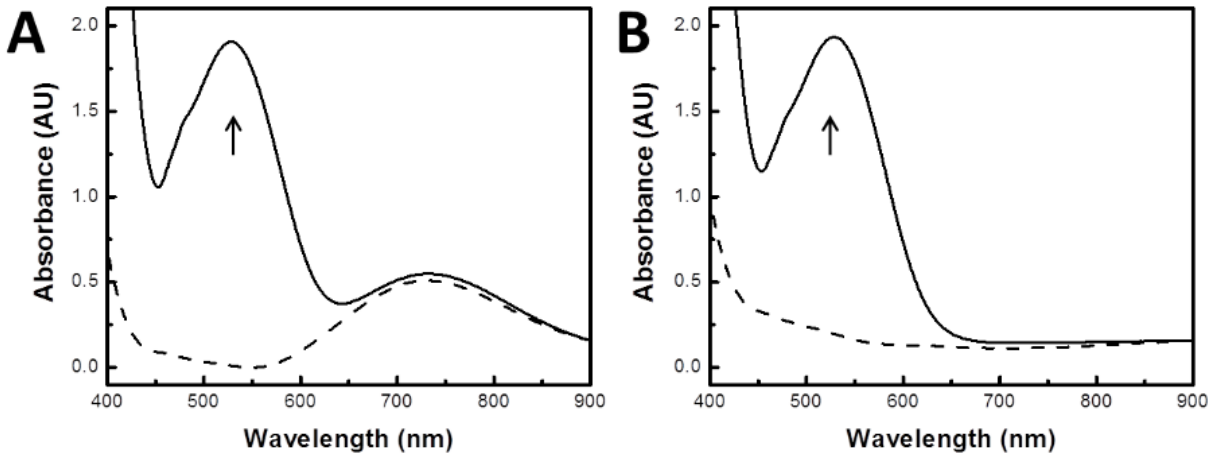

Figure 2.1: UV-vis spectra of fresh (dashed line) and matured (solid line) Vishniac & Santer trace metal solution. **(A)** complete trace element solution. **(B)** Cu_low trace element solution. The arrow denotes the wavelength for which maturation levels were determined. Reprinted from Laguna (2018).

2.2.1 Aerobic Batch Culture

Aerobic batch cultures were carried out using 50 mL of LB medium in sterile 250 mL conical flasks. These cultures were supplemented with the necessary antibiotics and inoculated with 1 mL/litre from an overnight culture of an optical density (OD_{600}) of 1. When conducting experiments requiring the pLMB509 overexpression vector, 10 mmol/litre taurine was added to the medium. To maximize aeration, the cultures were incubated at 30 °C with agitation at 200 rpm. Cell density was monitored by periodically extracting 1 mL samples from the cultures and measuring the OD_{600}. During extended growth phases, a 0.1 mL sample of the culture was diluted 1:10

(v/v) in LB before measuring the OD_{600} to reduce the cell density due to limitations of the spectrophotometer.

2.2.2 Anaerobic Batch Culture

To simulate denitrifying conditions, *P. denitrificans* was cultured anaerobically in a minimal medium, to achieve this, 250 mL Duran bottles with screw-cap lids and a gas-tight silica septa were used. Each bottle was filled with 200 ml of minimal medium, supplemented with 2 mL/litre Cu-low Vishniac and Santer trace elements solution, 4 mL taurine (prepared at 0.5 mol/litre to form a final media concentration of 10 mmol/litre), 100 µL of gentamicin (50 mg/litre) (Sigma-Aldrich, Darmstadt, Germany) and 1 mL/litre bacterial inoculant of OD_{600} 1. Once combined, the bottles were sparged with N_2 by inserting a 22 gauge spinal needle (BD, Vaud, Switzerland) through the silica septum and releasing N_2 into the media for 15 min, allowing pressure to remain stable by also inserting a 22 gauge Agani hypodermic needle (Terumo, Tokyo, Japan) through the septa allowing for the replacement of the bottled gas. Once sparged the bottles were stored upright in a 30 °C room and samples for OD_{600} and gas were taken at set time intervals from inoculation.

Gas samples were taken every 8 h by inserting a gas-tight, syringe (Hamilton, Reno, USA) through the silica septa. A 3 mL gas sample was taken from the headspace of each bottle and immediately transferred to a 20 mL headspace vial with a butyl septum (Greyhound, Birkenhead, UK) which had previously been sparged with N_2 for 5 min to remove ambient levels of N_2O. Gas samples were stored at 4 °C for up to three weeks before being analysed with a gas chromatographer.

2.3 Gas Chromatography

Gas chromatography is often used to determine the purity of a product that can be vaporised without destroying the sample, or in the case of these studies, to determine the concentrations of various gaseous components. To do this, a gas chromatograph instrument (GC) collects the sample into the mobile phase, usually an inert gas such as argon at a set pressure. This mobile phase is then pushed through a column around 5 to 150 m in length (the stationary phase), which is coiled within an oven of a set temperature. The length, diameter and material of the column determines the speed at which certain molecules can travel through the system due to their interactions with the column's material, and the physical properties of the molecule. At the end of the column is a detector which responds to the components eluting from the column, creating a signal based on the volume of the eluting component. Using gas chromatography for greenhouse gas headspace analysis is a standard procedure, with specific parameters being adapted to

provide data on specific greenhouse gases (Ferraz-Almeida et al., 2020). Chromatographs are the primary output from gas chromatography, a line graph displaying the retention time on the *x*-axis, and the signal on the *y*-axis. The value of the area of a peak is relative to the concentration of the chemical component in the sample, therefore with a calibration curve based on standards for the component of interest, the concentration of the component in the original sample can be calculated.

Gas chromatography was used to determine the concentration of N_2O in the headspace of the Duran bottle. A Clarus 500 Gas Chromatograph was used alongside a Turbomatrix Headspace Autosampler (both Perkin Elmer, Waltham, USA) to analyse the samples. The GC was set to the following parameters: Injector valve temperature, 115 °C; carrier gas pressure, 60 PSI; split total flow, 95 mm/m; oven temperature, 90 °C; oven run time, 6.5 min; electron capture detection (ECD) temperature, 350 °C; ECD gas flow make-up, 30 mL/m. In addition, - when initially turned on - the instrument was given several hours before running samples to ensure the background signal was stable and <2 mV.

Upon initiating the protocol the headspace autosampler would sequentially extract samples from the headspace vials, heating the vial and its contents to 30 °C, piercing the septa and extracting 50 µL, and channelling the sample to the GC via the helium carrier gas. Chromatographs for each sample were analysed using the TotalChrom Navigator software program. The peak representing N_2O was at approximately 4.5 min, determined from chromatographs where the samples were N_2O standards at the following concentrations: 5 ppm, 100 ppm, 1000 ppm and 5000 ppm. The peak area values of these standards in triplicate were used to construct a calibration curve from which the concentration of the headspace samples could be determined (Figure 2.2).

2.3.1 Calculating Total N_2O Concentration

To convert the peak area value to a concentration of N_2O the following equation was used:

$$C = \frac{Ad(V_H + BV_L)}{100cM_{r(N_2O)}} \tag{15}$$

A is the peak area value generated through gas chromatography of N_2O in the sample, measured in mV/sec. d is the dilution factor of the sample, in the case of this study it takes into account the dilution of 3 mL of headspace sample into a vial containing 20 mL of N_2. V are the volumes of the growth bottle, split into headspace V_H and liquid V_L, 50 and 200 mL respectively. B is Henry's law constant for N_2O at 30 °C (0.5392), which defines the proportion of dissolved gas between the gaseous and liquid phases. c is the calibration coefficient (Fig. 2.2). $M_{r(N_2O)}$ is the molar mass of N_2O; 44.013 g/mol. The output of Equation 15 is the number of moles of N_2O within the entire growth bottle at the time of sampling, given in µmol of N_2O.

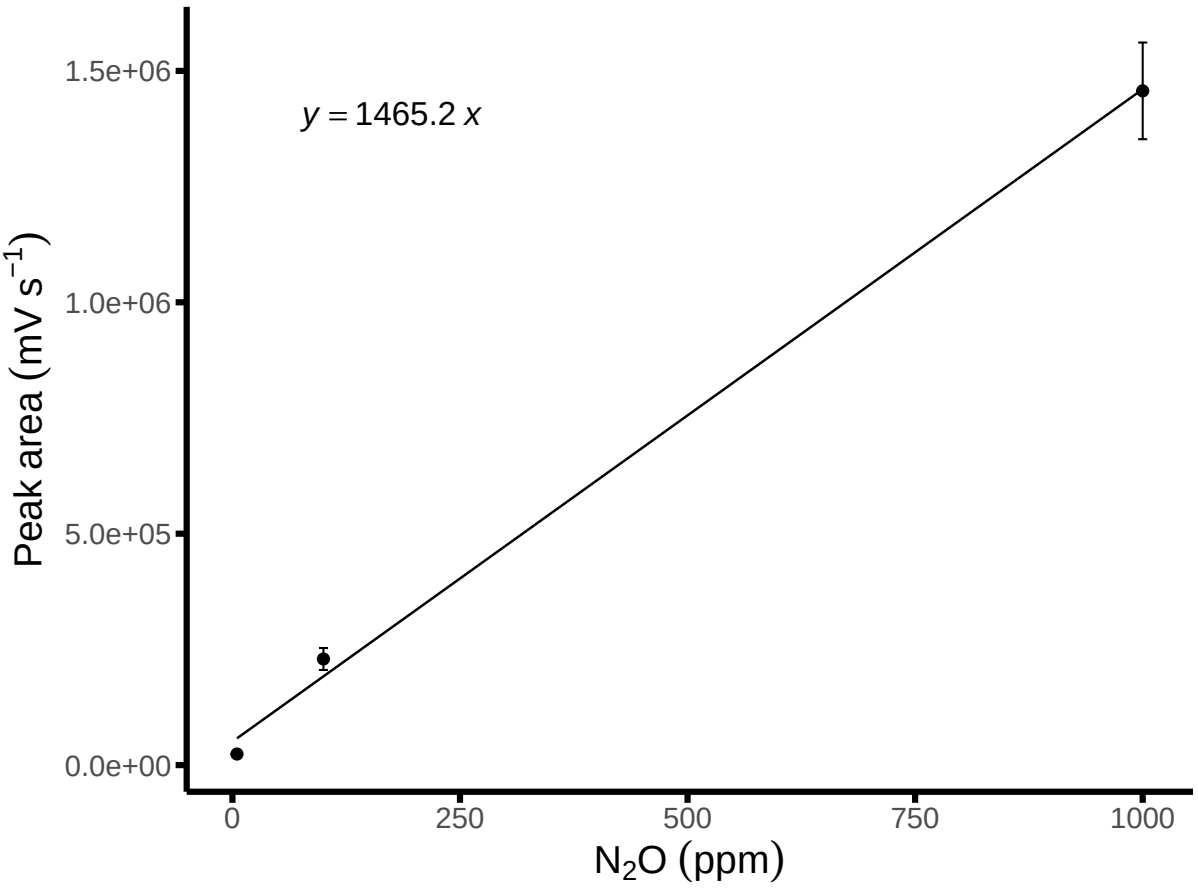

Figure 2.2: Calibration curve for N_2O using standards at 5 , 100 , and 1000 ppm. Peak area values were measured using a gas chromatograph.

2.4 Conjugation via Patch Crosses

Conjugation via the patch cross method was used to transfer plasmids from one strain of bacteria to another. This method requires the preparation of three strains; a donor, helper and recipient strain. The donor is the strain containing the relevant plasmid, this was often the Top10 strain of *Escherichia coli* due to the simplicity of working with the strain. The helper was the *E. coli* pRK2013 strain which contains the RK2 transfer gene allowing for the mobilisation of non-transmissible plasmids. The recipient is the bacterial strain intended to contain the plasmid, in most cases this was *P. denitrificans*.

These strains were independently cultured on solid LB-agar containing relevant antibiotics, an inoculation loop was used to retrieve cells from each plate and mix them on an LB-agar plate containing no antibiotics. This plate was incubated for two days before single colonies of the trans-conjugants were streaked onto plates containing antibiotics to select for both the recipient strain and the plasmid.

2.5 Polymerase Chain Reaction

When necessary, checks were made to ensure the correct strain was being cultured. To do this the polymerase chain reaction (PCR) was used to amplify specific sections of DNA. PCR allows for sections of DNA to be denatured and amplified so that detection of target sections via electrophoresis or sequencing is possible. PCR requires a DNA template from which a section is to be amplified; DNA polymerase to construct DNA molecules during the amplification stage; reaction buffer that maintains the solution at a suitable pH for polymerase activity; deoxynucleotide triphosphates (dNTPs) which are the nucleotides that form DNA; and both the $5'$ and $3'$ primers which hybridise with the DNA in a specific location to define a region for amplification. These constituents must then be mixed and inserted into a thermocycler. This instrument regulates temperature in a cyclical program, allowing the sample to go through the following stages: Denaturation, where a temperature of 94 °C was used to denature DNA from a double to a single strand; annealing, where temperature is set based on the melting points of the primers used (usually 45 to 60 °C) allowing them to bind to the complementary section of DNA, bracketing the amplicon; and extension, at 72 °C to provide optimal temperature for DNA polymerase to extend the primer sequences across the amplicon.

The first step of PCR was to extract the DNA from the sample. For this, several bacterial colonies from a single plate were mixed with 25 µL of PCR-grade H_2O (Sigma-Aldrich) in a 1.5 mL microcentrifuge tube (Eppendorf, Hamburg, Germany). The tube was then placed in a heat block for 5 min at 100 °C to lyse the cells, and centrifuged at 5000 RPM for 2 min to pellet the unrequired components of the cell at the bottom of the tube, allowing for the elution of water containing

Table 3: Table listing sequences of all forward and reverse primers used.

Primer	**Sequence**
pLMB509_Ndel_R	ATCACCTTCACCCTCTCCAC
pLMB509_Ndel_F	TTCTATTCAGCAAGCAAAGACC
nosZ_R	CTTTTCGACCTCCTACAACTCG
nosZ_F	CCGTTCAGTTCCTGATAGTCG
sRNA11_R	GCCGGCGAACCT
sRNA11_F	GGATCTTGCCT
Pda200_R	CCAGGAATCGATCTATGACAAGTT
Pda200_F	GGCCCATGCGATAGCAGG

DNA.

In a 0.2 mL Axygen PCR tube (Corning) the following components were added: 22.5 µL prepared H_2O containing DNA; 25 µL *Taq* 2X Master Mix; 1.25 µL of both the 5′ and 3′ primers from 10 µmol/litre stocks. These components were mixed by shaking and the PCR tube was placed into the thermocycler (Techne, Chelmsford, UK) and cycled for the determined duration and necessary settings. Once the sample had undergone the PCR it was kept on ice before gel electrophoresis.

2.6 Gel Electrophoresis

Gel electrophoresis is a process that separates molecules by their length. This process requires running an electric current through a permeable matrix (such as an agarose gel), forming a positive and negative charge at either end of the matrix. Due to the consistency of the matrix, smaller molecules will travel from the positive to negative currents faster than larger molecules. When combined with a DNA ladder running through the gel simultaneously to PCR samples, the approximate lengths of DNA molecules within the PCR sample can be determined.

The gel used for all electrophoresis attempts were made by combining 1 % (w/v) agarose with 50 mL TBE buffer and microwaving for 1 min. 2.5 mL SYBR Safe DNA Gel Stain (Invitrogen, Waltham, USA) was added once the agarose gel broth had cooled. This mixture was then poured into a template with an insert attached which formed wells in the gel and left for 20 min. To run the PCR sample on the gel electrophoresis instrument 5 µL of the PCR sample was pipetted into a

well in the gel alongside 5 μL of 1 kB DNA ladder (New England BioLabs, Ipswich, USA). The gel was then placed in the instrument and submerged in a TBE buffer. The instrument settings were set to the following parameters; 110 V; 400 mA; and runtime was set to 45 to 70 min depending on expected length of the PCR sample.

3 Characterisation of sRNA11; a *P. denitrificans* Growth Inhibiting sRNA

3.1 Introduction

As introduced in Section 1.6, non-coding small RNAs (sRNAs) are responsible for regulating many types of processes in microorganisms, often as a response to changing environmental conditions (Gottesman and Storz, 2011b). These sRNAs typically range from 50 to 200 nucleotides in length and are responsible for regulating gene expression post-transcriptionally. By base-pairing with target mRNAs, sRNAs can modulate mRNA stability and translation, allowing bacteria to swiftly respond to fluctuating conditions (Storz, 2002). This regulatory mechanism is particularly vital in stress responses, where sRNAs enable rapid adaptation to stressors such as oxidative stress, nutrient deprivation, and temperature changes (Holmqvist and Wagner, 2017). The involvement of sRNAs in these growth-related processes underlines their essential role in bacterial survival.

3.1.1 sRNA regulating growth processes

A substantial amount of research has been dedicated to better understanding the physiological roles of sRNAs across various bacterial species. sRNAs have recently been found to either directly, or indirectly impact bacterial growth rate.

An example of this would be OxyS in *Escherichia coli*. When *E. coli* encounters oxidative stress conditions, such as exposure to reactive oxygen species (ROS), it activates defence mechanisms to protect itself. In *E. coli* OxyS inhibits cell division through the repression of the essential transcription factor *nusG*. The presence of OxyS also promotes the expression of the *kilR* gene. The KilR protein interferes with the function of an important cell division protein, FtsZ, resulting in cell cycle arrest. Barshishat et al. (2018) found that the presence of OxyS induces cell cycle arrest to facilitate DNA damage repair. OxyS likely exerts its regulatory effects by base pair binding with the *nusG* mRNA. Their study found that two OxyS molecules could bind to separate binding sites on the mRNA at the same time. In total, 603 OxyS homologs were found across 10 genera and 27 bacterial species, showing its importance to many bacteria as a response to oxidative stress. OxyS-*nusG* binding is mostly the same between species, however, while OxyS in *Escherichia* regulates *nusG* and *mepS*, OxyS in *Salmonella* influences cell division through the regulation of *ftsZ*, *zapC*, and *mepS* (Elgrably-Weiss et al., 2024). Interestingly, *Escherichia* OxyS regulation of *nusG* is mediated by approximately the same region as *Salmonella* OxyS regulation of *ftsZ*. The regulation of *nusG* is predicted to be a more recent evolutionary adaptation by *Escherichia*.

Another oxygen-sensitive sRNA impacting growth is PhrS, in *Pseudomonas aeruginosa*. *phrS* is expressed when the oxygen-responsive anaerobic transcriptional regulator ANR is active. PhrS binds upstream of where the *pqsR* gene is translationally coupled, activating an open reading frame and promoting synthesis of the PqsR protein, a key regulator of quorum sensing in *P. aeruginosa* (Sonnleitner et al., 2011). A later study used RIL-seq with Hfq in *P. aeruginosa* to

examine mRNA targets for the previously identified sRNA landscape of 150-500 sRNAs (Ferrara et al., 2012; Gómez-Lozano et al., 2012; Gebhardta et al., 2023). They found that PhrS dominates the *P. aeruginosa* sRNA interaction network, capable of pairing with 741 different targets in exponential phase cells, accounting for 74% of all transcript pairings involving Hfq.

Another *Salmonella* sRNA of interest is GcvB, an Hfq-dependent sRNA that is highly conserved throughout Gammaproteobacteria (Urbanowski et al., 2000; Tjaden et al., 2006). Using a combination of pulse expression experiments and RNA-seq, GcvB was determined to post-transcriptionally control the expression of several amino acid metabolism genes. Resulting in growth inhibition to the culture. GcvB is most highly expressed in rich media and binds with the $5'$ UTR region of several ABC transporters, inhibiting translation by blocking ribosome binding sites (Sharma et al., 2007). Therefore, GcvB regulates the uptake of amino acids when nitrogen availability is high. It is suggested this process saves energy by focusing metabolism on synthesising amino acids when nutrient availability is high (Sharma et al., 2011).

sRNAs have also been found to regulate various biogeochemical cycles, which can often result in indirect regulation of growth rate, such as in the phosphorus cycle, where the trans-encoded SgrS sRNA present in both *Escherichia coli* and *Salmonella typhimurium* influences cellular phosphate uptake. SgrS becomes highly expressed when the bacteria are exposed to stress-inducing concentrations of the common cellular substrate; glucose 6-phosphate (Vanderpool and Gottesman, 2004). When activated through interaction with the Hfq chaperone, the trans-encoded SgrS molecule binds with the ptsG mRNA - a precursor to a key component of the glucose transporter EII, - inhibiting the synthesis of phosphate transporters, and stabilising the cell from glucose-phosphate stress (Wadler and Vanderpool, 2009; Papenfort and Vanderpool, 2015).

The Tricarboxylic acid (TCA) cycle is an important biological process for bacteria which rely on respiration for ATP production rather than solely on fermentation. GadY is an sRNA that is associated with the acetate production stage of the TCA cycle. In *E. coli* the Hfq-associated GadY sRNA is activated as an acid stress response at an acidity of around pH 4-6 (Opdyke et al., 2004). GadY downregulates the glutamate decarboxylase system (GDS), which is one of *E. coli*'s four proton-consuming acid resistance systems (Kashiwagi et al., 1991; Foster, 2004; Tetsch et al., 2008). GDS produces acetate, an acid, and is responsible for acid response in the late exponential phase (Biase et al., 1999). During a pH 6, high-cell density experiment, GadY deactivated the GDS system, resulting in reduced acetate production and improved growth of *E. coli* (Negrete and Shiloach, 2015).

3.1.2 Nitrogen cycle sRNA targets

Most pathways within the nitrogen cycle are regulated by sRNAs, with anammox and nitrification being the only pathways where no sRNA regulation has been identified. However, it has only been more recently that discoveries of sRNAs' role in responding to fluctuating environmental nitrogen availability or as regulators of nitrogen fixation have been unearthed (Prasse and Schmitz, 2018; Moeller et al., 2021).

NfiS is an Hfq-dependent sRNA directly involved in nitrogen fixation. NfiS was identified in *Pseudomonas stutzeri* and its presence was found to cause up to a 150% decrease in nitrogenase activity (Zhan et al., 2016). Based on computational predictions, NfiS is predicted to bind to the 5′ region of the *nifK* mRNA, responsible for a subunit of the nitrogenase enzyme, and improve the stability of the mRNA enough to increase translation efficiency (Prasse and Schmitz, 2018). NfiR was later identified by the same research group as another Hfq-dependent sRNA that is induced under nitrogen-fixing conditions(Zhan et al., 2019). NfiR binds with the *nifD* mRNA, which encodes another key subunit of the nitrogenase enzyme. This study proposed that NfiR and NfiS work in concert at the translational level to optimise nitrogenase production in *P. stutzeri*. Several other sRNAs have also since been identified as being involved in nitrogen fixation responses; Arrf in *Azotobacter vinelandii* (Muriel-Millán et al., 2014), $sRNA_{154}$ (Prasse et al., 2017) and $sRNA_{41}$ (Buddeweg et al., 2018) in *Methanosarcina mazei*, as well as the well conserved cyanobacterial sRNA NsiR1.

The *trans*-encoded sRNA NsiR1 found in the cyanobacteria *Anabaena* was the first known sRNA specifically upregulated in response to nitrogen depletion. NsiR1 controls the formation of nitrogen-fixing cells (heterocysts) when exposed to nitrogen starvation (Ionescu et al., 2010; Muro-Pastor and Hess, 2012; Zhao and Wolk, 2014). These heterocysts form from photosynthetic cells and provide fixed nitrogen to the surrounding non-differentiated cell mass. Located upstream of *hetF*, whose product is required for heterocyst formation, NsiR1 is predicted to regulate *hetF* expression and be an important regulator of nitrogen fixation and heterocyst formation in *Anabaena*. Another study computationally predicted the non-coding RNA transcriptome of several model cyanobacteria when under varying stress responses using RNA-seq and identified NsiR2, NsiR3 and NsiR4 (originally Syr10-12 respectively, (Voß et al., 2009). Along with NsiR1, sRNAs NsiR2 and NsiR3 have also been identified in *Anabaena*, however other than being responsive to nitrogen stresses their functions are unknown (Mitschke et al., 2011). NsiR4, identified from *Synechocystis* 6803, is an sRNA induced under nitrogen-depleted conditions and was the first identified sRNA regulating the assimilation of a macronutrient (Klähn et al., 2015). NsiR4 interacts with the 5′ UTR of the *gifA* mRNA, which encodes the inactivating factor (IF7) for glutamine synthetase, a key enzyme in biological nitrogen assimilation. Furthermore, a negative relationship was found in *Synechocystis*, whereby an increase in NsiR4 resulted in decreased

levels of IF7, leading to an increase in the assimilation of ammonium.

NalA is a cis-acting sRNA initially identified in *P. aeruginosa* through sRNAPredict2, a program developed to identify putative sRNA-encoding genes from bacterial genomes (Livny et al., 2006). With this prediction, NalA was experimentally found to have a putative binding site with both σ^{54}, a transcription initiator of nitrogen metabolism genes, and NtrC, a nitrogen response regulator (Romeo et al., 2012). Due to its putative targets, NalA was proposed as a regulator of nitrogen assimilation. This was further supported by the lower rate of *nalA* promoter activity when *P. aeruginosa* was cultured in the presence of ammonium or glutamine. However, this activity increased substantially when grown in the presence of nitrate, nitrite or glutamate, each required for a stage of nitrogen assimilation. A very similar acting sRNA, NrsZ, has also been identified in *P. aeruginosa* PAO1 (Wenner et al., 2014). It is also expressed most in nitrogen-limited conditions, however, rather than regulating, it is proposed to be regulated by σ^{54} and NtrC. NrsZ was shown to bind and upregulate the *rhlA* mRNA, a gene cluster responsible for the production of swarm motility apparatus. It is hypothesised that when *P. aeruginosa* is exposed to nitrogen-limited conditions, swarm mobility apparatus is produced through the activation of NrsZ, allowing the bacteria to adapt to environmental niches and prevent nitrogen starvation (Prasse and Schmitz, 2018). Another Pseudomonad, *P. pseudoalcaligenes* CECT5344; a model wastewater strain for studying nitrogen metabolism, contains $sRNA_{14}$, an sRNA expressed most in the presence of ammonium, with putative targets of NitH; an arsenate reductase gene, NitC, and glutamine synthetase based on analysis with the TargetRNA2 software (Olaya-Abril et al., 2019).

In a study researching P. aeruginosa PA14, RNA-Seq identified the sRNA PaiI as the strain's most highly expressed sRNA when cultured under anaerobic conditions (Tata et al., 2016). *paiI* transcription is predicted to be controlled by both nitrate accumulation and NarL, one-half of the NarXL two-component system responsible for activating the respiratory nitrate reductase operon, as well as repressing transcription of other respiration pathways (Darwin and Stewart, 1996; Tata et al., 2017). Although the study was uncertain as to how PaiI directly regulates nitrite reduction, they hypothesised that PaiI and DNR (nitric oxide-dependent regulator) work together to regulate an as-of-yet unknown accessory factor, required for the function of nitrite reductase.

The regulation of the denitrification pathway by sRNAs could provide an answer to the high N_2O emissions from environmental denitrifiers, however, moving laboratory findings of relevant sRNAs to implementable environmental action is not an easy task.

3.1.3 Identification of sRNA in *P. denitrificans*

An earlier study used RNA-Seq to identify 167 putative sRNAs from the model denitrifier *Paracoccus denitrificans* under nitrous oxide emitting (N_2O) conditions. As explained previously in Section 2.2.2, N_2O emitting conditions were achieved by culturing *P. denitrificans* anaerobically,

with or without the addition of Cu. Additionally, all strains were cultured in identical aerobic conditions (Gaimster et al., 2016). From this experiment RNA-seq analysis was run to quantify the variation of expression for these putative sRNAs between the previously mentioned treatments, highlighting sRNAs that were differentially expressed between anaerobic, and high N_2O emitting conditions. Under these conditions, more than a third of the putative sRNAs were differentially expressed between aerobic, and anaerobic N_2O emitting conditions. From these, RT-PCR was used to further analyse the expression of the putative sRNAs, confirming that 16 were non-coding sRNAs. These sRNAs were characterised due to their proposed function in *P. denitrificans*' response to denitrifying conditions. Of the 16 sRNAs, one was characterised in further detail in a follow-up study (Gaimster et al., 2019). Here, they identified DenR (formerly sRNA-29), an antisense sRNA, as a novel regulatory pathway which regulates denitrification. DenR was found to be most highly expressed under aerobic conditions. Initial experiments showed that when DenR was overexpressed in *P. denitrificans* under N_2O emitting conditions, N_2O emissions were reduced. This was due to DenR stabilising the GntR-type transcriptional regulator NirR, which is itself responsible for regulating the denitrification rate, by repressing the gene expression responsible for the nitrite reductase enzyme *nirS*. Cultures with *denR* overexpressed limited N_2O emissions by downregulating *nirS*, halting the denitrification pathway at nitrite and causing its accumulation in the cell (Gaimster et al., 2019). Based on computational predictions, it was hypothesised that DenR regulates denitrification by stabilising the *nirR* mRNA and increasing translation, binding to a complementary 7-bp seed within the coding DNA sequence of *nirR*.

Overexpression of *denR* was achieved in this experiment by inserting *denR* into the pLMB509 plasmid, a taurine-inducible promoter which induces a 15- to 20-fold increase in expression (Tett et al., 2012). pLMB509 is a His-tagged expression vector for *Alphaproteobacteria* and overexpresses the enclosed sequence when exposed to 10 mmol/litre taurine. The sequences of the sRNAs were synthesised into pLMB509 and then conjugated into *P. denitrificans*.

sRNA11, a 90-bp long antisense sRNA, is the focus of this chapter and was one of the 16 previously mentioned sRNAs identified in *P. denitrificans* when cultured under denitrifying conditions (Gaimster et al., 2016). It was identified as being of interest due to its greater than 2-fold increase in expression from low to high N_2O emitting conditions, as well as its 272-fold decrease in expression from low N_2O to aerobic conditions (Fig. 3.1). This shows that sRNA11 is increasingly expressed when culturing *P. denitrificans* in N_2O emitting conditions, making it an ideal candidate sRNA for further characterisation. This chapter aims to characterise sRNA11 further, ascertain whether it has a role in the regulation of denitrification and identify possible targets that would explain any observed phenotype.

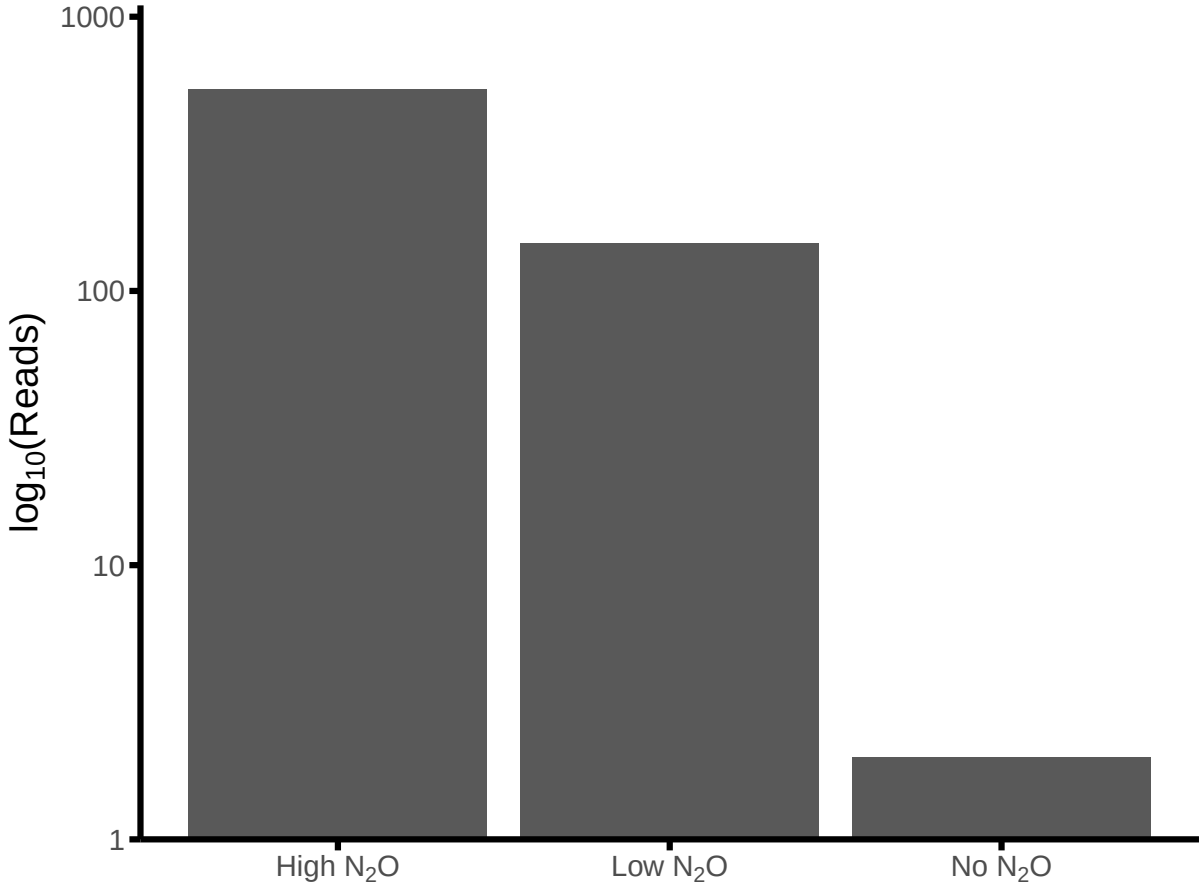

Figure 3.1: Normalised number of reads of sRNA11 in *P. denitrificans* when cultured under conditions producing; 2 to 3 mmol/litre N_2O (High N_2O), <1 mmol/litre N_2O (Low N_2O), and aerobic conditions where no N_2O was produced (No N_2O). Figure produced using RNA-seq data from Supplementary Table S1 from Gaimster et al. (2016).

3.2 Results

3.2.1 Aerobic growth of *P. denitrificans* with sRNA11 overexpressed

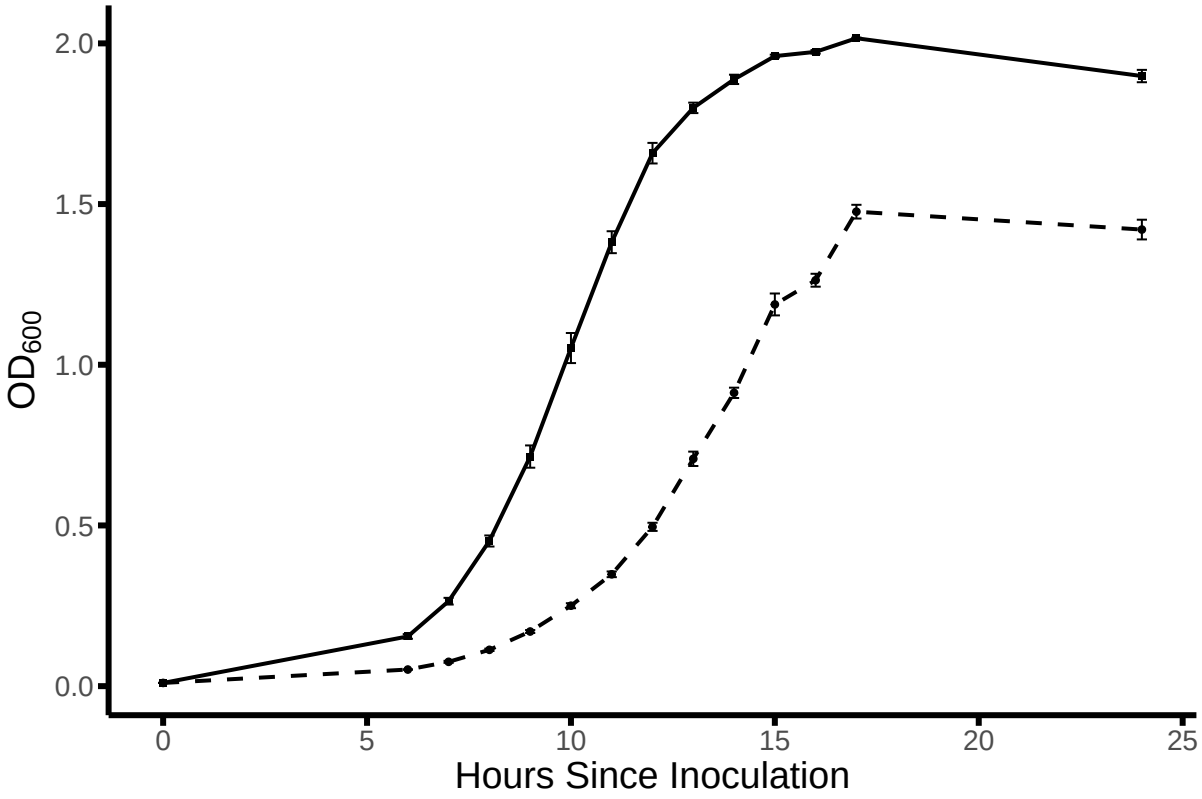

Figure 3.2: sRNA11 overexpression in *P. denitrificans* causes reduced growth when cultured in aerobic conditions on rich media. Six cultures of *P. denitrificans* were grown under aerobic conditions in Luria-Bertani broth at 30 °C. Three were cultured with *P. denitrificans* containing the pLM509 plasmid expressing sRNA11 (▪▪▪▪▪), and three were cultured containing an empty pLMB509 plasmid (—). Error bars represent the standard deviation of each result between triplicate experiments. Where not visible, error bars are smaller than the symbols.

To analyse whether sRNA11 has any impact on aerobic, high resource growth, *P. denitrificans* containing an empty pLMB509 plasmid (Pd_empty) and *P. denitrificans* containing the pLMB509 plasmid expressing sRNA11 (Pd_11) were cultured in triplicate aerobically on rich media (LB) as described in Section 2.2. Samples were taken at inoculation as well as every hour from 6 hours until 17 hours into the experiment. Figure 3.2 shows that sRNA11 overexpression slowed the growth rate of *P. denitrificans*, extending the lag phase by approximately three hours and reducing the rate of growth throughout the experiment, when compared to the control group. It also reduced the final mean peak cell density from an OD_{600} of 2.016 ± 0.001 to 1.476 ± 0.017.

3.2.2 Anaerobic growth and N_2O production of *P. denitrificans* with sRNA11 overexpressed

With the aerobic reduced growth phenotype identified, the next step was to analyse how Pd_11 responded to anaerobic growth on a minimal media, this would also allow for the extraction of headspace gas samples to measure N_2O concentration. Pd_empty and Pd_11 were both cultured anaerobically on minimal media (Fig. 3.3). In this experiment, Pd_11 displayed a significantly reduced growth rate within 2 hours of inoculation when compared to Pd_empty. Pd_empty peaked after 27 hours of growth at a cell density (OD_{600}) of 1.1 ± 0.22, compared to Pd_11 at the same time point with a cell density of 0.04 ± 0.005. This growth profile was one of four observed growth phenotypes of Pd_11, labelled as "Minimal Growth" due to the inability of the strain to culture beyond an OD_{600} of 0.1 (Table A.1). This phenotype had a 19% frequency rate from 21 growth experiments, with the most common being "No Growth" (52%), where the strain dies during the experiment and does not recover. This phenotype will not facilitate the exploration of the role of sRNA11 on the denitrification pathway due to the inability of the small population to reduce sufficient amounts of nitrate for N_2O analysis. As such, focus will be given to the observed "Strong Growth" phenotype which requires a growth density of at least 0.5 OD_{600} being reached during the experiment. This phenotype had a 14% occurrence rate across all anaerobic growth experiments.

Pd_empty and Pd_11 were both cultured anaerobically alongside a *P. denitrificans* strain lacking the *nosZ* gene and containing the empty pLMB509 plasmid (PdΔ*nosZ*). Therefore, PdΔ*nosZ* could not synthesise the NosZ enzyme and would not be able to reduce N_2O, acting as a control for the maximum concentration of N_2O that *P. denitrificans* can produce. These were cultured in triplicate under denitrifying conditions on minimal media containing 20 mmol/litre nitrate, 30 mmol/litre succinate, 10 mmol/litre taurine and with Cu-low Vishniac and Santer trace element solution (Section 2.2) (Sullivan et al., 2013). Media and gas samples were taken at 0, 8, 11, 14, 17, 20 and 32 hours from the point of inoculation (Fig. 3.4). Similarly to aerobic growth, Pd_11 cultures at a slower rate than Pd_empty, however, it reaches a similar peak cell density to Pd_empty after 32 hours from inoculation, this only occurs in the "Strong Growth" phenotype of Pd_11 (Table A.1). PdΔ*nosZ* cultured at a variable rate, all recovering to the same peak value of growth during the stationary phase.

Gas samples were immediately stored and analysed using gas chromatography as described in Section 2.3. N_2O production was found to increase for Pd_empty and PdΔ*nosZ* over the initial 30 hours, peaking at 32 hours from inoculation (Fig. 3.5). PdΔ*nosZ* produced the most N_2O throughout the experiment, peaking at 3.83 ± 1.655 mmol/litre N_2O, compared to 2.18 ± 1.471 mmol/litre for Pd_empty. Pd_11 peaked at 0.42 ± 0.121 mmol/litre N_2O 16 hours from inoculation before reducing to negligible values throughout the remainder of the experiment. A paired *t*-test was performed to test whether there was a significant difference in N_2O accumulation

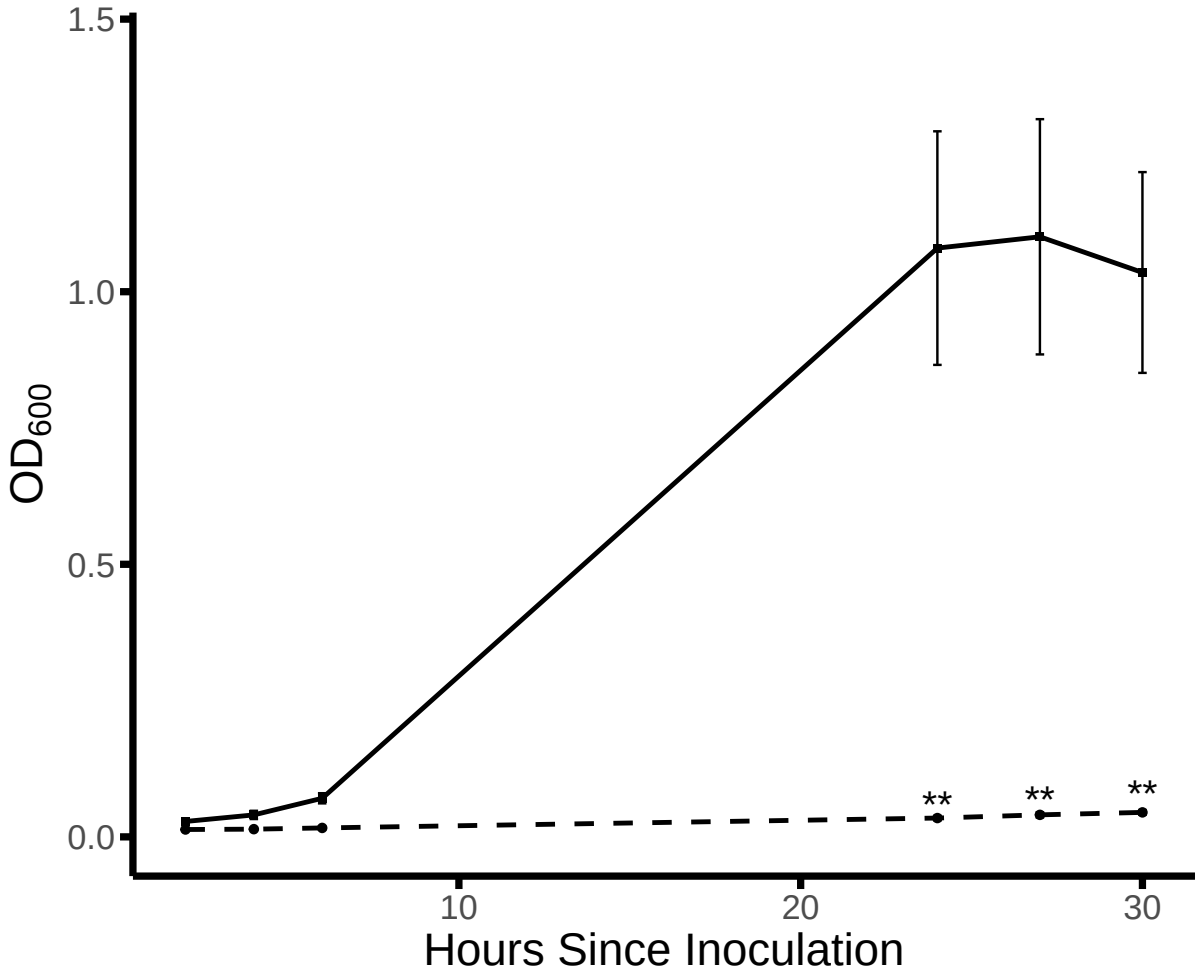

Figure 3.3: Example of how sRNA11 overexpression in *P. denitrificans* can cause extremely reduced growth when cultured in anaerobic, denitrifying conditions. Six cultures of *P. denitrificans* were grown under denitrifying conditions in minimal media at 30 °C. Three were cultured containing an empty pLMB509 plasmid (━━), three were cultured with *P. denitrificans* containing the pLM509 plasmid expressing sRNA11 (▪▪▪▪). Asterisks indicate statistical significance between treatment and Pd_empty: $^{*}p < 0.05$, $^{**}p < 0.005$, non-significant differences ($p \geq 0.05$) are not marked. Error bars represent the standard deviation of each result between triplicate experiments. Where not visible, error bars are smaller than the symbols.

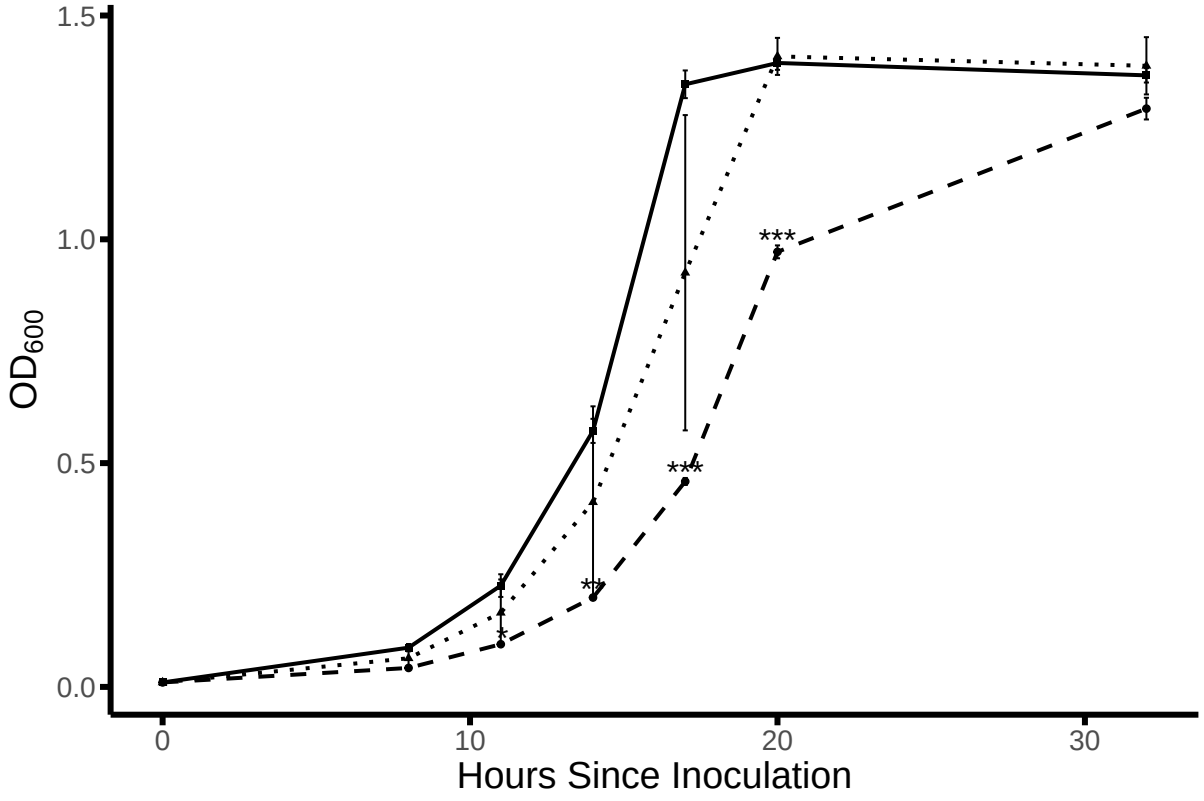

Figure 3.4: sRNA11 overexpression in *P. denitrificans* causes reduced growth when cultured in anaerobic, denitrifying conditions. Nine cultures of *P. denitrificans* were grown under denitrifying conditions in minimal media at 30 °C. Three were cultured containing an empty pLMB509 plasmid (—), three were cultured with *P. denitrificans* containing the pLM509 plasmid expressing sRNA11 (▪▪▪▪▪), and three were cultured in a Δ*nosZ* knockout mutant containing an empty pLMB509 plasmid (▪ ▪ ▪). Asterisks indicate statistical significance between a treatment and Pd_empty: $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$, non-significant differences ($p \geq 0.05$) are not marked. Error bars represent the standard deviation of each result between triplicate experiments. Where not visible, error bars are smaller than the symbols.

between the sRNA11 and (PdΔ*nosZ*) after 32 hours of growth ($t(2) = -4.79$, $p < 0.05$). The mean difference was −4.10 (95% CI: −7.77 to −0.42). These results suggest that *srna11* and *nosZ* exhibit significantly different mean values for N_2O accumulation. No other stages of N_2O accumulation were statistically significant. Up to that point, Pd_11 was producing N_2O at a similar rate to the other strains. All strains reduced their levels of N_2O from 32 to 40 hours from inoculation.

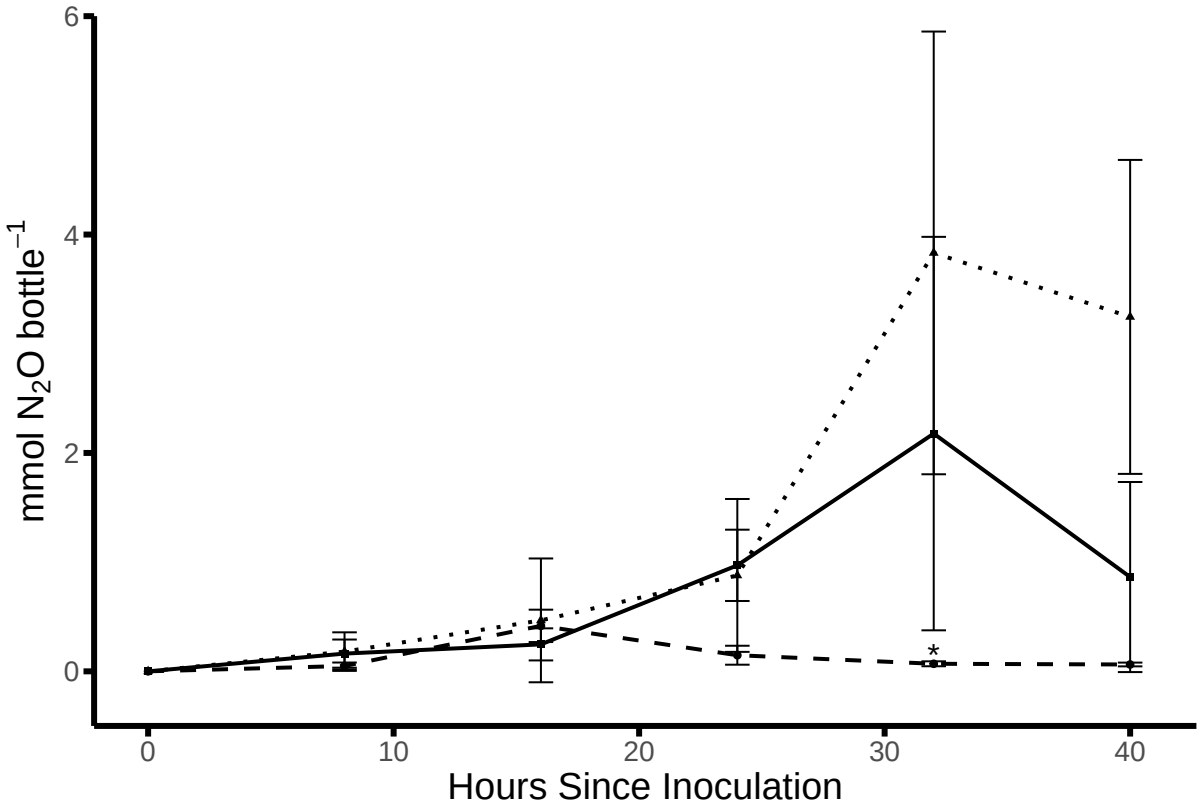

Figure 3.5: Accumulation of N_2O during growth in minimal media under denitrifying conditions. *P. denitrificans* with an empty pLMB509 vector (▬), Δ*nosZ* mutant *P. denitrificans* containing an empty pLMB509 vector (▪ ▪ ▪), and *P. denitrificans* with pLMB509 cloned with sRNA11 into the overexpression site (▪▪▪▪▪). N_2O production measured in mmol/litre. Asterisks indicate statistical significance: $^*p < 0.05$, non-significant differences ($p \geq 0.05$) are not marked. Error bars represent the standard deviation of each result between triplicate experiments. Where not visible, error bars are smaller than the symbols.

3.2.3 Impact of taurine on sRNA11 growth inhibition

To understand whether the growth regulation of sRNA11 was taurine dependent Pd_11 was cultured anaerobically in minimal media containing 20 mmol/litre nitrate, 30 mmol/litre succinate

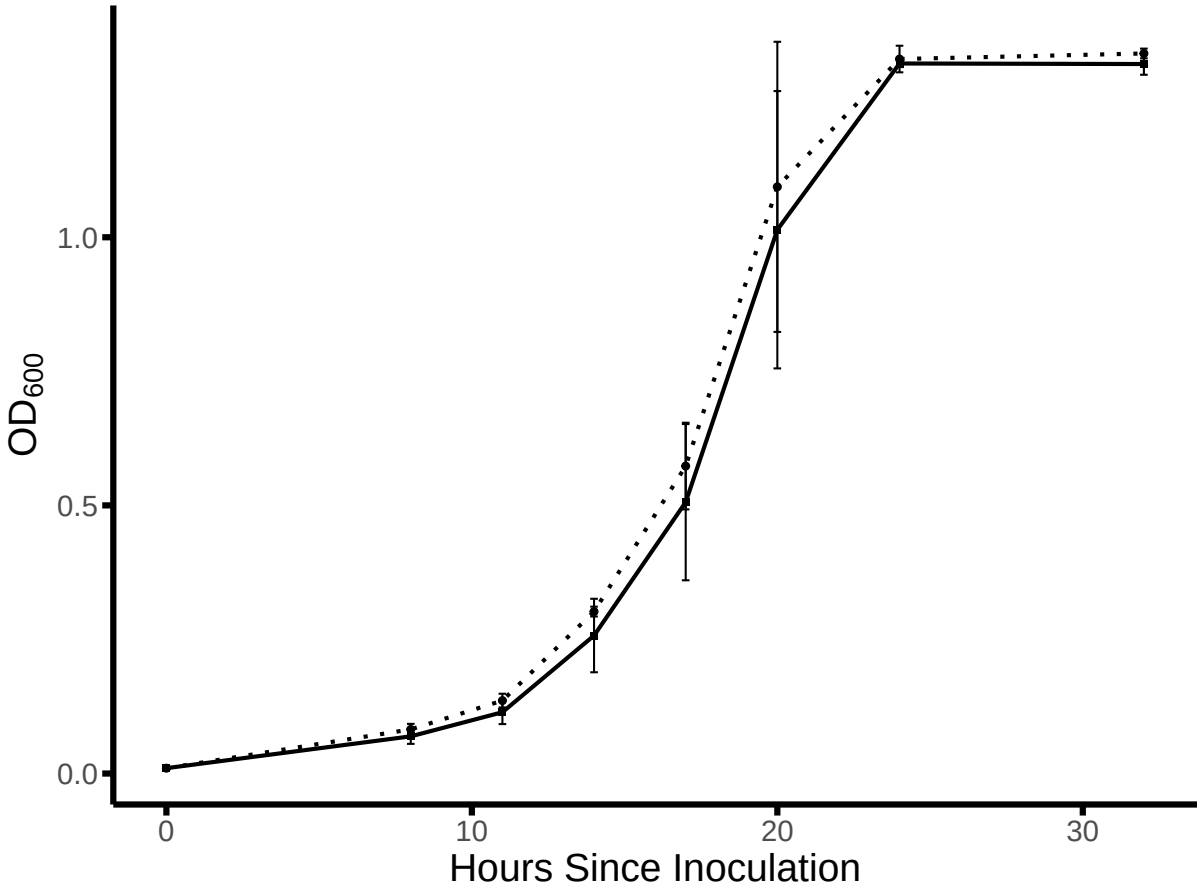

Figure 3.6: Six cultures of *P. denitrificans* with pLMB509 plasmid containing sRNA_11 were grown under denitrifying conditions (20 mmol/litre nitrate as electron acceptor) for 32 hours at 30 °C. Three cultures had 10 mmol/litre taurine added at 0 hours (—), whilst other 3 were cultured in the absence of taurine (▪▪▪▪). Error bars represent the standard deviation of each result between triplicate experiments. Where not visible, error bars are smaller than the symbols.

and with Cu-low Vishniac and Santer trace element solution (Section 2.2). Treatments were run in triplicate and were treated with or without 10 mmol/litre taurine present. There was no significant difference between the two treatments in their growth rate (Fig. 3.6). However, the pLMB509 expression vector can leak low concentrations of the overexpression target. This has been the case in this experiment as the growth phenotype is visible even when overexpression is not induced. Therefore sRNA11 will cause a significant growth phenotype even at low expression.

3.2.4 Computational approaches to sRNA11 target prediction

With a growth and N_2O accumulation phenotype, further analysis was needed to identify possible mRNA targets for sRNA11. To do this, computational prediction programs were used which could search the *P. denitrificans* genome for mRNA with complementary seed regions. Table 4 shows the five likeliest predicted gene targets of sRNA11 using TargetRNA2 (Kery et al., 2014). Pden_4486 was identified by the program as *lpxC* and was predicted to be the most likely gene targeted by sRNA11 due to the energy value of −13.11 kcal/mol. None of the five listed TargetRNA2 predicted targets were also predicted targets when using sRNARFTarget, with Pden_2958 being the most likely with a probability of 0.44095. Similarly, of the top five candidate interactions for sRNA11 using sRNARFTarget, none of them was recognised as a possible target by TargetRNA2. Of those identified by sRNARFTarget, the most likely was Pden_1962 (Tab. 5), a hypothetical protein that shares 97% sequence similarity to a gene responsible for quorum sensing.

3.3 Discussion

3.3.1 The role of sRNA11 in growth and N_2O production

Figures 3.2 & 3.4 both show that the overexpression of sRNA11 in *P. denitrificans* results in a significantly reduced growth rate, we have also shown that leakage from the pLMB509 plasmid with no taurine present is still sufficient to impact the growth of the bacteria (Fig. 3.6). This implies that the presence of sRNA11 in the cell is not beneficial to the survivability of the cell and may be responsible for initiating apoptotic cell death. This would be the most extreme function of sRNA11, however, the observed impact on both aerobic and anaerobic growth means that it may not be directly involved in the regulation of respiration pathways when under oxic stress. However, this does not answer why in *P. denitrificans*, sRNA11 shows a 272-fold change increase in expression from aerobic to high N_2O producing anaerobic conditions (Fig.3.1) (Gaimster et al., 2016). Such a dramatic reduction in expression when in aerobic conditions implies that sRNA11 has an important role in the cellular response to changing oxic environments. There is some likelihood that this role involves reducing N_2O accumulation rates, and therefore interaction

Table 4: Top five predicted gene targets of *Paracoccus denitrificans* sRNA11 using the TargetRNA2 software (Kery et al., 2014). Gene refers to the relevant gene name in the *P. denitrificans* genome. Name is given when a study has designated the gene with a name, Hyp. protein is shown when listed as a hypothetical protein in the genome annotation file. Energy refers to the thermodynamic energy (kcal/mol) of the hybridisation between sRNA11 and the mRNA molecule of the listed gene. *p*-value is the probability of whether this interaction would form by chance. Function is determined by gene annotation or NCBI BLAST analysis (Altschul et al., 1990).

Gene	**Name**	**Energy**	***p*-Value**	**Function**
Pden_4486	*lpxC*	-13.11	0.003	Zinc-dependent metalloamidase (Whittington et al., 2003)
Pden_1370	Hyp. protein	-12.9	0.004	Unknown
Pden_2562	Hyp. protein	-12.69	0.005	Unknown
Pden_2958	*iscR*	-12.29	0.006	Rrf2-type transcriptional regulator (Midorikawa et al., 2009)
Pden_0183		-11.89	0.008	D-amino acid dehydrogenase

with the denitrification pathway, as after the exponential phase of growth, Pd_11 stopped accumulating N_2O in the flasks (Fig.3.5). In comparison, both Pd_empty and PdΔ*nosZ* produced substantially more N_2O once entering the stationary phase of growth, although the diverse range of values from these triplicate experiments does leave some uncertainty on just how much N_2O is being produced by these strains. A previous study recorded peak N_2O of 3 to 4 mmol/litre for Pd_empty after 48 hours of growth (Gaimster et al., 2019), however, their data shows a continued increase in N_2O accumulation throughout the experiment. In comparison, the data presented here shows a drop-off in N_2O accumulation from 32 to 40 hours. This is unlikely a result of N_2O reduction, as this is also found in the PdΔ*nosZ* strain which lacks the gene encoding for the NosZ enzyme, making it incapable of reducing N_2O to N_2. Instead, this is predicted to be a result of minor leakage from the Duran bottles used as growth chambers. This could be due to internal pressure rising beyond their capabilities or, more likely, due to gas escaping through the small holes perforated into the silica septa throughout the experiment. The internal pressure of the flasks was an issue during the experiment due to its role on N_2O calculations, as the flasks increased pressure throughout the experiment. This will have had impacts on the calculated N_2O concentrations as 3 mL of headspace was always extracted. As such, the reported N_2O concentrations may be higher than the actual values. This likely impacted readings, as 200 mL

Table 5: Top five predicted gene targets of *Paracoccus denitrificans* sRNA11 using the sRNARFTarget software (Naskulwar and Peña-Castillo, 2022). Gene refers to the relevant gene name in the *P. denitrificans* genome. Name is given when a study has designated the gene with a name, Hyp. protein is shown when listed as a hypothetical protein in the genome annotation file. Probability refers to the likelihood of interaction between the sRNA-mRNA pair. Function is determined by gene annotation or NCBI BLAST analysis (Altschul et al., 1990).

Gene	**Name**	**Probability**	**Function**
Pden_1962	Hyp. Protein	0.5915	Quorum sensing (Yasuda et al., 2022)
Pden_0580	*mraZ*	0.5793	Transcriptional regulator (Eraso et al., 2014)
Pden_2214		0.57743	Unknown Function
Pden_3733		0.56877	Unknown Function
Pden_4395		0.56787	Sulfate adenylyltransferase

of 20 mmol NO_3^-, is equivalent to 4 mmol NO_3^- per flask. The stoichiometry would then suggest that this could only produce a maximum of 2 mmol of N_2O per flask. This suggests that some part of the calculation used to determine N_2O concentration is incorrect, and I believe that the most likely source is the pressurisation of the headspace. The septa we used are designed to be punctured with 22G needles or thinner to reduce the chance of leakage, however, at the end of an experiment, these septa have been punctured 15 times due to sparging, media removal, and gas extraction. As such, there is the possibility that a loss of internal pressure would occur, causing the observed dip in N_2O levels towards the end of the experiment, however, it is unlikely that this dramatically impacted Pd_11.

To reduce the number of punctures to the septa throughout an experiment, several tests were run using 16.5 mL anaerobic Hungate tubes, however, culturing *P. denitrificans* in Hungates resulted in a substantially higher rate of experimental failure due to inoculant death. Cultures would often fail to grow, or culture at extremely varied rates, resulting in low precision and unrepeatable results. We believe this is a result of the lower media volumes within the Hungates compared to the Duran bottles. Operating with a lower volume of media meant that any contaminants attached to the Hungate tubes from washing or previous experiments would have a greater impact on the growth media, and due to *P. denitrificans*' temperamental growth, often resulted in stunted or unsustainable growth.

Additionally, to analyse exactly how sRNA11 is causing decreased N_2O accumulation, further research is required to measure the levels of denitrification intermediates throughout the experiment. This would elucidate whether N_2O is either not being produced, or being completely

reduced when sRNA11 is overexpressed. Quantification of sRNA11 overexpression should also be carried out, to ascertain how much of the molecule is leaking from the pLMB509 vector when no taurine is added.

3.3.2 Difficulties of working with sRNA11

A major issue of working with Pd_11 is a result of sRNA11's clear impact as a growth inhibitor. Pd_11 was often unable to culture on all types of media and had a shorter deep freeze (−80 °C) lifetime. This presented constant issues when it came to collecting the data in this chapter. Many experiments were cut short due to overnight cultures not growing, or due to the strain not taking to the LB broth or minimal media. The data presented in this chapter is focused on one of the main phenotypes of sRNA11 overexpression, the "Strong Growth" phenotype as it allows for the analysis of N_2O accumulation data which is not possible with the other growth phenotypes. The frequency of these phenotypes is recorded in Table A.1. The other observed phenotypes all had a reduced growth rate than the "Strong Growth" phenotype, often resulting in Pd_11 colonies dying early into experiments.

These difficulties may be due to sRNA11 having a cytotoxic impact on the cell, and small concentrations of the molecule can kill enough of the cells to impact the community. sRNAs responsible for regulating cytotoxic processes have been identified before, such as IstR1 in *E. coli* which is an important, constitutively expressed sRNA responsible for inhibiting the *tisB* gene. TisB is a peptide toxin with a cytotoxic effect where it targets its own cell's membrane. Its production is triggered by DNA damage and is part of the final stages of the SOS response in *E. coli* should DNA repair not be achievable, resulting in programmed cell death (Gerhart et al., 2012). In the circumstances that sRNA11 is instead an activator of programmed cell death then it would explain why its production by the cell results in a growth inhibition.

3.3.3 Have prediction software offered possible candidates?

The predicted mRNA candidates for sRNA11 determined by TargetRNA2 and sRNARFTarget offer interesting options for possible targets. TargetRNA2 is a web server capable of identifying sRNA-mRNA interactions, it uses the predicted sRNA secondary structure, conservation of the sRNA in other bacteria, and the hybridization energy between the sRNA and the secondary structure of any mRNAs to predict likely candidates (Kery et al., 2014). TargetRNA2 predicted the most likely candidate mRNA interaction for sRNA11 was Pden_4486 (Tab. 4). Pden_4486 also known as *lpxC* encodes the enzyme UDP-(3-O-(*R*-3-hydroxymyristoyl))-*N*-acetylglucosamine deacetylase (LpxC). LpxC is an essential, single-copy gene that is found in almost all gram-negative bacteria and as such has been considered as a possible antibiotic target (Zhou and Barb, 2008). Studies

have previously targeted LpxC to successfully inhibit growth in *E. coli*. This is because of LpxC's role in lipid A biosynthesis (Luo et al., 2006). Interestingly when *E. coli* LpxC activity is increased or decreased through the use of knockout mutants or overexpression vectors, it was found to always be fatal to the cell (Führer et al., 2006). This makes the *lpxC* mRNA an interesting target for sRNA11 and would fit with the observed phenotype.

TargetRNA2 also identified Pden_2958 (*iscR*), an Rrf2-type transcriptional regulator as the fourth most likely target for sRNA11. This family of regulators are poorly characterised in *P. denitrificans*. Identified initially as an iron-responsive regulator, it is closely tied to other Rrf2-type regulators which have functions in the denitrification pathway (Olaya-Abril et al., 2022). In *E. coli*, IscR represses the *iscRSUA* operon, which is partly responsible for the assembly of Fe-S clusters in many proteins, including IscR (Tokumoto and Takahashi, 2001). Studies have also found that IscR will form a regulatory response to nitrosative stress, a result of the sensitivity of the Fe-S cluster (Py et al., 2011; Choi et al., 2020). This relationship with Fe-S makes it particularly interesting, as several enzymes relevant to the denitrification process require Fe-S clusters. One of these is the transcriptional regulator FnrP, which is responsible for switching *P. denitrificans* to anaerobic respiration in response to oxygen (Section 1.4.1. Fe-S cluster-containing proteins also include NosR, responsible for regulating the *nos* operon, the nitrate reductases NarGHI and many other proteins important for cellular processes.

sRNARFTarget takes a more modern approach to target prediction, relying on machine learning techniques (Naskulwar and Peña-Castillo, 2022). This tool is built using the Random Forest Model (RFM) and is trained from a trinucleotide frequency difference dataset, ensuring it identifies high likelihood sRNA-mRNA interactions. This dataset contains the 745 confirmed sRNA-mRNA pairs from 37 bacterial species, gathered using a variety of techniques (RNA-seq, MAPs, GRIL-seq, CLASH-seq, and RIL-seq). The core function of an RFM is to build multiple decision trees, determined by the feature, where each branch of the tree is close to 50% probability. The max depth of these trees is often varied according to the function of the research, however, for this model it was limited to nine. A review of all the current computational sRNA target tools found that sRNARFTarget was the most accurate software when working with sRNAs lacking three or more homologs, making it the ideal software for sRNA11 (Grešová et al., 2022). The mRNA target with the highest predicted probability given by sRNARFTarget was Pden_1962, which encodes for a hypothetical protein that has been associated with a range of quorum sensing genes in *P. denitrificans* (Yasuda et al., 2022). There is little information about this gene and how it functions in *P. denitrificans*, however, regulation of quorum sensing could impact the growth rate as some quorum sensing proteins are responsible for telling the cell that nearby populations are low and therefore growth should be prioritised (Dubern et al., 2023). If Pden_1962 were to be involved in this process, then it could explain the growth phenotype observed.

Of more interest is the second predicted mRNA target for sRNA11, Pden_0580 (*mraZ*). MraZ

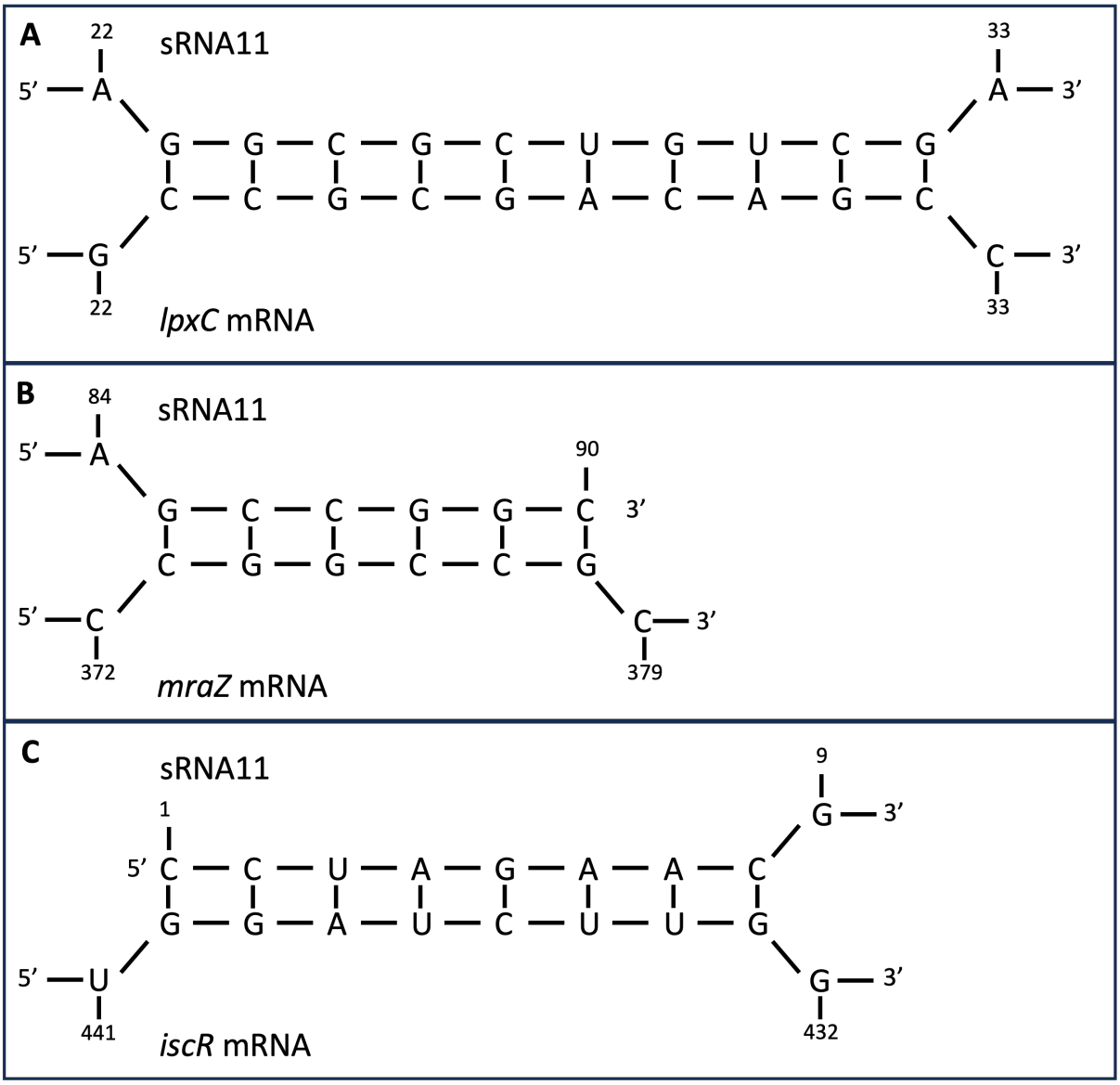

Figure 3.7: Complementary binding sites of three computationally predicted mRNA targets of the *P. denitrificans* sRNA11. A) 10-bp complementary region between sRNA11 and the *lpxC* mRNA. B) 6-bp complementary region between sRNA11 and the *mraZ* mRNA. C) 6-bp complementary region between sRNA11 and the *iscR* mRNA.

is an enzyme thought to have responsibilities for cell division and cell envelope biosynthesis, due to its location as the first gene in the *dcw* gene cluster (Hara et al., 1997). When MraZ was overproduced in *E. coli*, it was found to inhibit cell division and even be lethal in rich media when greatly reduced and minimal media when slightly reduced (Eraso et al., 2014). These findings link particularly well with what has been shown in this chapter and could therefore be the most convincing argument for how sRNA11 regulates anaerobic growth. This is made even more promising by the identification of a 6-bp long seed region between sRNA11 and the *mraZ* mRNA (Fig. 3.7B). Generally, a contiguous seed region of at least 6-8 base pairs in length is sufficient for sRNA-mRNA complex formation (Gottesman and Storz, 2011a). One issue may be that bacterial sRNAs have usually been found to base pair with the 5′ end of mRNAs, not the 3′ end. However, a recent review of bacterial sRNA regulation found that far more sRNAs are derived from mRNA 3′ ends than previously thought (Ponath et al., 2022). Of the three discussed targets of sRNA11, only the *lpxC* mRNA is being bound with at the 5′ end (Fig. 3.7A), with the others favouring the 3′ end. The longer contiguous seed regions identified between sRNA11 and *lpxC* of 10-bp does not definitively make it more likely to form a complex, as it is rare for seed regions to reach such a length. However, it may show that there are more opportunities for the RNAs to form a complex.

Despite these strong findings from sRNARFTarget it has been identified as having an aversion to sRNAs with many homologs. This does seem to have a major impact on the predicted probability of interaction as sRNARFTarget predicted only a 0.36904 probability of the *denR* sRNA interacting with its experimentally proven target NirR (Gaimster et al., 2019). *denR* has nine known homologs which may show the difficulty that sRNARFTarget has with highly conserved sRNAs. sRNARFTargets' focus on trinucleotide frequency simplifies the target prediction process and unlike many other target prediction models it does not consider secondary structures when predicting targets. They found that when the RFM also used secondary structure distances in the feature set, the model performance did not improve, but the analysis time drastically increased.

Preliminary analysis using the CopraRNA and IntaRNA sRNA target prediction tools also suggested mRNA targets that did not support findings from TargetRNA2 or sRNARFTarget, offering mRNAs that were considered extremely unlikely targets in these programs. As such it is difficult to say with any certainty that the use of these programs should be for confirming any experimentally found sRNA-mRNA interaction, but only as a guide to identifying possible targets to further analyse experimentally. This would be done by looking at the expression levels of these putative targets when overexpressing or knocking out sRNA11 from *P. denitrificans* through qRT-PCR. Or by measuring the interactions between sRNA11 and the target mRNAs, which could be done with EMSA or CLASH experiments.

4 Contribution of Hfq to sRNA mediated regulation of denitrification.

4.1 Introduction

4.1.1 Role of RNA chaperones

RNA chaperones are a form of RNA-binding proteins that are found in nearly all forms of life and are often responsible for influencing RNA-RNA interactions (Jarmoskaite and Russell, 2014). They can assist in the folding and structural rearrangements of RNA molecules in a similar way to how molecular chaperones act on protein folding. RNA chaperones have significant importance in ensuring the proper folding, stability and function of RNA molecules, which are essential in various cellular processes, including gene expression (Yoon et al., 2008), RNA processing (Rajkowitsch et al., 2007) and RNA-based regulation (Woodson et al., 2018). Furthermore, the loss of RNA chaperones can cause reduced stress tolerance (Gottesman et al., 2006), slowed bacterial growth (Sobrero and Valverde, 2012) and reduced virulence (Chao and Vogel, 2010).

The function of RNA chaperones depends on the family of chaperones as well as the RNA they are interacting with. For instance, RNA chaperones can facilitate the folding of RNA secondary and tertiary structures, forcing RNA into a required shape and unlocking their functionality. An example is the peptide Tat(44–61), a fragment of the HIV-1 Tat protein, which efficiently anneals to RNA and DNA of varying sizes (Kuciak et al., 2008). Once annealed, its interaction with RNA phosphates results in alterations to the structure of the RNA, increasing the likelihood of RNA-RNA binding in the future (Doetsch et al., 2011). RNA chaperones also function by preventing misfolding and mispairing of RNA molecules, reducing the chance of non-functional or harmful structures forming. An example of this is the Hfq RNA chaperone, which is necessary to regulate the *Escherichia coli* DsrA untranslated RNA. DsrA itself regulates the global transcription factors RpoS and H-NS. A Hfq mutant showed DsrA was unstable due to Hfq acting as a cofactor for the regulatory activities of DrsA through altering the substrate's structure (Sledjeski et al., 2001). Also, RNA chaperones can assist in RNA-RNA interactions, such as by annealing complementary RNA strands. This is also shown in *E. coli* by Hfq, whereby Hfq cooperates with the sRNA Spot 42 and its target mRNA to encourage intermolecular base pairing between the pair (Moller et al., 2002).

4.1.2 FinO-family of RNA chaperones

FinO is an RNA binding protein that makes up half of the well-documented FinOP two-component system in *E. coli*, the other half being the antisense RNA; *finP*. Together this system regulates gene expression in the *E. coli* F-like plasmid, a 100-kbp long circular conjugative plasmid also known as the fertility factor due to its role in transferring genetic material between bacterium (Koraimann, 2018). *finP* is complementary to the 5′ UTR of the *traJ* mRNA, blocking ribosomal entry when bound to *traJ* (Koraimann et al., 1996). When FinO is absent during *E. coli* growth, FinP quickly

degrades allowing TraJ synthesis (Lee et al., 1992). With FinO present, FinP is stabilised against degradation and the *finP-traJ* duplex can form, blocking *traJ* translation (van Biesen and Frost, 1994). Unlike other RNA chaperones, FinO is believed to destabilise the base pairing within the stem of a single RNA (Arthur et al., 2003). These initial studies showed FinO in a class of its own for nearly two decades, however, recent studies have shown that there is instead a family of FinO-like RNA chaperones that are found in a wide range of bacterial species (Glover et al., 2015). One example is the *Neisseria meningitidis* protein, NMB1681, which, besides having structural and functional similarity to FinO, can also repress the conjugation transfer of the F-plasmid through interaction with the *finP* RNA (Chaulk et al., 2010). ProQ is another FinO family RNA chaperone, responsible for regulating the *E. coli* proline transporter ProP. The N-terminal half of ProQ is 35% identical to FinO, and various structure prediction servers claim a high similarity in crystal structures between the two proteins (Smith et al., 2004). ProQ also contains an Hfq-like C-terminal domain, allowing for increased duplexing activity by the protein (Chaulk et al., 2011). ProQ's primary purpose is to regulate ProP and ensure that *E. coli* maintains growth in high osmolality media by regulating osmotic response, but may also impact biofilm formation (Sheidy and Zielke, 2013). The role of ProQ may extend beyond the regulation of ProP as they are not linked amongst bacterial genomes, showing up in some species without the other (Smith et al., 2004). Additionally, the absence of ProQ in *E. coli* altered the uptake rate of other amino acid transporters, ProP was just the largest detected (Kunte et al., 1999).

4.1.3 Other RNA chaperones

StpA is a DNA-binding protein found in *E. coli*, known best as a suppressor of a mutant form of the phage T4 thymidylate-synthase gene (Zhang and Belfort, 1992). StpA was identified as an RNA chaperone with a possible role in promoting the assembly of RNA precursors into an active configuration, allowing RNA-mRNA binding to occur at a higher rate (Zhang et al., 1995; Clodi et al., 1999). StpA can even be removed after folding the RNA molecules without degradation to the RNA. Later studies observed that StpA can bind to RNAs with poor structural stability at a higher affinity (Grossberger et al., 2005). StpA prefers binding to unstructured RNA molecules, forming weak bonds and relying on transient interactions to chaperone the RNA (Mayer et al., 2007).

Carbon storage regulator A (CsrA) is a key global regulator of many bacterial cellular processes in *E. coli* and *Salmonella* as well as other species. It is also an RNA-binding protein that is often repressed by the well-conserved sRNAs CsrB and CsrC (Babitzke and Romeo, 2007). It was first identified as a repressor of glycogenesis in *E. coli* (Romeo et al., 1993), with later studies observing regulatory roles in biofilm formation and quorum sensing in *Vibrio cholerae* (Lenz et al., 2005). CsrA has structural homologs in RsmA and RsmE found in both *Pseudomonas* and

Erwinia species which perform similar post-transcriptional regulation (Cui et al., 1995; Pessi et al., 2001). In *Salmonella*, CsrA acts as a post-transcriptional regulator and its presence modulates mRNA translation of over 8% of all genes (Lawhon et al., 2003). Beyond this, CsrA has also been observed regulating the *Salmonella* pathogenicity islands (SPI)-1 a set of invasion genes required for successful host invasion (Altier et al., 2000). When the suppressant sRNAs *csrB* and *csrC* are absent, *S. enterica* Serovar Typhimurium had a near 100-fold decrease in host cell invasion in mice (Fortune et al., 2006). CsrA often works by binding to an mRNA's ribosome loading site, inhibiting translation. However, it can also be a positive regulator, shielding mRNA from RNase E degradation through repression of the RNase E-based degrader CsrD (Yakhnin et al., 2011). CsrD is a targeted degrader of the sRNAs CsrB and CsrC. Therefore, by repressing the expression of CsrD, CsrA indirectly contributes to the stabilization of its sRNA antagonists. Based on these various functions of CsrA it has been designated chaperone-like (Katsuya-Gaviria et al., 2022).

Similarly to ProQ, CsrA also interacts with Hfq synthesis, directly binding to and regulating the *hfq* mRNA (Stenum and Holmqvist, 2022). In this instance, it also acts by blocking the ribosome from binding to the mRNA, though it is the only occasion known of a CsrA-regulated gene containing only one CsrA binding site (Baker et al., 2007). Other similarities with Hfq include its ability to bind to the *E. coli* sRNA Spot 42, a regulator of carbohydrate metabolism and uptake (Bækkedal and Haugen, 2015). When bound to the sRNA, it prevents cleavage of Spot 42 leading to enhanced repression of Spot 42's regulatory targets (Lai et al., 2022).

4.1.4 Hfq: The most ubiquitous RNA chaperone

Hfq is the most ubiquitous and recognised RNA chaperone and is capable of binding to both sRNA and mRNAs (reviewed by Updegrove et al. (2016)). It was first identified in *E. coli* where it acts as a host factor for bacteriophage QB replication (Hfq), however, over half of all bacterial genomes encode for Hfq or its homologs (Sun et al., 2002). It has also been identified in Cyanobacteria where it forms fairly weak bonds with A/U-rich sections of regulatory RNAs (Bøggild et al., 2009). Hfq is encoded in gram-negative and gram-positive bacteria as well as in α, β, and γ-Proteobacteria (Sobrero and Valverde, 2012). The presence of Hfq has been correlated with genomes of high GC content due to the greater stability of RNA in these genomes. Hfq would be of increased use, as its role as a chaperone would ensure the RNA are activated into their functional states. This is assumed to be the reason that the majority of γ-proteobacteria encode *hfq* as their genomic GC content is 50-67% (Sonnleitner et al., 2008). Despite this, some bacterial species with low GC content still rely on Hfq as an RNA chaperone, such as *Staphylococcus aureus* (Liu et al., 2010), *Bacillus subtilis* (Hämmerle et al., 2014), and *Neisseria gonorrhoeae* (Dietrich et al., 2009).

Hfq is possibly the broadest-reaching RNA-binding protein currently known as it not only

protects and transports free sRNAs, but can also accelerate sRNA-mRNA complex degradation (Moeller et al., 2021). Its role as a key bacterial regulator of RNA activity is supported by research stating that it was present in the common ancestor of Bacteria, Eukaryotes and Archaeal lineages (Vogel and Luisi, 2011). This post-transcriptional regulation of gene expression by Hfq is seen most in Enterobacteriaceae, where it has been observed to regulate cell size, growth rates and yields, osmosensitivity, oxidation of carbon sources, and susceptibility to ultraviolet light (Tsui et al., 1994). This is usually achieved through stabilising sRNAs and increasing the rate of interaction with their target mRNA resulting in either enhanced complex stability or translation of the targets (Vogel and Luisi, 2011).

In the cell, Hfq is produced in great quantities. A study working with *E. coli* found that during the early exponential growth phase, there were up to 55,000 molecules of Hfq per cell with levels plateauing at approximately 17,500 molecules per cell during the stationary phase (Azam et al., 1999). Despite this abundance, it is predicted that RNA is at a molar excess in the cell resulting in a majority of the Hfq molecules being RNA-bound during the exponential growth phase (Moon and Gottesman, 2011; Gerhart et al., 2012). An impact of this supply-demand issue is that many RNA degrades before forming a required RNA-chaperone complex. To mitigate this happening to RNAs required for critical cellular functions there is a form of RNA hierarchy for the active site of Hfq (Moon and Gottesman, 2011). Therefore, high-priority RNAs can outcompete other RNAs for Hfq binding. Recent research has shown how important this hierarchy is, as a study observed that some sRNAs, when overexpressed within the cell, did not result in increased mRNA translation, whilst other sRNAs did (Faigenbaum-Romm et al., 2020). This is hypothesised to be a result of these over-expressed sRNAs not having any priority for Hfq binding and therefore being outcompeted by endogenous sRNAs. Another experiment, this time completed *in vivo*, found that during regular growth most Hfq are bound with mRNAs. Only high Hfq-binding affinity sRNA molecules interacting with the Hfq-mRNA complex would be able to replace the bound mRNA, whilst other sRNAs could sometimes co-bind alongside it (Park et al., 2021).

4.1.5 Hfq mechanisms of acting

Since Hfq was first identified as being involved in sRNA-mRNA interactions there has been much experimental interest in understanding how it mechanically functions. Hfq has four exposed regions which can act as RNA binding sites, the proximal face, the distal face, the rim and the C-terminal tail. The use rate of each of these regions varies between organisms due to changes in electrostatic surfaces and tertiary structure changes (reviewed in Sobrero and Valverde (2012)). However, a complete mechanistic model for how Hfq functions has not yet been completed. Despite this, two main modes of action have been identified for how Hfq interacts with RNAs. One of these mechanisms involves Hfq simultaneously binding to an sRNA and its target mRNA.

This has been experimentally proven in several papers such as for the *rpoS* mRNA which formed complexes with Hfq and the DsrA sRNA simultaneously (Soper and Woodson, 2008), and the OxyS sRNA which acted similarly with Hfq and its *fhlA* mRNA target (Salim and Feig, 2010).

Another method of acting by Hfq is more relevant to its chaperoning potential, whereby it can restructure bound RNAs to allow for an sRNA-mRNA complex to form. This was identified for the RyhB sRNA, whereby the RNase binding site on the *sodB* mRNA was significantly altered through binding with Hfq (Geissmann and Touati, 2004). This also opened up the seed region for the RhyB sRNA allowing for the sRNA-mRNA complex to form (Afonyushkin et al., 2005).

These two methods of Hfq action have been identified in *E. coli* and *Salmonella*, which is also where the majority of sRNA research has been focused. This means there is a clear lack of understanding of how Hfq operates in non-pathogenic organisms. Especially considering that *hfq* sequences are likely present in most bacterial species in a similar way to how sRNAs are being found across the majority of taxa (Sobrero and Valverde, 2012). Despite this, some species which have identified sRNAs have been found to lack the *hfq* gene (Swiercz et al., 2008). It is thought that this could be because the Hfq tertiary structure has been better preserved than its nucleotide sequence, something that was uncovered in many cyanobacteria species (Bøggild et al., 2009). Hfq has been identified in several environmental bacteria but not yet in the model soil denitrifier *P. denitrificans*. Despite this, Hfq is predicted to be present in *P. denitrificans*. Pden_4124 has a 54% similarity to Hfq in *Pseudomonas aeruginonsa*, and a 95% sequence similarity to the Hfq protein in *Rhodobacter sphaeroides* (Gaimster et al., 2016).

4.1.6 Aims

As Hfq is known to have an important role in regulating sRNA function, this chapter aims to research how elevating Hfq levels in the cell beyond regular concentrations impacts cellular functions when under denitrifying conditions. This is particularly interesting for the denitrification pathway, where the pathway's final stage (N_2O to N_2), has been shown to not always be complete in the environment. In this chapter, we look into the impact of the overexpression of *hfq* within *P. denitrificans* and how the resultant abundance of endogenous RNA chaperone impacts the production of N_2O.

4.2 Results

4.2.1 Anaerobic growth of *hfq* mutant

To determine whether an abundance of Hfq in the cell impacts growth rate, *P. denitrificans* containing the pLMB509 plasmid with the gene encoding the hypothetical *hfq* (Pden_4124) in the expression site (Pd_hfq) was cultured (as described in Section 2.2.2). It was cultured in triplicate under denitrifying conditions on minimal media containing 20 mmol/litre nitrate, 30 mmol/litre succinate, 10 mmol/litre taurine and with Cu-low Vishniac and Santer trace element solution (Section 2.2). This was compared to *P. denitrificans* containing an empty pLMB509 plasmid (Pd_empty). When culturing *P. denitrificans* anaerobically, *hfq* overexpression resulted in no significant changes to growth rate or stationary cell density (Fig. 4.1). Pd_hfq showed a slightly lower mean growth density as it entered the stationary phase (20 hours) than Pd_empty, OD_{600} of 1.204 ± 0.171 and 1.279 ± 0.196 respectively.

4.2.2 N_2O accumulation under *hfq* overexpression

Pd_hfq initially showed no difference in N_2O accumulation during the exponential growth phase compared to Pd_empty, slowly increasing over the initial 16 hours (Fig. 4.2). Pd_hfq steadily accumulated N_2O throughout the experiment. At 32 hours the N_2O concentration in Pd_empty growth vessels peaked at 2.177 mmol/litre compared to 0.604 mmol/litre in Pd_hfq, a greater than 3-fold decrease. In the final 8 hours of growth, N_2O levels in Pd_hfq plateaued to just 0.218 mmol/litre lower than Pd_empty. The triplicate mean data point for Pd_empty at 32 hours had a standard deviation of 1.802 mmol/litre, a result of one of the included data points not accumulating N_2O at a discernible rate. This is also why range bars for Pd_empty at 24 and 40 hours are broad. As a result, the difference in N_2O accumulation between the treatments is not significantly different.

Figure 4.1: Growth curve of *P. denitrificans* containing an empty pLMB509 vector (▪▪▪▪▪), and *P. denitrificans* with pLMB509 cloned with the *hfq* gene cloned into the overexpression site (▬). Cultured under denitrifying conditions in minimal media and incubated at 30 °C. Error bars represent standard deviations of results based on triplicate experiments.

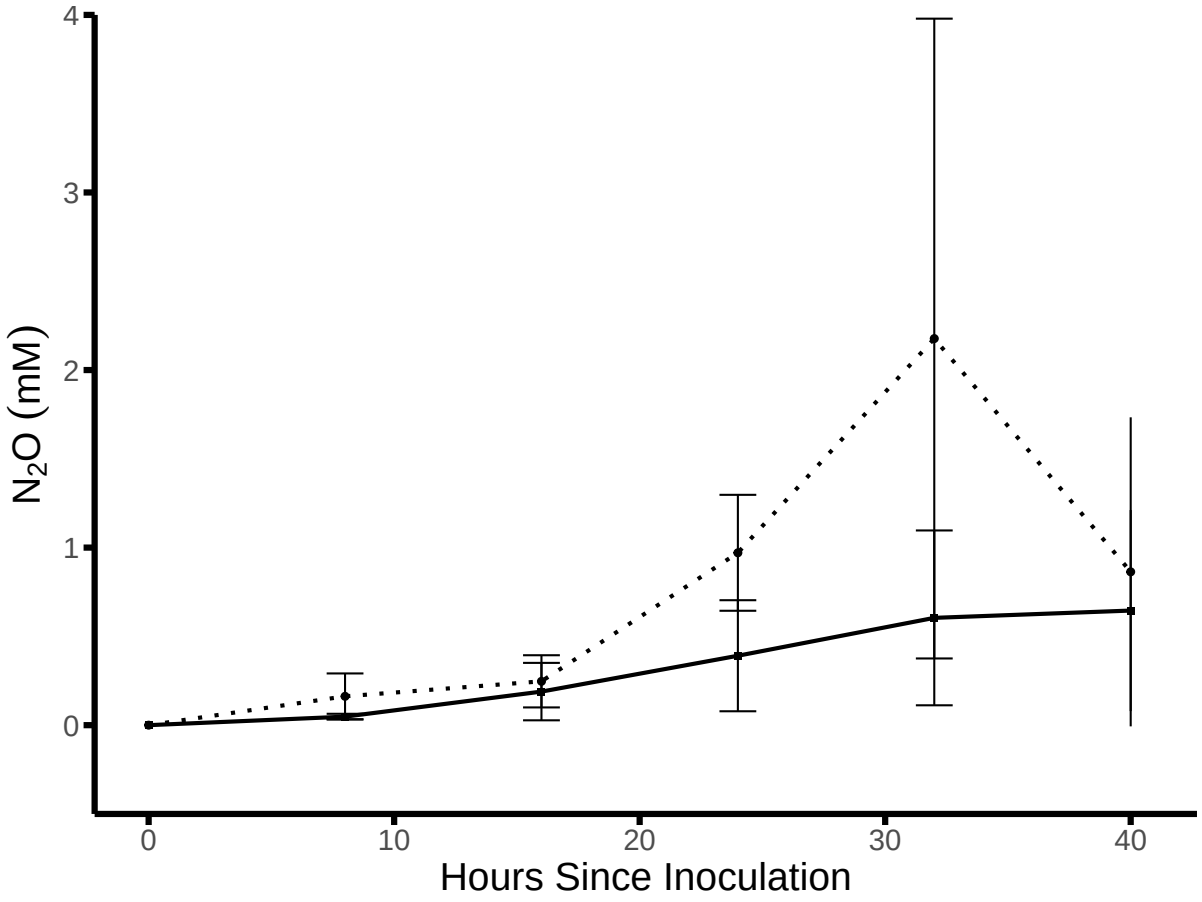

Figure 4.2: Accumulation of N_2O during growth in minimal media under denitrifying conditions. *P. denitrificans* with an empty pLMB509 vector (▪▪▪▪▪), and *P. denitrificans* plus pLMB509 cloned with *hfq* into the overexpression site(▬). Cultured under denitrifying conditions in minimal media and incubated at 30 °C. N_2O production measured in mmol/litre. Error bars represent standard deviations of results based on triplicate experiments.

4.3 Discussion

4.3.1 *hfq* overexpression impacts on *P. denitrificans* growth rate

Overexpression of *hfq* in the model denitrifier *P. denitrificans* showed no substantial difference in growth rate from the control (Fig. 4.1). A slight decrease in growth rate was observed although not significantly different. As sRNAs regulate cellular processes of many bacterial functions, enabling their activation through increased chaperone activity could be presumed to enhance cell density. Hfq's role in growth rate has previously been observed in *E. coli*, where mutants were formed to eliminate Hfq functionality, this mutant also showed a reduced growth rate compared to their control (Tsui et al., 1994). Specifically, it cultured slower during the lag and late-exponential phases, before plateauing at a decreased maximum cell density than the control. These minor cell density decreases seen with *hfq* overexpression are of added interest when considering that N_2O has been found to have a cytotoxic effect on *P. denitrificans* at concentrations of just 0.1 mmol/litre (Sullivan et al., 2013). A result of N_2O's affinity to bind to and inactivate vitamin B12, an essential molecule for DNA synthesis (Drummond and Matthews, 1994b,a; Sullivan et al., 2013). This shows that despite the observed lower concentrations of N_2O in Pd_hfq compared to the control (Fig. 4.2), Pd_hfq cultured as well, if not slightly worse than Pd_empty.

4.3.2 Impacts on N_2O emissions

For the first 16 hours of culturing, there were no clear differences to N_2O accumulation in the growth vessel between Pd_hfq and Pd_empty. However, from 16 to 32 hours, Pd_hfq continued a steady increase in N_2O accumulation, compared to the dramatic increase seen when *hfq* was not overexpressed. This increase began approximately when the cells entered their stationary phase of growth. This is of particular interest when discussing *hfq* overexpression as past studies have found that sRNA activity peaks during the exponential phase of growth, so to see a clear deviation in N_2O accumulation from this point in Pd_hfq poses the question of how Hfq is influencing this trend. A possible reason is that under standard growth conditions, endogenous Hfq concentration is 3-fold lower during the stationary growth phase compared to the exponential growth phase (Azam et al., 1999). Therefore, due to the continued overexpression of *hfq*, the stationary phase is no longer as chaperone-limited and can engage with more sRNAs. This requires the assumption that this normal drop in Hfq from the exponential to stationary phase is not a programmed cellular response to decreasing sRNA concentration and is instead a preservation of life process that reduces the required energetic load to ensure the colony does not outgrow its energy source. This could be linked with the observed slight decrease in cell density during *hfq* overexpression, as it might show that increasing the abundance of Hfq in the cell does not allow it to outgrow the

control.

The observed profile of N_2O accumulation shows that *hfq* is a biologically relevant protein in the denitrification pathway. Its abundance above normal cellular levels resulted in dramatically decreased, and stable N_2O levels when compared to the control. This implies that *hfq* may chaperone important regulatory sRNAs within *P. denitrificans*, activating or repressing the functions of these molecules. The exact manner in which this *hfq* overexpression results in decreased N_2O production requires further research. Such as measuring levels of all denitrification intermediates within the growth media to determine whether N_2O levels are abnormally high, or if *hfq* overexpression is slowing the reduction of nitric oxide to N_2O.

It is important to consider that not all sRNAs are Hfq-dependent, however, studies have found that as many as half of all sRNAs in *Salmonella* are Hfq-dependent (Sittka et al., 2008). Many of the RNAs that are Hfq-dependent all share a similar structural motif, primarily being that these sRNAs will have three stem-loops (Zhang et al., 2003). Although useful information, it does not offer any guarantees of Hfq-dependence of an sRNA, this can only be ascertained through analysis such as ChIP-seq (Kambara et al., 2018), Hfq CLASH (Iosub et al., 2020) or RIL-seq (Bar et al., 2021). Each of these provides different methods of identifying sRNAs that rely on Hfq for their regulatory properties.

Although we have no evidence that overexpression of *hfq* has resulted in a greater rate of sRNA-mRNA binding complexes forming, previous research has shown that the amount of complementary sRNAs and mRNAs is not the only relevant factor impacting mRNA regulation (Faigenbaum-Romm et al., 2020). They found that the regulation of some mRNAs, particularly those with high Hfq-binding affinity, was not influenced by an overabundance of their complementary sRNA. Based on this, the abundance of endogenous Hfq is made available to *P. denitrificans* in this study might be an important step in assuring that sRNA-mRNA complexes with low Hfq-binding affinity are not outcompeted for a chaperone. I believe this process provides the clearest concept of how *hfq* overexpression could result in the low N_2O accumulating phenotype seen in Pd_hfq. The over-availability of this RNA chaperone allows for low Hfq-binding affinity mRNAs to be translated through regulation from complementary sRNAs. It is, however, impossible from this work to be certain of where in the denitrification pathway the overexpression of *hfq* is impacting, or whether this abundance of Hfq is positively or negatively regulating said process. An example though of what could be occurring is that a hypothetical sRNA responsible for positively acting on an mRNA, such as nitrous oxide reductase (NosZ), could under normal Hfq levels have very low Hfq-binding affinity, and as such is usually outcompeted by other sRNA-mRNA complexes for Hfq's active site, resulting in the NosZ mRNA not being translated, and N_2O not being reduced into N_2. The difference would occur when we overexpress *hfq*, there are now sufficient Hfq molecules in the cell that Hfq's active site no longer outcompetes the NosZ mRNA-sRNA complex. Therefore, this hypothetical sRNA can positively act on the

nosZ mRNA, resulting in increased production of the NosZ enzyme, causing N_2O production to be limited. Possibilities such as these highlight how Hfq abundance can play a role in various cellular processes when the abundance of the protein is increased.

Future research into this topic should focus on how Hfq is causing this slowed accumulation of N_2O production. One way could be to utilise an additional overexpression vector within Pd_hfq that is overexpressing a characterised Hfq-dependent sRNA. The expectation would be that the overabundance of both the chaperone and sRNA causes a stronger phenotype. Furthermore, an analysis of which regulatory processes Hfq is interacting with to cause the observed decrease in N_2O accumulation from *P. denitrificans* would be useful. As well as quantifying the amount of Hfq that is produced by the pLMB509 plasmid through leakage when taurine is not added to the growth solution.

5 Pda200 Regulation Beyond Florfenicol Stress Response

5.1 Introduction

The denitrification pathway, discussed in Section 1.3, is mostly completed by biological denitrification, through a step-wise reduction from NO_3^- to N_2. The final stage of this, reducing the greenhouse gas N_2O to N_2, is often incomplete in the natural environment. Known factors causing this include copper availability (Felgate et al., 2012), vitamin B_{12} deficiency (Sullivan et al., 2013) and small RNAs (sRNAs) regulating gene expression (Gaimster et al., 2019). This chapter focuses on two research papers from Guangdong Provincial Key Laboratory of Veterinary Pharmaceutics Development and Safety Evaluation at the South China Agricultural University in Guangzhou, Wang et al. (2021) and Wang et al. (2022). They found that antibiotics commonly used in aquatic farming can impact *nosZ* expression by regulating sRNAs. Their findings present a potential sRNA that could be a key regulator of nitrous oxide reduction under denitrifying conditions.

5.1.1 Antibiotics in aquaculture affecting denitrification rates

Aquaculture has been the fastest-growing food production sector in the world (Troell et al., 2014), with China accounting for 62% of global aquaculture by volume as of 2008 (Bostock et al., 2010). With this, there has been a 25-fold increase in N_2O emissions from Chinese aquaculture systems over the past 41 years (Zhou et al., 2021). This may be a result of nitrogen leaching, a process whereby excess nitrogen in soils drains into nearby water sources. This is most common near intensive farmland where fertilisers high in nitrogen run off the soils during rainfall and flooding. Amongst the many ecological and environmental impacts this process causes, it is also a major problem for the aquatic organisms being cultivated in aquacultures, due to the resultant health effects and water quality issues. As such, biological denitrification is often relied upon to remove nitrogen from aquacultures to increase productivity, much like how it is utilised in wastewater treatment (Lu et al., 2014). However, many antibiotics have been found to affect various stages of the denitrification pathway, with several increasing N_2O emissions (reviewed in Zhou et al. (2024)).

The first of the two aforementioned papers is focused on the antibiotic florfenicol, often used to treat a range of diseases in aquatic animals (Wang et al., 2021). As of 2013, florfenicol was the second most used antibiotic in China, despite not being used in humans at all (Zhang et al., 2015). A previous study from the research group in question found that the presence of florfenicol reduced the abundance of denitrifying genes in the soil environment, with *nirS*-type denitrifiers being more sensitive than *nirK*-type denitrifiers (Wang et al., 2020). As a result of this finding, Wang et al. (2021) focused on determining the effects of florfenicol on *nirS*-type denitrifiers and investigating the reasons for the inhibition or delay of bacterial denitrification.

5.1.2 Impact of florfenicol stress on *nirS*-type denitrifier *Paracoccus denitrificans*

To research this, Wang et al. (2021) cultured the model denitrifier *Paracoccus denitrificans* strain ATCC 19367 anaerobically in minimal media (Section 2.2.2) with or without the presence of 1 μg/mL florfenicol. Growth was measured at OD_{600} and verified through colony-forming units (CFU). The addition of the florfenicol resulted in a delay to the stationary phase of approximately 4 hours and a reduced peak growth density of ~ 1 OD_{600} compared to the control which reached ~ 1.15 OD_{600}. Showing that florfenicol had an obvious inhibition of *P. denitrificans* growth. During this experiment, they also measured nitrate and nitrite reduction capability by extracting the culture supernatant and using spectrophotometry. They found that the addition of florfenicol delayed nitrate removal and caused a later and greater peak in accumulation when compared to the control. This led to the conclusion that the growth inhibition of *nirS*-type denitrifiers by florfenicol is the main reason for reducing nitrogen removal efficiency and damaging their wastewater bioremediation ability. RNA was also extracted during the exponential phase of growth for both RNA-seq and qRT-PCR analysis to determine differential gene expression caused by florfenicol presence. This showed that a total of 433 genes were differentially expressed (292 upregulated and 141 downregulated) by florfenicol exposure. Amongst those genes significantly inhibited were *nirS* (2.21-fold), *norB* (1.27-fold), *nosD* (1.02-fold) and *nosZ* (1.63-fold), all of which are crucial enzymes in the denitrification pathway. Fold changes are represented as log2. As nitrate-reducing enzymes were found to have a reduced fold change through RNA-seq analysis it was further confirmed that in *P. denitrificans*, *nirS* is more susceptible to florfenicol stress than *narG* and *narH*.

Also of interest from the RNA-seq analysis was the identification of 292 candidate sRNAs. sRNAs are short, non-coding regulatory RNAs that usually bind with mRNAs to posttranscriptionally regulate gene expression in bacteria. Of the 292 candidate sRNAs, 14% were differentially expressed in the presence of florfenicol. From these, they identified Pda200, a 413 base pair, highly structured sRNA that had a >10-fold increase in expression under florfenicol exposure. They also stated that through the use of target prediction, Pda200 showed complementary base pairing to the *nosZ* mRNA from base 143 to 170 in Pda200 (Wang et al., 2022). Based on these findings, they further analysed Pda200 as a novel denitrification regulator and aimed to identify its potential as a method of enhancing denitrification within a bacterial consortium.

5.1.3 Pda200 as a novel denitrification regulator

To further analyse Pda200, they continued their research into their second paper, Wang et al. (2022). They continued with the same experimental procedure as previously, this time comparing the florfenicol response of *P. denitrificans* ATCC 19367 to other antibiotics: doxycycline hydrochlo-

ride (DOH), neomycin sulfate (NS), sulfadimidine (SM) and flumequine (FQ). These were all added at the same concentration as florfenicol during the exponential phase (1 μg/mL), except for DOH which was at a concentration of 0.01 μg/mL. When analysing the nitrogen levels in the cultures, DOH caused a nearly 3-fold increase in nitrite accumulation rate, whilst florfenicol reduced nitrate removal rate by 27% when both compared to the control. The qRT-PCR data from this study found that Pda200 was the only sRNA of those differentially regulated whose expression levels were impacted by multiple antibiotics (DOH, FQ and florfenicol). Further analysis also found that under florfenicol treatment, Pda200 expression was positively correlated with that of *nosZ*, however, the timing of their expression during *P. denitrificans* growth was slightly different, with *nosZ* expression no longer inhibited after two hours and thirty minutes. Whilst Pda200 inhibition was consistent throughout growth compared to when florfenicol was absent from the growth media. These findings proved to them that the computationally predicted interaction between Pda200 and *nosZ* could be observed as a regulatory effect by Pda200.

To further test the role of Pda200 they designed a consortium experiment. Two bacterial strains were isolated from sediments in contaminated water, SB-1 (*Pseudomonas* sp.) and WB-2 (*Thauera* sp.). These strains were chosen as they could completely remove nitrate during growth on minimal media and complete genomic sequencing suggested that they both carried the genes necessary to carry out complete denitrification. These two strains were combined with *P. denitrificans* ATCC 19367 to form a synthetic microbial consortium suited to denitrifying conditions. The control group contained only *P. denitrificans* ATCC 19367, florfenicol concentration was varied from 0.1 to 10 μg/mL to assess antibiotic tolerance of the consortium and single denitrifer. These were cultured at 30 °C and samples were taken throughout their growth to measure nitrate removal efficiency. They stated that "*the experimental consortium displayed its optimal denitrification performance in the presence of antibiotic pressure*". Specifically, after 48 hours, at the highest florfenicol concentration of 10 μg/mL, cell densities in the treatment group were 7 orders of magnitude greater than the control group. They also found that the consortium had a final nitrate removal efficiency of 91.66% whilst *P. denitrificans* by itself could not remove any nitrate due to the antibiotic pressure. They suggest that these results show how denitrifying consortiums with antibiotic-tolerant traits are far more suitable for treating contaminated water than a single strain. With this, they expanded on their consortium experiment to determine whether Pda200 could regulate denitrification within a whole synthetic microbial consortium.

Within this experiment, they aimed to explore how Pda200 could enhance the denitrification potential of the consortium. To do this, they expanded on the original consortium, inserting Pda200 into the recombinant plasmid pBBR1MCS-2, a kanamycin-resistant, IPTG-induced expression vector (Kovach et al., 1995), which was then introduced into *E. coli* Top10 cells (Ec_Pda200). Single colonies of SB-1, WB-2, *P. denitrificans* ATCC 19367, Ec_Pda200 and the helper strain *E. coli* Top10 pK2013 were cultured separately in LB media containing kanamycin

where necessary. For the treatment group, these were all combined and cultured in minimal media (Sullivan et al., 2013) containing 1 μg/mL florfenicol to simulate the antibiotic pressure of the aquaculture environment. The control group was identical except for culturing an *E. coli* Top10 strain containing an empty plasmid. The growth bottles were sealed and cultured at 30 °C. IPTG was added to both treatments once they reached exponential growth to induce Pda200 expression. The presence of Pda200 within the denitrifying consortium significantly increased the expression of *napA. napB* and *norB* when compared to the control group. These key denitrification genes were increased in SB-1 by 2.16- to 3.65-fold and 2.18- to 3.04-fold in WB-2 when treated with Pda200. They stated that " *Pda200 can enhance the expression of denitrifying genes in multiple denitrifier species under antibiotic pressure*". This would make Pda200 a key denitrification regulator in *P. denitrificans* that can also invoke posttranscriptional gene expression influence on other families of denitrifiers. However, issues with how this study utilized the denitrifying consortium and their identification of the *nosZ* mRNA as complementary to Pda200, among other concerns, make many of their findings at least partially unfounded. These problems are discussed in depth throughout Section 5.3.

Based on these findings we intend to further test the denitrifying capability of the Pda200 sRNA, focusing on its possibility as a first-to-be-discovered sRNA interacting directly with the *nosZ* mRNA. Pda200 from *P. denitrificans* ATCC 19367 shares 100% identity with *P. denitrificans* PD1222, which will be used as the denitrifier for my experiments. These strains share 4738 core genes with 428 genes being specific to ATCC 19367 (Si et al., 2019). Pda200 will be overexpressed using our expression system to further analyse the purported regulatory role of Pda200 on N_2O production.

To achieve this, Pda200 was synthesised and cloned into pLMB509 (GenScript), a taurine inducible expression vector used for its high copy number and ease of conjugation (Tett et al., 2012). This was then transformed into *E. coli* Top10 prior to conjugation into *P. denitrificans* PD1222 to form our Pd_Pda200 strain. Pd_Pda200 was then cultured anaerobically, in triplicate, under denitrifying conditions on minimal media containing 20 mmol/litre nitrate, 30 mmol/litre succinate, 10 mmol/litre taurine and with Cu-low Vishniac and Santer trace element solution. Pd_empty, an identical strain containing an empty pLMB509 plasmid was used as the control for growth rate. Alongside measurements of cell density, samples were extracted for later gas chromatography analysis to measure N_2O levels in the sealed growth chambers. PdΔ*nosZ*, identical to Pd_empty except for having the gene encoding for *nosZ* removed was used as the upper control for N_2O accumulation. The data collected during these experiments is shown in the following sections.

5.2 Results

5.2.1 Anaerobic growth when overexpressing Pda200

The growth rate was measured to ensure that all treatments were growing at similar rates whilst headspace samples were being extracted for later gas chromatography. When Pd_Pda200 was cultured anaerobically in the presence of taurine it showed a slightly delayed growth rate than the control of approximately two hours (Fig. 5.1). Peak cell density reached an OD_{600} of 1.28 ± 0.22 compared to 1.39 ± 0.15 for the empty vector. Pd_Pda200 growth was not as reliable as the control, showing larger error bars, especially during the late exponential phase, when the Pd_Pda200 growth rate was highly varied.

5.2.2 Overexpression of Pda200 causes variable N_2O response

Throughout the anaerobic growth experiment, headspace samples were extracted and processed as described in Section 2.2.2 to determine N_2O accumulation in the growth chambers. This would be key in elucidating whether Pda200 displays its reported phenotype of increasing *nosZ* activity, which should result in a reduction to N_2O concentration in the growth chamber.

When Pda200 was overexpressed, N_2O accumulation was highly varied, however, it followed similar trends to both Pd_empty and PdΔ*nosZ* with a steady increase throughout the exponential phase of growth, followed by a dramatic increase as the cells entered the stationary growth phase (Fig. 5.2). Pd_Pda200 N_2O concentration peaked after 32 hours with an average of 1.34 ± 1.11 mmol/litre lower than both Pd_empty and PdΔ*nosZ* which reached peaks of 1.88 ± 1.80 mmol/litre and 3.83 ± 2.03 mmol/litre respectively. The initial 24 hours of growth, which aligns with the majority of pre-stationary phase growth shows all three strains having an average of between 0.44 to 0.97 mmol/litre. The decrease in N_2O accumulation at 40 hours represents only a 15% loss from its peak of stored N_2O for PdΔ*nosZ*, compared to 61% and 90% for Pd_empty and Pd_Pda200 respectively.

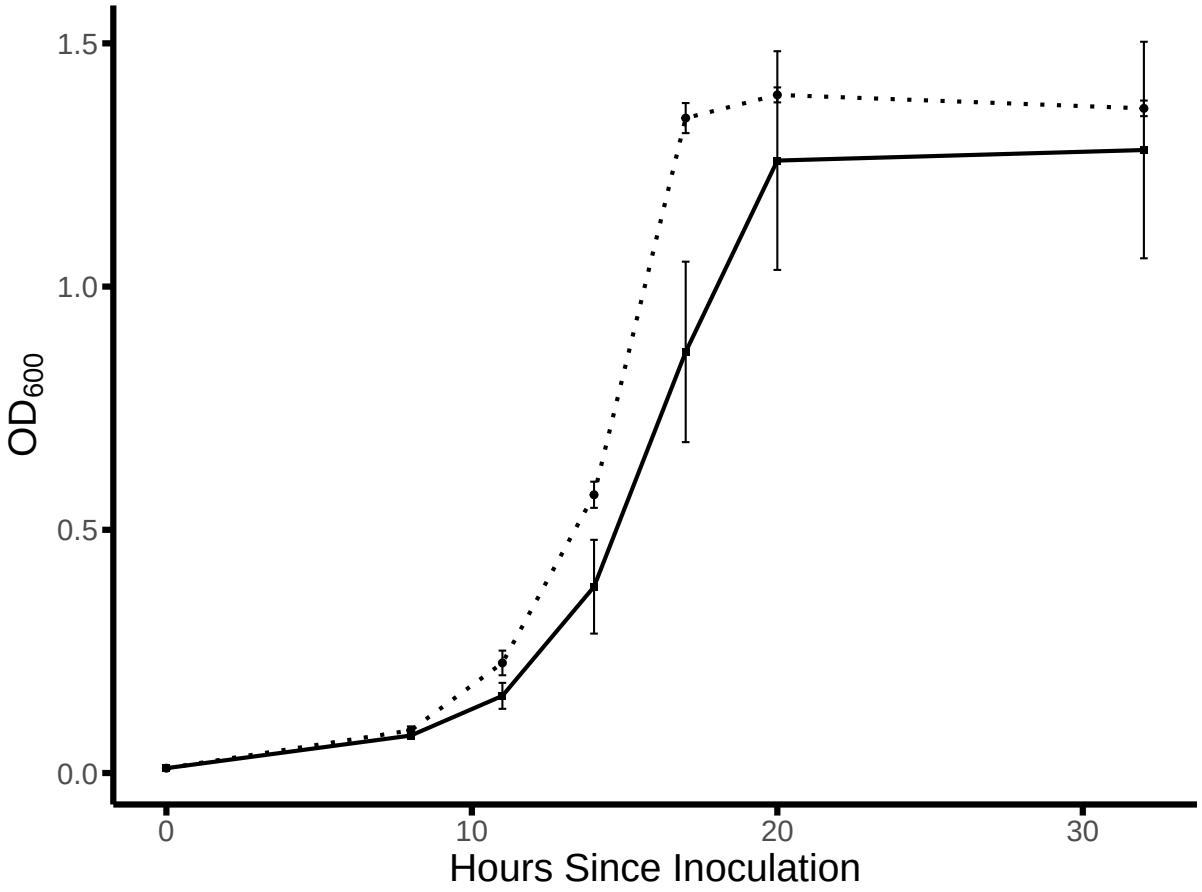

Figure 5.1: Pda200 overexpression in *P. denitrificans* causes slightly reduced growth when cultured in anaerobic, denitrifying conditions. Six cultures of *P. denitrificans* were grown under denitrifying conditions in minimal media at 30 °C. Three were cultured with *P. denitrificans* containing the pLMB509 plasmid expressing Pda200 (—), and three were cultured containing an empty pLMB509 plasmid (▪▪▪▪). Error bars represent the standard deviation of each result between triplicate experiments. Where not visible, error bars are smaller than the symbols.

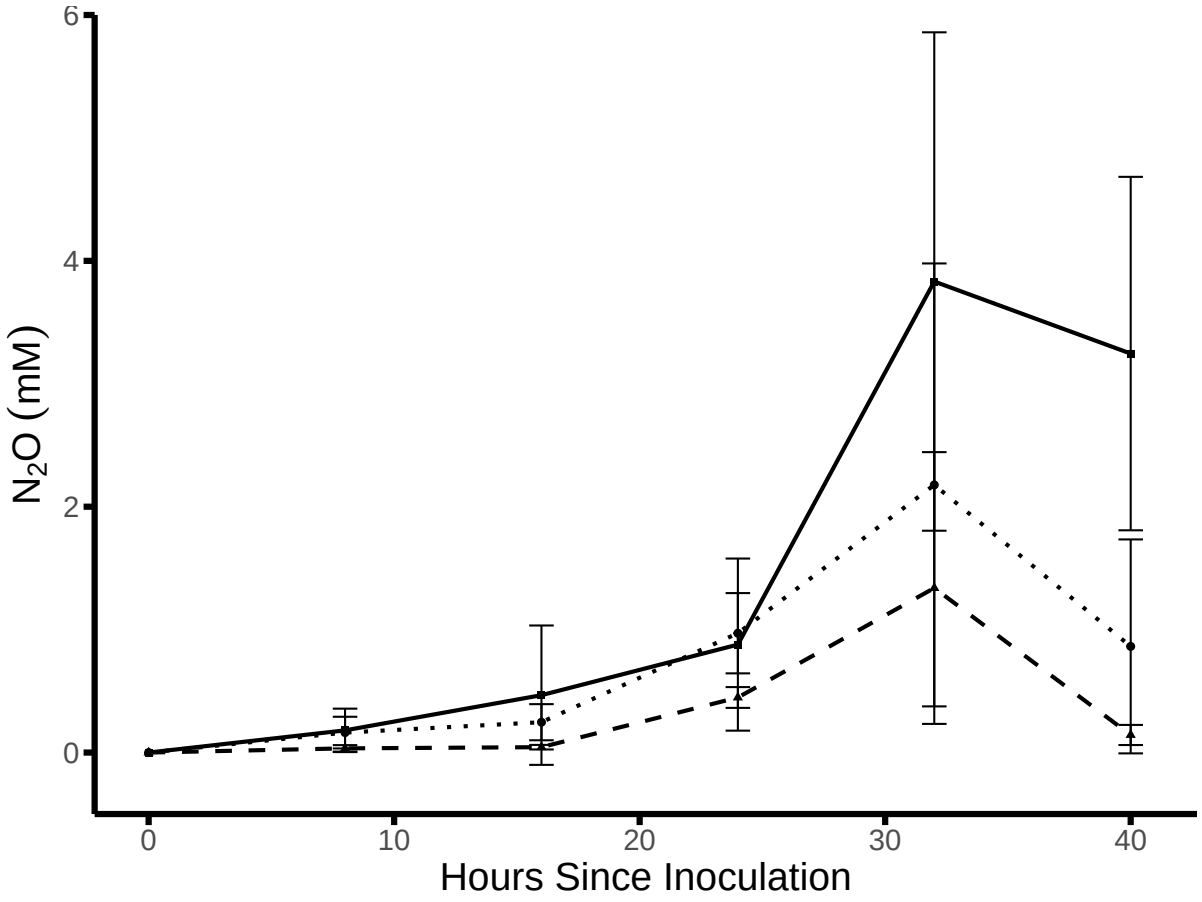

Figure 5.2: Accumulation of N_2O during growth in minimal media under denitrifying conditions. *P. denitrificans* with an empty pLMB509 vector (▪▪▪▪▪), *nosZ* mutant *P. denitrificans* containing an empty pLMB509 vector (▬▬), and *P. denitrificans* with pLMB509 containing Pda200 cloned into the overexpression site (▪ ▪ ▪). N_2O production measured in mmol/litre. Asterisks indicate statistical significance: $^*p < 0.05$, non-significant differences ($p \geq 0.05$) are not marked. Error bars represent the standard deviation of each result between triplicate experiments. Where not visible, error bars are smaller than the symbols.

5.3 Discussion

5.3.1 Relevance of Pda200 on N_2O emissions

Pda200, a 413bp-long sRNA found in *P. denitrificans* has previously been identified as having an integral role in the regulation of denitrification, specifically of interest is its purported function as a posttranscriptional regulator of *nosZ*, a gene responsible for producing the N_2O reducing enzyme, NosZ (Wang et al., 2021, 2022). This is of particular interest due to the well-established issues for the denitrification pathway in the environment, where the final step, the reduction of N_2O to N_2 is often not completed.

Pda200 was initially isolated from the *P. denitrificans* ATCC 19367 strain, the complete nucleotide structure of the sRNA was not provided within either of the relevant papers, however, using the primers provided we found that Pda200 in *P. denitrificans* ATCC 19367 shares an identical similarity with a portion of the *P. denitrificans* PD1222 strain genome. Located on chromosome 1 within the Pden_2366 gene, a purported acetaldehyde dehydrogenase (Felux et al., 2013), it also is not found in synteny to any genes directly related to the denitrification pathway. Other than the expression-based data that shows how Pda200 concentrations adapt with and can regulate various denitrification enzymes, the main function of the sRNA is believed to be its direct interactivity with the *nosZ* mRNA (Wang et al., 2022). They found that Pda200 shares a 25-nucleotide-long section of close complementary base pairing with the *nosZ* mRNA, responsible for the translation of the nitrous oxide reductase enzyme. This region is displayed as a figure in their paper as having perfect complementarity across 22 of 25 nucleotides with the *nosZ* mRNA (Wang et al. (2022); Figure 3b), however, on inspection, 4 of the 22 do not match as stated, mostly G-U pairs that although can bind, rarely do in the circumstances of sRNA-mRNA complexes. They also do not propose a seed region or discuss what mechanism of regulation Pda200 could potentially be using. Information from them on how they perceive the mechanism of action between these molecules would go a long way towards understanding how they came to their conclusions. However, based on what we know of Pda200 and of the varying known mechanisms of sRNA action (reviewed in Moeller et al. (2021)), I would presume they saw Pda200 as a positively acting sRNA which functions by unmasking ribosome binding sites (RBS) on the *nosZ* mRNA, as there are several Shine-Dalgarno sequences within Pda200 (Section 1.6. These sites are required to align the ribosome with the start codon of the mRNA, ensuring translation. This assumption is aided by the low AU content in Pda200 (33.7%) and the lack of AU-rich regions which are required for RNase E activation, reducing the likelihood of Pda200-mediated inhibition of RNase E, which is the other commonly observed mechanism for positive-acting sRNA.

Whether or not Pda200 has a highly complementary region to the *nosZ* mRNA may not be of utmost importance as it seems to be a *trans*-acting sRNA due to its positioning being

unrelated to that of the *nosZ* gene. As *trans*-acting sRNAs have been found to not require high complementarity, it is still possible for Pda200 to act on the *nosZ* mRNA. Therefore, one way to determine whether Pda200 is regulating the translation of the *nosZ* mRNA is to analyse how its overexpression within the model denitrifier *P. denitrificans* PD1222 impacts the final stage of denitrification. The overexpression of Pda200 caused highly variable N_2O accumulation within Pd_Pda200 (Fig. 5.2). The result of the triplicate experiment showed that on average Pda200 overexpression caused reduced N_2O accumulation compared to the empty vector control (Pd_empty). This shows that there may be some regulatory role of Pda200 on the denitrification pathway. If it is interacting with the *nosZ* mRNA as predicted in Wang et al. (2022) then it would be through a positively acting mechanism, as an enhanced translation of the *nosZ* mRNA through Pda200 regulation would cause a higher concentration of endogenous NosZ enzyme, resulting in the observed drop in N_2O accumulation. This lines up with the findings from the previous study. On average, N_2O accumulation when Pda200 was overexpressed was lower than the control and all strains displayed low levels of reliability across the triplicate experiment. The high standard deviations were a result of highly variable measurements within each treatment. The most likely reason that this was observed, is due to the issue of ensuring a complete seal on the growth chambers (mentioned in Section 3.3.2). This is especially likely as these impacts were seen across each of the strains cultured through this experiment. It would be very useful to run this experiment again and see not just whether variability in the N_2O accumulation continued when culturing in other anaerobic container options, but also to analyse and compare the changing concentrations of nitrogen intermediates within the growth media throughout the experiment. Unfortunately, the observed slight reduction in growth rate for Pd_Pda200 compared to Pd_empty is impossible to pull any information from without knowing how these nitrogen intermediates are changing between treatments (Fig. 5.1). For instance, should Pda200 decrease the rate of NO reduction through regulation of the *norB* mRNA, something that has been previously described (Wang et al., 2022), then the resultant lack of accumulating N_2O would not be a direct impact of Pda200-*nosZ* mRNA interaction. The problem with this is that this data has not been collected and therefore further experiments would be needed to find out where this observed change to the denitrification pathway is occurring. Further data which would be of use is to quantify the amount of Pda200 produced through the addition of taurine as this would prove that Pda200 is being produced in relevant concentrations by the cells.

5.3.2 Does Pda200 belong to the small RNA family?

Alongside Pda200's documented role as a purported denitrification regulator, another aspect that led to further analysis was that it shared no similarity to any of the 167 putative sRNAs identified in *P. denitrificans* PD1222 from a previous study (Gaimster et al., 2016). This study was

performed using the same experimental methodology established in Sullivan et al. (2013) and was specifically designed to identify sRNAs involved in the denitrification process. However, in Gaimster et al. (2016), they specifically isolated RNA molecules smaller than 200 nucleotides as this fits with the majority of sRNAs. This also reduces the risk of false positives being identified, however, the caveat of this approach was that they would lose the very few putative sRNAs which were larger than 200 nucleotides. sRNAs of lengths up to 400 nucleotides have been identified, however, there is quite a range of how these cases are noted in the literature. The issue arises in what differentiates sRNAs from other RNA molecules. The consensus seems to come from the early studies of bacterial sRNAs which describe them as being shorter than 200 nucleotides long. This was decided as a cut-off to reduce the risk of accidentally including mRNA when searching for these regulatory RNAs. Based on this alone, questions should be asked as to how and why this study categorised Pda200 as an sRNA and what would be the correct family of RNA to define it as.

One possible category Pda200 could fit within is as an mRNA. mRNA range in length from 300 to 5000 nucleotides in length, although this can depend on the species (Hurowitz and Brown, 2003). Pda200 being an mRNA initially seems unlikely as mRNAs are commonly seen as having the sole purpose of carrying protein information from the cell's DNA to protein-forming machinery. However, mRNA can also perform regulatory roles, as the line between regulatory RNAs and protein synthesis RNAs is much blurrier than many studies claim it to be (Karapetyan et al., 2013; Meyer, 2017). An important aspect of mRNAs is that most are adenylated, providing the RNA with stability and a reduced risk of degradation by exonucleases.

An alternative to sRNAs is long non-coding RNAs (lncRNAs), which are forms of RNA that are of at least 200 nucleotides in length (Uszczynska-Ratajczak et al., 2018). On initial discovery, lncRNAs were seen as influencers of gene expression primarily by guiding protein complexes to specific DNA loci, often modifying the abundance of mRNA in the cell as a result of their actions (Ernst and Morton, 2013). However, some studies view lncRNA as a more broad-reaching regulator with highly diverse functionality, capable of acting at many different levels of gene expression (Dykes and Emanueli, 2017). lncRNA are very rare in the cell, making up approximately 0.03% to 0.20% mass of eukaryotic cellular RNA, with this proportion predicted to be even lower in prokaryotes (Palazzo and Lee, 2015). Studies have found that in eukaryotic cells, lncRNA can act as primary transcripts for miRNA production. miRNAs are small non-coding RNA molecules that can target multiple mRNAs in both eukaryotic and viral DNA (Cai et al., 2009). Importantly, no similar process of lncRNA splitting has yet been identified in prokaryotes, however, if it did occur the production of functional sRNAs should not be ruled out due to their functional similarity to miRNA. Many, but not all lncRNA are polyadenylated at the 3′ end of the transcript, as mentioned before this is also common in mRNA as a form of structure stabilisation (Hangauer et al., 2013). Polyadenylation refers to when a structure contains a poly(A) tail, which stabilises the molecule and aids in its export from the nucleus to the cytoplasm. It can also be responsible for signalling

the termination of transcription and regulating an mRNA's lifespan. Identifying these structures is done by searching for a poly(A) signal in the transcript, often a nucleotide sequence of AAUAAA or similar. However, Pda200 does not contain any similar signal, which would point towards it being a non-polyadenylated lncRNA.

The lack of consideration for what constitutes an sRNA is an oversight of these studies, especially considering how the majority of bacterial sRNA literature states the length constraints of an RNA to be an sRNA as at most 200 nucleotides. Considering what has been previously described, I would class Pda200 as a non-polyadenylated lncRNA. This differentiation is important as sRNA are generally seen as having a global primary function as posttranscriptional regulators of gene expression. Whilst lncRNA can also have this function, they have also been found to regulate transcription by acting as a scaffold or decoy to modulate RNA transcription. This opens up many more options for Pda200 regulation mechanisms than those expected of an sRNA.

5.3.3 Accuracy of previously documented Pda200 studies

Previous paragraphs have picked up on issues with Pda200 and how it had been presented throughout the papers this chapter is focused on, such as its misrepresented complementary binding site with the *nosZ* mRNA and uncertainty on being classified as an sRNA. These are not the only issues present in these studies and as such should be addressed alongside the new data presented here on the function of Pda200.

When documenting the impacts of Pda200 on anaerobic growth, Wang et al. (2021) showed that florfenicol exposure slowed the growth of *P. denitrificans* ATCC 19367 and delayed the stationary phase by approximately four hours (Fig. 5.3A). This is a very similar response to what has been shown in our study although *P. denitrificans* PD1222 took a few hours longer to reach its peak population and the delay was reduced to two hours (Fig. 5.1). They also stated that "*CFU (colony forming units) counting showed a similar result and confirmed that FF (florfenicol) had an obvious inhibition on P. denitrificans growth*". This is another potential issue with their findings, as Figure S2 from their supplementary information shows no clear delay to the stationary phase (Fig. 5.3B). There is a reduced bacterial population for the initial 10 hours, however, the sharp increase in growth seen in the CFU data from the point of inoculation seems very odd and is not explained. A possible reason could be that rather than measuring CFU at a time of 0 hours, they miscalculated it based on the addition of their initial inoculant. This could then cause one treatment, in this case, the control, to begin the experiment at a higher cell density than the florfenicol treatment. After the stationary phase, the data formed from the CFU concentration seems to follow very similarly between the control and florfenicol treatment, not supporting their claim of reduced growth shown in the OD_{600} data.

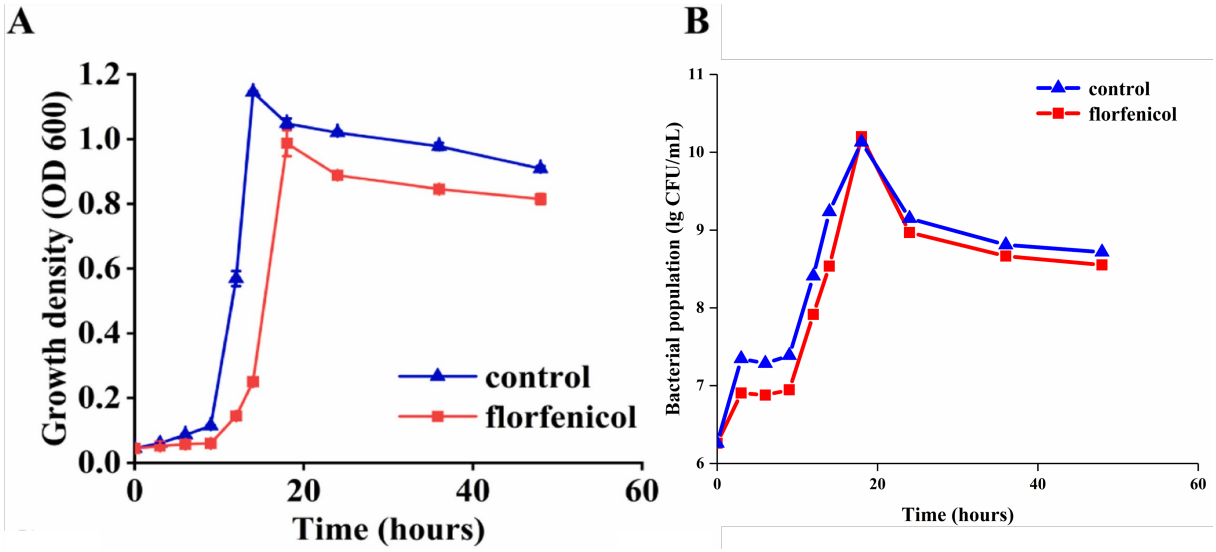

Figure 5.3: Impact of florfenicol exposure to *P. denitrificans* ATCC 19367 growth. Measured using (A) Optical density (OD_{600}) and (B) Colony forming units (CFU) (expressed in logCFU/mL). Figures used from Wang et al. (2021) (A) Figure 1A and (B) Supplementary Figure S2.

There were also several examples of poor outlining of their methodology. For instance, no reasoning was provided for the choice of using *P. denitrificans* in the study other than it being a model *nirS*-type denitrifier. *P. denitrificans* is not mentioned in bacterial community studies focused on aquaculture environments, instead being commonly associated with soil environments, predominantly farmland. The closest mentions come from a bacterial community analysis of aquaculture systems where strains belonging to the *Rhodobacteraceae* family were found at <1% sample proportions of a breeding pool, the same family that contains *P. denitrificans* (Brailo et al., 2019). Another similar study found that *Rhodobacterales* never formed more than 1.5% of the bacterial community in recirculating aquaculture (Qiu et al., 2016). Thirdly, a recent study looking at an intensive shrimp aquaculture pond in China looked at *nosZ*-bacteria within this aquaculture system and found that the *Paracoccus* genus was present, however, only at negligible relative abundance, whilst the dominant genus was *Ruegeria*, a well-documented marine denitrifier (Zheng et al., 2023). Examples like these show that there are likely to be more relevant bacterial strains to base their experiment on than *P. denitrificans*, as it seems so loosely linked to marine and specifically aquaculture systems.

5.3.4 Validity of using the bacterial consortium experiments

In the forming of the synthetic bacterial consortium, two denitrifying strains were combined with *P. denitrificans*. Bacterial consortiums have recently been shown as a useful strategy to mimic

environmental conditions in a laboratory, as single strains can sometimes fail to complete biological processes *in situ*, despite being genetically capable (John et al., 2020; Bhatt et al., 2021; Liu et al., 2021). The decision in Wang et al. (2022) to use a bacterial consortium makes sense based on their intent to further analyse the impacts of florfenicol on denitrification rates. However, there seem to be several issues with how they approached and executed this methodology. One problem with the approach to the consortium is it lacks sufficient control experiments. They should have provided data showing whether or not Pda200 impacted denitrification rates of either SB-1 (*Pseudomonas* sp.) or WB-2 (*Thauera* sp.) when grown independently as one of these strains may be far more resistant to antibiotic pressure and was performing all the nitrate removal itself. To add to this, during the final consortium experiment both Pda200 and florfenicol presence were used as the treatment, with no experiments using only one or the other. This means that their claims of the consortium enhancing the expression of several denitrifying genes when under antibiotic pressure are not entirely true. Neither are the claims that compared to the control group *napA*, *napB* and *norB* expression levels were significantly increased when treated with Pda200, as both Pda200 and florfenicol were acting on the consortium at the same time. This latter statement is even more questionable as their basis for this is that Pda200 is acting on denitrification enzymes in both SB-1 and WB-2 from outside of the outer membrane of these bacteria (Wang et al. (2022); Figures 6B and 6C). This conclusion makes sense based on their methodology, as the source of Pda200 within the consortium is not from *P. denitrificans* but from *E. coli* Top10 containing the pBBR1MCS-2 plasmid with Pda200 in the expression site. Therefore, their consortium experiment has Pda200 being overexpressed within Top10. There is no explanation within their paper on how the sRNA can leave the *E. coli* cell, or then alter gene expression in the denitrifiers. Bacterial sRNAs have a short lifespan when compared to most cellular components due to their fragile nature, especially when not chaperoned by the likes of Hfq, making the act of an sRNA surviving long enough to travel between cells very unlikely. There is also the question of how sRNAs would pass through the outer membrane, an area of *E. coli* cells that are found to have a high concentration of RNA degrading enzymes (Miczak et al., 1991). Outer membrane vesicles (OMVs) have recently been discovered to contain sRNAs (reviewed in Diallo and Provost (2020)). With sRNAs within OMVs from *P. aeruginosa* directly modulating a host's immune response (Koeppen et al., 2016). However, no examples exist of these for non-pathogenic organisms (Hoy and Buck, 2012). Additionally, as earlier stated, Pda200 is likely to be a non-polyadenylated lncRNA. Lacking a poly(A)-tail makes Pda200 highly degradable and therefore further decreases the likelihood of Pda200 being capable of being stable long enough to perform any of these proposed extracellular functions. Overall, I do not see how Pda200 could be acting extracellularly on denitrification gene expression levels and these observed changes would much more likely be caused by the florfenicol exposure.

Alternatively, the pBBR1MCS-2 plasmid containing Pda200 could be conjugated into any

or all of SB-1, WB-2 and *P. denitrificans*. *E. coli* Top10, containing the helper plasmid pK2013 is also present in the consortium and would be able to transfer the pBBR1MCS-2 containing Pda200 into the denitrifiers. With Pda200 being overexpressed within the denitrifier cells then the observed gene expression changes could be a result of Pda200 acting intracellularly. However, this study provided no data showing that the plasmid was present in these strains at any point of their experiments, therefore it is impossible to know whether any, some or all of the cells are capable of producing Pda200 intracellularly during the consortium experiments. This could have been solved by conjugating the plasmid into each strain before the experiment and maintaining all cultures on kanamycin, to select for the conjugated strain. Then a PCR using the plasmids previously designed could identify whether the plasmid containing Pda200 was successfully transformed into the strains. If this had been done there would be no doubt that the entire population of the consortium contained the plasmid and could therefore produce its own Pda200.

With this in mind, these papers still present some very interesting information. Pda200 is a denitrification-relevant lncRNA which we showed can decrease N_2O emissions when overexpressed in *P. denitrificans*. It shares a significant complementary binding site with the *nosZ* mRNA (Wang et al., 2022), and has been found to positively correlate with *nosZ* expression when under antibiotic stress (Wang et al., 2021). However, the further claims of Pda200 directly regulating the expression of other denitrification genes when grown in the consortium are unfounded based on the information provided in Wang et al. (2022).

Further research should determine whether Pda200 can form a complex with the *nosZ* mRNA. For this, an electrophoretic mobility shift assay (EMSA) could be utilised to indicate whether RNA-RNA complex formation occurs. If dimerisation is occurring then further work would be to elucidate what mechanism Pda200 is acting by on the mRNA. We have predicted here that this is a positive relationship, however, it is not certain as additional data tracking the nitrogen intermediates would be required. This also would point towards what section of the denitrification pathway is being directly affected.

6 Discussion and Future Prospects

6.1 Summaries of chapters

N_2O is continuing to enter the atmosphere and contribute towards global warming and the damaging of the ozone layer. N_2O is being released from agricultural soils at an alarming rate as a result of huge amounts of nitrogen-rich fertilisers being added to croplands. These nitrogen species should be reduced through the stepwise reaction to form N_2, however, in the environment, this process is often stopping one step early and emitting vast quantities of N_2O. When considering N_2O emissions, denitrification is the most important pathway of all biogeochemical cycles, as it is the prominent source of this greenhouse gas and also its only natural sink. Denitrification also provides an incredible opportunity with the final stage of this pathway, facilitated by NosZ, allowing for the reduction of N_2O, into the inert gas N_2, which does not impact global warming. Therefore, a focus, such as the data presented in this thesis, on how bacteria regulate their denitrification pathway is imperative to solving this part of the climate change puzzle.

This research has shown that both sRNAs and their chaperones can have a substantial effect on N_2O emissions in the model denitrifying organism *P. denitrificans* and are therefore potential targets for future mitigation strategies to reduce N_2O emissions.

6.1.1 Characterisation of sRNA11; a *P. denitrificans* growth inhibiting small RNA

Our investigation into the role of sRNA11 in *P. denitrificans* has uncovered significant insights into its regulatory functions and impact on bacterial growth and N_2O production. Overexpression of sRNA11 resulted in growth inhibition under both aerobic and anaerobic conditions, suggesting a cytotoxic role potentially linked to apoptotic cell death. This finding is particularly intriguing given the previously found 272-fold increase in sRNA11 expression from aerobic to high N_2O-producing anaerobic conditions (Gaimster et al., 2016), indicating a critical role in the cellular response to fluctuating oxygen levels. Our results show that Pd_11 strains ceased N_2O production post-exponential growth phase, in contrast to Pd_empty and PdΔ*nosZ* strains, suggesting a unique interaction between sRNA11 and the denitrification pathway.

The difficulties in working with sRNA11, including its negative impact on cell viability and growth inconsistencies, underscore the complexity of its function. These challenges possibly link sRNA11 to other sRNAs with cytotoxic effects, such as IstR1 in *E. coli*, which inhibits the toxin-encoding *tisB* gene as part of the cell's SOS response. The possibility that sRNA11 acts similarly, perhaps activating programmed cell death, presents an exciting avenue for further research.

Future research should focus on the precise molecular mechanisms of sRNA11-mediated regulation. Techniques such as RNA-seq combined with the computational predictions from TargetRNA2 and sRNARFTarget can help identify direct mRNA targets and their roles in growth

and N_2O production. Experimental validation of these predicted interactions, particularly with *lpxC* and other mentioned candidates, will be useful in understanding how accurate these computational prediction software are at identifying sRNA targets. Additionally, exploring the role of sRNA11 in natural and fluctuating environmental conditions will provide insights into its ecological significance and broader impact on microbial communities involved in nitrogen cycling.

These future studies will enhance our understanding of sRNA-mediated regulation and its implications for microbial physiology and environmental processes. Deepening our understanding between sRNA11 and denitrification pathways could offer novel strategies for managing N_2O emissions, a critical issue in mitigating climate change.

6.1.2 Contribution of Hfq to sRNA mediated regulation of denitrification

Our study on the overexpression of *hfq* in *P. denitrificans* has revealed impacts on bacterial growth and N_2O production. Although *hfq* overexpression did not significantly alter growth rates, a minor reduction in cell density was observed, indicating a potential but not drastic effect on growth. Notably, the overexpression of *hfq* resulted in a change to N_2O accumulation, with a slower increase in N_2O accumulation levels during the stationary phase compared to controls. This suggests that Hfq plays a significant role in regulating the denitrification pathway, potentially through its chaperone activity with sRNAs.

The change in N_2O accumulation rate seen in Pd_hfq implies that Hfq may facilitate the activity of sRNAs involved in denitrification. Previous studies have shown that sRNA with a low Hfq-binding affinity are often outcompeted by Hfq, suggesting that overexpressing *hfq* might enhance the formation of sRNA-mRNA complexes with lower Hfq-binding affinity. This could explain the observed phenotypes, as an increased Hfq availability may enable sRNAs to more effectively regulate target mRNAs involved in N_2O reduction, such as those relevant to the *nos* operon. Understanding this interaction could provide insights into how sRNA chaperones like Hfq modulate key environmental processes such as denitrification and impact global N_2O emissions.

Future research should focus on the specific molecular mechanisms by which Hfq influences denitrification. One approach could involve overexpressing both Hfq and a known Hfq-dependent sRNA to determine if this amplifies the observed phenotypes. Additionally, investigating the full range of denitrification intermediates and the specific stages impacted by Hfq overexpression will be useful. Techniques like ChIP-seq and Hfq CLASH can identify Hfq-dependent sRNAs and their target mRNAs, providing a comprehensive view of Hfq's regulatory network in *P. denitrificans*. Further work on these interactions will not only improve our understanding of Hfq's role in bacterial growth and N_2O regulation but also offer potential strategies for mitigating

N_2O emissions, contributing to managing greenhouse gases and combatting climate change. This could arise through the identification of novel sRNAs relevant to the denitrification pathway which are Hfq-dependent. These Hfq-dependent sRNAs have been well-researched in various model organisms and often share similarities in how they are regulated within the cell (Kavita et al., 2018), potentially allowing for broader regulation techniques which could focus on certain clades of Hfq-dependent sRNAs.

6.1.3 Pda200 regulation beyond florfenicol stress response

Pda200, a 413bp-long sRNA from *Paracoccus denitrificans*, was identified as a key regulator of denitrification by Wang et al. (2021), particularly as a posttranscriptional regulator of the *nosZ* gene responsible for the N_2O-reducing enzyme NosZ. This is significant due to environmental issues where the final step of denitrification, the reduction of N_2O to N_2, often remains incomplete.

Pda200 was initially isolated from the *P. denitrificans* ATCC 19367 strain, sharing identical similarity with a portion of the *P. denitrificans* PD1222 strain genome within the Pden_2366 gene. Despite its lack of proximity to denitrification-related genes, the paper that identified it suggested that Pda200 may regulate denitrification enzymes, likely interacting directly with *nosZ* mRNA. However, the reported perfect complementarity of 22 base pairs between Pda200 and the *nosZ* mRNA was questionable due to mismatches and contained no mention of a seed region. The mechanism for how this sRNA-mRNA complex would form remains unclear, but there is a possibility that Pda200 could unmask ribosome binding sites on *nosZ* mRNA to enhance translation, supported by its low AU content and absence of RNase E activation sites. The classification of Pda200 as an sRNA is also debated due to its length exceeding typical sRNA limits. Pda200 might better fit as a non-polyadenylated lncRNA, which, while also regulatory, has broader functions than sRNAs. This reclassification could open new avenues for understanding its regulatory mechanisms.

Experimental data produced for this thesis indicated that Pda200 overexpression in *P. denitrificans* PD1222 results in variable N_2O accumulation, generally showing reduced levels compared to controls, suggesting a regulatory role aligning with previous findings. However, high variability in the results, likely due to sealing issues in growth chambers, necessitates further experiments under different conditions and with detailed tracking of nitrogen intermediates to confirm Pda200's function and impact.

Critically, the prior studies detailing Pda200 have inconsistencies. For instance, claims of florfenicol impacting growth and CFU counts are not consistently supported by their data. Also, the methodology and choice of *P. denitrificans* for environmental relevance also appear questionable given its low presence in bacterial communities relevant to their studies. Future research should verify Pda200-*nosZ* mRNA interaction through electrophoretic mobility shift assays (EMSA) and

determine its precise regulatory mechanism, given the current data's limitations and inconsistencies in previous studies.

In conclusion, Pda200 has been shown to have potential implications on the N_2O emissions of *P. denitrificans*, however, we do not have any certainty that this relationship is a result of direct pairing with the *nosZ* mRNA.

6.2 Next steps for environmental and transcriptional regulation of denitrification

In the first year of this PhD, many preliminary experiments were conducted on several interesting topics related to the content of this thesis, however, due to timing constraints and prioritisation, these experiments were put on hold. For instance, much effort went into adapting the Vishniac and Santer trace elements solution described in Section 2.1.1 to vary in concentration of zinc rather than copper. This was based on research that found that the application of zinc chelates to agricultural soil increased *nosZ* copy number by 31.2% and reduced N_2O emissions from the same soil by 21.4% (Montoya et al., 2018). A later study by the same group found that in soils of different environmental conditions, the addition of a zinc-nitrogen fertiliser resulted in a 75% reduction in *nosZ* gene abundance and an increase in N_2O emissions (Montoya et al., 2021). These findings formed the basis of a study into how a range of zinc concentrations in anaerobic, denitrifying growth media could impact N_2O production in *P. denitrificans*. The methodology was to follow along with the same techniques used in Sullivan et al. (2013) when they identified how extracellular copper regulates *nosZ* gene expression. However, due to the time constraints involved in correctly producing the zinc-replete Vishniac and Santer trace element solution and the difficulty in ensuring complete maturation of the zinc, this part of the project was dropped.

Alongside this, much time was spent early in the PhD researching clade II *nosZ* organisms (*nosZII*). As described in Section 1.3.4, *nosZII* denitrifiers are incomplete denitrifiers that can sometimes be genetically incapable of producing N_2O, therefore acting as N_2O sinks. An example of this is *Dyadobacter fermentans*, a species that can only perform the reduction of N_2O stage of denitrification. One study found that when *D. fermentans* was inoculated into established soil samples N_2O consumption increased by 189% (Domeignoz-Horta et al., 2016). Findings like this led to a desire to examine not just the RNA transcriptome of this species, but also whether the NosZII enzyme activity responded to copper and vitamin B_{12} in the same manner as the NosZI enzyme activity does. This part of the project stopped because of lacking access to N_2O gas as an electron acceptor for culturing the bacteria in denitrifying conditions. Following previous methodologies of Sullivan et al. (2013) and Gaimster et al. (2016), with only a change from NO_3 to N_2O as the electron acceptor would provide information on how different the transcriptional

and environmental regulation of these clades of organisms is. Understanding how effective these *nosZII* organisms are at reducing N_2O under variable conditions will further any possible mitigation strategies planning to utilise this unique clade of bacteria.

Beyond *P. denitrificans*, further work should be done to create sRNA transcriptome libraries under denitrifying conditions of the dominant microbial species found in agricultural soils and aquacultures. Then identify sRNAs that share homology across the species and find out how they regulate the denitrification pathway and whether they could be utilised for reducing N_2O emissions. If this library also contained information on many sRNA's proven mRNA targets, then it could provide better training for the various sRNA target prediction software available, which at the moment are inaccurate and complex to use (Kumar et al., 2021). This could be combined with studies into environmental sRNA, whereby sRNAs are collected from all organisms residing in an environment. Should a particularly important sRNA be identified for global NosZ activity, then we would look to find ways to influence its activity in its environment. For instance, fertiliser contents could be adapted to add a compound that elevates or represses transcription of the relevant sRNA, this would make sense if working with an sRNA that could activate NosZ activity. Alternatively, you could generate an ideal synthetic sRNA which is introduced into the environment by broad host phages. It would have to be tested to ensure it is unable to function in non-denitrifiers, and that its function will not cause the target bacterial strains to become nonviable. These ideas are all extremely large scale, especially when the goal is to create a global warming relevant difference in N_2O emissions, however, the microbial denitrification pathway is currently the clearest target for mitigating N_2O emissions and as such, any sufficient strategy will need to target it.

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Paracoccus denitrificans has a nirS gene.

Dyadobacter fermentans has none of the 3 genes.

atypical nosZ occur in Bacteria and Archaea that denitrify (44

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A Supplementary Data

Table A.1: Summary of growth phenotypes observed on minimal media under anaerobic conditions of *Paracoccus denitrificans* when sRNA11 is overexpressed through the pLMB509 vector (Pd_11). Strong Growth refers to experiments where the cell density of the strain surpasses an OD_{600} of 0.5 within 40 hours and follows an exponential growth curve. Poor Growth refers to experiments where the cell density of the strain surpasses an OD_{600} of 0.1 but not an OD_{600} of 0.5 within 40 hours, usually displaying a linear growth profile. Minimal Growth refers to experiments where the OD_{600} never surpasses 0.05 within 40 hours. No Growth refers to experiments where the strain dies during the experiment and does not recover within 40 hours.

Growth Phenotype	**Count**
Strong Growth	3
Poor Growth	3
Minimal Growth	4
No Growth	11